Foreword: a historical overview of advocacy for research in sex-based biology

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Throughout history, science has been influenced by the political climate. The emerging field of sex-based medicine reflects such political forces.

On April 15, 2001, the Institute of Medicine (IOM) announced the publication of a landmark report, *Exploring the Biological Contributions to Human Health: Does Sex Matter?* [21]. This event was the culmination of 6 years of work by the Society for Women's Health Research to obtain independent, unbiased validation of research on sex differences. Since that date, interest in sex as a biological variable has increased among all participants in the biomedical research enterprise: funding agencies, basic and clinical researchers, publishers, health care providers, and health care consumers. While this textbook is further evidence of the breadth of research into physiological sex differences, the need for further research into how sex hormones and chromosomes affect etiology, prevention and treatment of disease is clear. This foreword will provide a brief overview of scientific, social and political events that lead up to these concepts. Researchers have found sex differences in every tissue and organ system.

1. The male norm

Until the late 20th century, biomedical research and medical practice were characterized by the general acceptance of the "male norm" and a misdirected sense of protectionism. Courses in human physiology, anatomy, or pharmacology were replete with references to the "typical 70-kg man" [12]. Males were studied as representative of the species, and it was assumed that sex differences (outside of reproductive functions) could be explained by, for example, differences in body or organ size, body weight, or percent body fat. Anything that differed from the male norm was labeled "atypical" or even abnormal. A common rationale for the use of males (animal or human) as the prototypical research subject is that ovarian (estrous or menstrual) cycles made females too difficult to study, and that adding female study subjects would only increase expenses and complicate the data.

For the most part, biomedical researchers used male animals in preclinical studies, and men in clinical studies. Researchers working on cell and tissue culture systems did not

consider sex as a variable and rarely reported the sex (i.e. chromosomal makeup) of their experimental material. A Medline search shows that the male norm remains, as practice guidelines and research examples are still often expressed in terms of the typical 70-kg man. Research results from men have been routinely extrapolated to women and incorporated into treatment guidelines, disregarding even the commonly acknowledged male/female differences such as body fat and hormonal status.

Acceptance of the "male norm" was bolstered by a misdirected sense of protectionism. In the US, this preference for male subjects was reinforced by policies, guidelines, and regulations aimed at protecting the fetus and a women's reproductive potential. In 1977 the US Food and Drug Administration (FDA), responding to the thalidomide tragedy of the 1960s[1] [8] and the discovery that the daughters of women who took diethylstilbestrol (DES) during pregnancy had an increased risk of vaginal cancer [14], issued guidelines that required women of childbearing potential be excluded from drug trials (except for drugs used in the treatment of life-threatening or serious diseases for which there was no effective treatment) until teratogenicity data from animal studies were available [10]. Because teratogenicity studies were usually performed at the same time as clinical trials in humans, these guidelines had the effect of excluding women from most drug trials. Given the acceptance of the male norm at the time, this exclusion was unquestioned. It was not until the mid-1980s that the medical research community began to recognize that the knowledge gap created by these policies had a detrimental effect on women's health [20].

The wide acceptance of the male norm and the paternalism behind the 1977 guidelines did women more harm than good. Women in the US were routinely prescribed drugs that had not been tested for safety or efficacy in women, and there was little understanding of the differences between men and women that might result in differences in safety or efficacy of any medical intervention. In 1985 the US Public Health Service Task Force on Women's Health Issues concluded that: "The historical lack of research focus on women's health concerns has compromised the quality of health information available to women as well as the health care they receive" [20]. The report's recommendations prompted the US National Institutes of Health (NIH) to announce a policy recommending the inclusion of women in federally funded clinical research [18]. However, this did not affect the FDA Guidelines, and researchers and women's health advocates soon became aware that the inclusion policy was not being enforced and that women were not being routinely included in medical research studies.

In 1990, these researchers and advocates organized the Society for Women's Health Research. One of the first efforts of the Society led to a request from Congress that the Government Accounting Office (GAO) conduct a study into NIH's policies and practices regarding the inclusion of women and minorities in clinical trials. The GAO auditors concluded that the NIH policy on the inclusion of women in clinical trials had not been well communicated or understood within NIH or the research community, was applied

[1] Thalidomide, given to pregnant women in Australia and Europe during the 1950s and early 1960s to treat morning sickness, caused severely deformed limbs in babies exposed to the drug in utero.

inconsistently across institutes, and was applied to extramural research only. Moreover, the GAO report concluded that there was "…no readily accessible source of data on the demographics of NIH study populations," so that it was impossible to determine if the NIH were enforcing its own recommendations [17].

One month after the GAO report was released, the Congressional Caucus on Women's Issues introduced the Women's Health Equity Act (WHEA) of 1990 [23]. This legislation comprised 20 separate bills to improve women's health research, access to health care, and disease prevention services. WHEA's chief Senate sponsor, Senator Barbara Mikulski (D-MD), attached three provisions to legislation reauthorizing NIH: creating an office specifically devoted to women's health research at NIH, requiring that women be included in NIH-funded clinical trials, and establishing five extramural contraceptive and infertility research centers.

Although President George H. W. Bush vetoed the WHEA, faced with the prospect of further congressional action, the NIH quickly strengthened its policy on inclusion of women in research studies and created the Office of Research on Women's Health to coordinate women's health research activities across the NIH institutes and centers [13]. The NIH instituted guidelines for grant submission that required inclusion of women and minorities as human subjects unless there was a clear justification for their exclusion. These guidelines became supported by law in 1993 with enactment of the NIH Revitalization Act, and became effective on publication in the Federal Register in early 1994 [19]. Specifically, the 1994 NIH guidelines state that "the NIH must ensure that women and members of minorities and their subpopulations are included in all human subject research" and "for Phase III clinical trials,[2] [the NIH must] ensure that women and minorities and their subpopulations must be included such that valid analysis of differences in intervention effect can be accomplished" [19].

The 1990 GAO report on the inclusion of women in NIH-sponsored research was followed by a 1993 GAO report on the inclusion of women in the clinical trials used by the FDA in evaluating drugs for marketing approval [24]. This report found that while women were sometimes included in drug trials, they were significantly underrepresented. Even when women were included, data were not analyzed to determine if women's responses to drugs differed from those of men. Further, drug manufacturers often failed to study whether or not the cycle and lifespan changes in the hormonal environment of a woman's body affected the drug's action. The report concluded by recommending that the FDA should ensure that drug companies consistently include "sufficient numbers of women in drug testing to identify gender-related differences in drug response and that such sex differences are explored and studied" [24].

In 1993, the FDA reversed its 1977 guidelines, publishing a new "Guideline for the Study and Evaluation of Gender (sic) Differences in the Clinical Evaluation of Drugs" [11]. The new guideline encouraged the participation of women in Phase 1 and Phase 2 safety and dosing studies, required that study sponsors collect and analyze data on sex differences, and encouraged the consideration of the effects of the menstrual cycle,

[2] The NIH defines a Phase III clinical trial as "a broadly based…clinical investigation usually involving several hundred or more research subjects, for the purpose of evaluating an experimental intervention in comparison with a standard or control intervention or comparing two or more existing treatments" [10].

exogenous hormone therapy, and effect of the drug on oral contraceptives, when feasible [7].

These changes began a shift in biomedical research away from the male norm toward inclusion of female subjects in studies, and the analysis of data to look for sex differences. As a result, the number of women included in medical research studies has increased. A follow-up audit of NIH efforts in this area by the GAO in 2000 found that women were being included in clinical trials at rates proportionate to their numbers in the general population [9]. The report found that "the review for extramural research now treats the inclusion of women and minorities as a matter of scientific merit…and it appears that NIH staff and researchers are working to ensure that, when appropriate, study findings will apply to both women and men." However, the GAO auditors also concluded that "NIH has made less progress in implementing the requirement that certain clinical trials be designed and carried out to permit valid analysis by sex, which could reveal whether interventions affect women and men differently" [9].

Shortly before release of the 2001 IOM report on sex-based biology, drug manufacturers and the FDA received the message that sex analysis of clinical trial data is crucial to the development of safe and effective drugs. In January 2001, the General Accounting Office released an interim report on their investigation of FDA practices in reviewing clinical trials data with respect to the inclusion of women in studies, and analysis of data by sex. The GAO looked at the sex breakdown of the incidence of adverse events that led to the FDA to withdraw approval of 10 drugs. Of these drugs, eight caused adverse events more often in women than in men. Four of these drugs were more often prescribed to women than to men, so the higher number of adverse events in women was not unexpected. For the other four, however, the drugs were prescribed equally to both men and women, yet the number of adverse events was higher in women, suggesting a true sex difference in the incidence of adverse events [5].

The final report on the 2001 GAO audit of FDA records said that about one-third of the study documents examined failed to fulfill the requirements for presentation of outcome data by sex, and that nearly 40% did not include the required demographic information. The auditors concluded that "The FDA has not effectively overseen the presentation and analysis of data related to sex differences in drug development" [22].

2. Does sex matter?

In the mid-1990s, the Society for Women's Health adopted a new strategic priority: to promote research on physiological sex differences and the effects of sex differences on disease prevention, diagnosis, and treatment. The Society initiated a campaign to engender public and private sponsors for the formation of the IOM Committee on Understanding the Biology of Sex and Gender Differences (The IOM Committee). The IOM Committee was charged with reviewing and evaluating the current state of knowledge about, and scientific evidence for, sex and gender differences and their determinants; to identify gaps in research on sex and gender differences, and to make recommendations for filling those gaps. In April 2001, the Committee released its report,

Exploring the Biological Contributions to Human Health: Does Sex Matter? [21]. This IOM report concluded that:

> There is now sufficient knowledge of the biological basis of sex differences to validate the scientific study of sex differences and to allow the generation of hypotheses with regard to health.…Naturally occurring variations in sex differentiation can provide unique opportunities to obtain a better understanding of basic differences and similarities between and within the sexes [21].

The report conveys three main messages:

- Sex matters. Sex is a crucial biological variable that "should be considered when designing and analyzing studies in all areas and at all levels of biomedical and health-related research."
- The study of sex differences is evolving into a mature science. "There is now sufficient knowledge of the biological bases of sex differences to validate the scientific study of sex differences and to allow the generation of hypotheses. The next step is to move from the observational to the experimental …"
- Barriers to the advancement of knowledge about sex differences in health and illness exist and must be eliminated.

The IOM Committee also reported that:

- "Every cell has a sex." Sex begins with the chromosomal complement that normally includes either a pair of X chromosomes, or one X and one Y chromosome. It was once thought that this chromosomal difference was significant only for its importance in the determination of phenotypic sex. Advances in molecular biology have taught us otherwise, as researchers have identified a number of differences attributable to sexual genotype (XX or XY). The significance of theses differences to cell and tissue physiology is not completely understood, and progress is impeded by the lack of information on the sex of origin of cell and tissue culture material in published reports.
- "Sex begins in the womb." Sex differences appear in the organization of tissues in the embryo, and continue to manifest throughout the lifespan, and there is a lack of data on sex differences from longitudinal studies across the lifespan.
- Sex affects behavior and perception. It is in this area that the interplay between genetic and biological factors and environmental factors is most evident.
- Sex affects health in all areas, including health promotion and disease prevention, diagnosis, and treatment. Men and women have different patterns of illness and statistically different lifespans. Again, this is an area where both biological and environmental factors come into play.

The IOM Committee made several recommendations regarding research on sex differences. They noted that sex at the most basic level is determined (in humans) by the presence of two X chromosomes, or an XY pair, and that research is needed

- on the functions and effects of the proteins encoded by genes on these chromosomes, and the effects of gene dosage for genes that escape X-inactivation;
- on how sex differences at the genetic level manifest at the level of the cells, tissues, organs, and whole organism; and
- to distinguish the effects of gene products from the effects of hormones on gene expression.

The IOM Committee emphasized that sex should be included as a variable in research design, including laboratory as well as clinical and epidemiologic research. They noted that sex differences must be studied across the lifespan, as the effect of sex differences on health and disease may change with age. They pointed out the broad interdisciplinary nature of this research and the need for institutional and funding support for interdisciplinary approaches.

The IOM Committee also considered the usefulness of animal models and of so-called "experiments of nature" in exploring sex differences. They identified a need for animal models that reflect human sex differences. For example, primate models may be required to study the effects of hormonal cycling. They noted that human variations due to genetic or endocrine disorders, or environmental exposures, may also be useful in elucidating the hormonal effects underlying sex differences. An example is the behavioral research being done on girls with congenital adrenal hyperplasia, who are exposed to high levels of androgens in utero and have the condition corrected at birth. This work is exploring the role of hormones in neonatal development.

The potential for use of sex-based research in defending discrimination based on such differences was noted by the IOM Committee. They recommended that the scientific community be aware of this potential and to increase the public's awareness that human beings are not just the sum of their genetics and physiology; that often the end results of our genetic programming are amenable to environmental influences and conscious efforts to change.

3. Sex differences in the scientific and medical literature

Among the barriers to progress in sex-based biology identified by the IOM Committee is the relative lack of sex-specific data in the scientific and medical literature. The IOM Committee recommended that investigators and scientific publishers make sex-specific data more readily available and accessible, in all areas of biomedical research. This includes:

- Determining and disclosing the sex of origin of biological research materials – cells, tissues, organs, and experimental animals
- Identifying the endocrine status of research subjects, i.e. cycling or not, and, if cycling, stage of cycle. Treat endocrine status as an important variable that should be considered when possible in analysis.
- Constructing and conducting longitudinal studies so that the results can be analyzed by sex.

Once the studies are done and the data appropriately analyzed by sex and endocrine status, there is a need to make the data and analysis more readily accessible in the literature, particularly with regard to database search methods. The inconsistent use of the terms "sex" and "gender," in particular the use of "gender" as a euphemism for "sex," exacerbates this problem.[3] At present there is no consistent use of these terms abstracts or as key words in bibliographic databases, and it takes persistent and creative use of these databases to track down publications that report sex differences (or lack thereof).

The Society for Women's Health Research has encouraged the consistent reporting of data by sex, and several prominent journals have begun requiring authors to include sex analysis in their manuscripts. *The Journal of the National Cancer Institute (JNCI)* specifically states in its information for authors that "…where appropriate, clinical and epidemiological studies should be analyzed to see if there is an effect of sex or any of the major ethnic groups. If there is no effect, it should be so stated in Results" [15]. The wording of the editorial policy of the *JNCI* is of note in that it specifically states that negative results must be reported. This is the antithesis of the more common practice of suppressing negative results [1,6]. It is interesting to note that several studies have found that publication bias (failure to publish negative findings) is initiated by the investigator and is not due to editorial decisions [2–4]. These studies found that most unpublished negative findings remained so because the investigators thought the results were uninteresting, or they did not have enough space. By requiring investigators to include sex analysis results, even negative ones, in their manuscripts, journals such as *JNCI* are reinforcing the message of the IOM report: sex does matter.

4. Sex differences at the NIH

Despite the fact that sex differences are recognized as crucial to understanding human biology and the etiology, diagnosis, prevention, and treatment of disease in men and women, there is currently no effective coordination of sex differences research efforts at the NIH. Several institutes, including the National Institute on Drug Abuse, the National Institute for Mental Health, the National Institute for Environmental Health Sciences, the National Institute on Aging, and the National Institute for Dental and Craniofacial Research have recognized the need for such research within their own disciplines, and have programs in place to fund research on sex differences. However, the majority of NIH institutes have not made research into sex differences an explicit part of their research portfolio. At present, there is no entity (Institute, Center, Division, or Office) charged with coordination of sex-based biology research programs across the NIH. This is in contrast to the Canadian Institutes of Health Research, which include the Institute of Gender and Health. The mission of this institute is to support research on "… how sex and gender

[3] The IOM committee developed the following working definitions of these terms: "*Sex* refers to the classification of living things, generally as male or female, according to their reproductive organs and functions assigned by their chromosomal compliment. *Gender* refers to a person's self-representation as male or female, or how that person is responded to by social institutions based on the individual's gender presentation."

interact with other factors that influence health to create conditions and problems that are unique, more prevalent, more serious or different with respect to risk factors or effective interventions for women and for men" [16].

At present, the only organizational entity to take on the question of sex differences on an NIH-wide basis is the Office of Research on Women's Health. Although coordination of NIH research on sex and gender differences was not an explicit part of the original ORWH mandate, the increased recognition of the importance of sex differences research to women's health has led the ORWH to include sex differences in their list of research priorities for 2002. Among ORWH's efforts in 2002 was coordinating the award of eleven grants for Specialized Centers of Research on Sex and Gender Factors Affecting Women's Health, a program administered by the National Institute of Arthritis and Musculoskeletal, and Skin Diseases.

Progress in sex-based biology is fundamental to furthering our understanding of male and female biology, and to furthering our ability to prevent, diagnose and treat disease in men and women. Such progress in sex-based biology will require collaboration across research disciplines and medical specialties, and among all research approaches, from molecular biology to epidemiology. The importance of sex-based biology to health care necessitates a "bench-to-bedside" translation that requires integration of research findings from studies at the cellular level, in animals, and in human subjects. Timely and accurate reporting of research results by sex, and support and funding for sex-based biology research and research resources, will be crucial as the field matures.

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Sex chromosomes

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1. Introduction

Phenotypic differences between the sexes have traditionally been attributed to either genetics or the effects of sex hormones. Recent studies have suggested a diversity of sexual dimorphisms that influence physiological systems in ways that project beyond the reproductive axis. There are many physiological responses, disorders and diseases that affect one sex more or differently from the other. As the human genome is explored and science advances to an era of gene-based physiology, we are better positioned to explore the mechanisms and origins of these differences. Important questions can be asked about how research and information on sex differences will be translated into preventative, diagnostic, and therapeutic practice. This chapter focuses on the sex chromosomes, basic genetic mechanisms for sex determination and the involvement of the sex chromosomes in non-reproductive physiology.

2. Background

2.1. Mammalian sex chromosomes

In mammals one pair of chromosomes is sexually dimorphic and designated as the sex chromosomes. The mammalian sex chromosomes are the X chromosome and the Y chromosome. Females have two copies of the X chromosome while males have one copy of the X and one copy of the Y chromosome. In males the X and Y chromosome act as homologous chromosomes and pair in the beginning of meiosis and separate from each other at the end of the first division. Meiotic pairing and separation ensures in males that half of the gametes have an X chromosome (fertilization results in a female) and half of the gametes contain the Y chromosome (fertilization results in a male).

In contrast to males who have one X chromosome and thus one copy of X chromosome genes in each somatic cell, females, with two copies of the X chromosome, have two copies of each gene. The X and Y chromosomes are unique in being present in only one copy in males; a single copy of any other chromosome in humans is not compatible with life. X inactivation compensates for the different gene dosages in females (two copies)

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compared to males (one copy). In female embryos one of the X chromosomes is randomly inactivated early in development. Thus males, and for most of their life females, have one active X chromosome. The inactivation of the X chromosome is random as it originally occurs, that is in a female of genotype X1/X2 half the cells have X1 off and half the cells have X2 off. Once an X is turned off, then all cells derived from that cell by mitosis have the same X chromosome turned off. This results in the classic mosaic appearance in females heterozygous for X-linked traits.

The genes producing familiar phenotypes such as color-blindness, hemophilia and muscular dystrophy are mutant alleles of genes located on the X chromosome. There are no classic phenotypes apart from sex determination and fertility on the human Y chromosome that have been identified at the present time.

2.2. *The Y chromosome*

In the human genome project, the Y chromosome was the first chromosome to have its complete DNA sequence assembled [16]. The mammalian Y chromosome contains very few genes compared to the X and autosomal chromosomes. Results from the human genome project identify 211 genes on the human Y chromosome, compared to 780 genes on chromosome 22 (about the same size as the Y) and 1363 genes on the X chromosome [14]. There are two major regions of the mammalian Y chromosome: a small pseudoautosomal region (PAR) and a larger Y unique region. The PAR has loci that are homologous to a region on the X chromosome. These regions pair during meiosis, resulting in the homologous pairing and separation of the X and Y chromosomes during meiosis I. The unique region of the Y does not recombine with the X chromosome resulting in the majority of the Y chromosome being inherited without variation from father to son. The unique region also contains the testes determining locus (*SRY*). Transgenic studies and chromosome defects have demonstrated that the presence of *SRY* is necessary for testis determination. The *SRY* location need not be restricted to the Y chromosome as translocations elsewhere in the genome still results in testis development.

2.3. *Inheritance patterns of the sex chromosomes*

Y-linkage or holandric inheritance is a pattern of inheritance of a trait from father to all sons. The phenotype of all sons matches the phenotype of the father and there is no inheritance through a female lineage. In a pedigree this would be indicated by phenotypes of all sons matching both their father and all their sons. In addition, because Y linkage is rare, a Y chromosome location for a gene would need to be confirmed with other crosses or molecular data. Because the majority of the Y chromosome does not cross over, the classic genetic tools of linkage analysis to locate a specific gene of interest cannot differentiate between loci on the Y chromosome. This makes identifying and mapping genes on the Y chromosome different and more difficult than for any other chromosome.

The X chromosome inheritance pattern is the classic Mendelian X-linked or sex-linked inheritance pattern. This pattern is well known and will not be discussed in great detail in this chapter. Important to the present discussion is that the copy number differences

between males (one copy) and females (two copies) of the X chromosome. This difference requires dominant and recessive inheritance patterns to explain female X chromosome phenotypes, but these are not necessary for male X chromosome phenotypes.

The only exception to the two patterns is for genes located in the PAR of the X and Y chromosomes. Because of the pairing and recombination for this region their inheritance pattern is that of an autosomal locus. Since the inheritance pattern is not unique, there must be other mapping or molecular data to confirm a pseudoautosomal location for a gene.

These inheritance patterns of the X and Y chromosomes are the result of genetic mechanisms necessary for sex determination and meiotic consistency. The assumption that all genes for sex determination or phenotypic sex or only genes involved in sex are on the sex chromosomes is wrong. The X and Y chromosomes contain many genes not involved in sex determination or other sex-related functions. These genes still have the sex chromosome inheritance patterns because of their location not because of any functional relationship to sex. For example, for an X-linked recessive trait like colorblindness, more men are colorblind because they only need one copy of the gene for expression; there is no functional or physiological reason that connects colorblindness to sex.

The gene encoding the androgen receptor is located on the X chromosome while the enzymes required for synthesizing testosterone are on autosomes. For example, 17 beta hydroxysteroid dehydrogenase (the enzyme that converts androstenedione to testosterone) is on human chromosome 17 and the steroid 5 alpha-reductase (converts testosterone to dihydrotestosterone) genes are on human chromosomes 2 and 5. Estrogen receptor genes are on human chromosomes 6 and 14.

2.4. Sex determination

Mechanisms of mammalian sex determination are complicated and mostly unresolved. Two types of data have been used to investigate mechanisms of mammalian sex determination. First, the study of mutations that have disrupted pathways so that phenotypic sex is either not obvious or inconsistent with chromosomal or gonadal determinations (gonadal dysgenesis, Fig. 1). These types of studies have come primarily from human sources identified originally by infertility. Transgenic or studies using genetic mouse populations of experimental animals have provided a second type of data. For the purpose of this chapter, a general overview will be presented of the basic processes involved in sex determination.

Sex is determined by two major components: chromosomes and genes. All of the genes involved in sex determination are not identified completely. The primary testis determining gene is *SRY*, although other loci can override or replace mutant *SRY* activity. The genes involved in ovary determination are not identified. Therefore, gonadal differentiation is a result of the expression of either testis determining loci or ovary determining loci in the bipotential-developing gonad. There is evidence that each pathway is able to inhibit the other. The historical idea of a single testis determination gene, with ovarian development occurring only when the testis gene is missing, is overly simplistic and does not fit current observations (see Ref. [17] for a recent review of mammalian sex determination).

2.5. *Phenotypic patterns and mechanisms*

Fig. 1 summarizes and illustrates the interplay between genes, chromosomes, tissues and hormones involved in determining the phenotype of sex. Sex can be analyzed at several levels: genetic, chromosomal, gonadal, hormonal or phenotypic (Table 1). Genetic models and mutations where these levels of sex are not consistent have been instrumental in elucidating the mechanisms and interactions involved in sex determination and sex

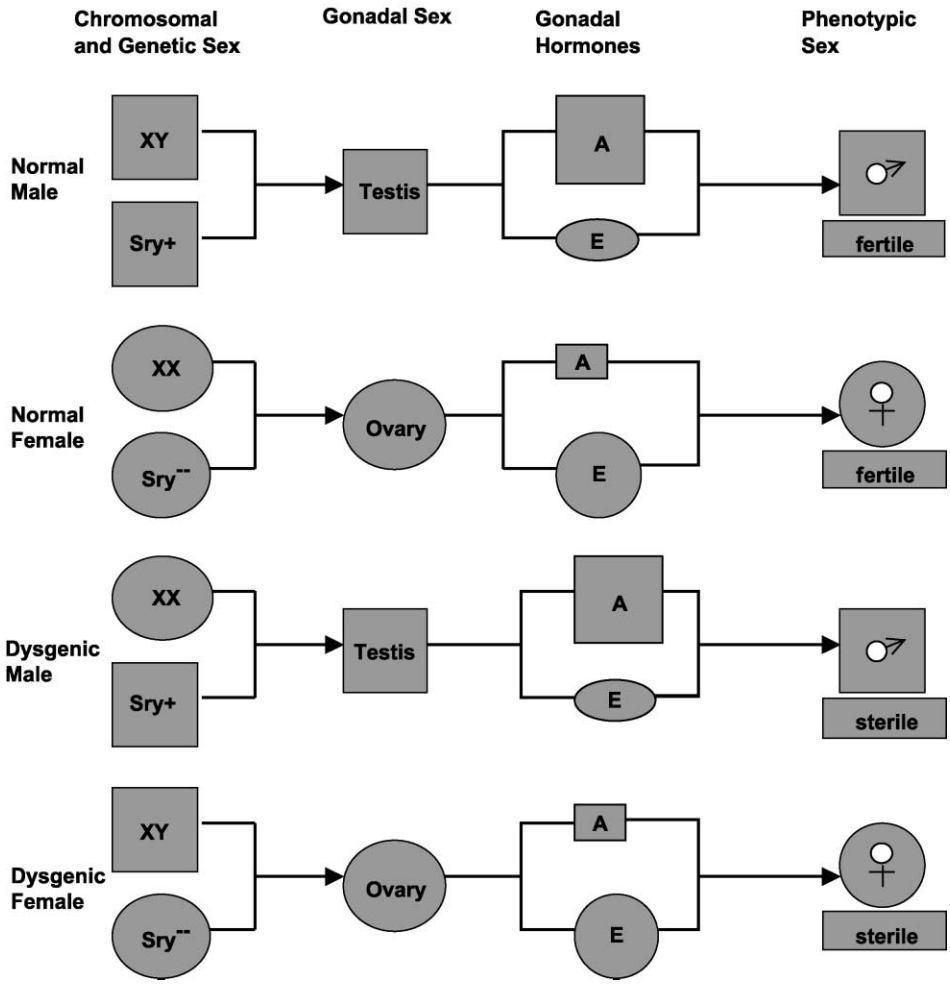

Fig. 1. Summary of the components and levels of phenotypic sex determination. Boxes or rectangles indicate male components and circles or ovals are female components. Genetic sex is only diagrammed in terms of Sry for simplicity and because identity of comparable ovarian determinants are unknown. The sizes of the gonadal hormone rectangles or ovals (A, androgens; E, estrogens) are indicative of the different amounts of these products from male and female gonads.

Table 1
Vocabulary list for this chapter

Autosomal chromosomes	All chromosomes but the sex chromosomes
Autosomal trait	A phenotype caused from a locus on an autosomal chromosome
Chromosomal sex	Sex determined by the presence of the sex chromosomes, in mammals no Y is female and presence of Y is male
Dosage compensation (X inactivation)	The process whereby the difference in numbers of the X chromosome in males and females is equilibrated; in mammals this occurs by the turning off of one X chromosome in females so only one X is active
Genetic sex	Sex determined by whether the male or female specific genes are present. The ovary specific genes have not been identified and a primary testis specific gene is SRY (although it is not the only one)
Gender	A phenotype dependent on whether an individual perceives himself or herself as male or female
Gonadal dysgenesis	Phenotype where gonadal development or differentiation is abnormal and not consistent with genetic, chromosomal or phenotypic sex
Gonadal sex	Sex determined by the presence of male (testis) or female (ovary) gonads
Holandric trait	A phenotype caused by a locus found on the Y chromosome
Hormonal sex	Sex determined by whether androgens (male) or estrogens (female) are the primary hormone produced by the gonads, gonadal tissue or other organs
Imprinting	The control or expression of a gene is modified depending on whether it was inherited from the maternal or paternal gamete
Phenotypic sex	Sex determined by the overall phenotype of the individual, primarily the secondary sexual characteristics
Pseudoautosomal region	The portions of the X and Y chromosomes that pair and cross over during meiosis. Loci in this area demonstrate neither X or Y linked inheritance patterns
Sex chromosomes	That set of chromosomes that are sexually dimorphic, in mammals the X and Y chromosomes
Sex linkage	The inheritance pattern of genes on the sex chromosomes and in mammals this usually refers to those patterns of X chromosome inheritance

(*continued*)

Table 1
Continued

Sex-linked locus (trait)	Phenotype caused by a locus that is on the sex chromosomes, but usually in mammalian genetics this refers to genes on the X chromosome
Sex-influenced trait	A trait that has a different inheritance or phenotypic pattern depending on whether the individual is male or female, these loci can be autosomal, sex-linked or holandric
Sex-limited trait	A phenotype that is limited to either male or female but not in both, these traits can be the result of autosomal, sex-linked or holandric genes

physiology. Just as sex can be categorized at different levels, the interactions can occur at different levels.

The different levels of sex and sex determination can have both direct and indirect effects on the physiology of an individual. One direct effect is through the products and hormones made and secreted by the gonads. The many organism-wide effects of androgens and estrogens are the well-known sex effects that influence the physiology of many organ systems. The gonad determination genes, such as the testis determining gene *SRY*, may have functions other than sex determination. The evidence supporting this is that the *SRY* locus is expressed in some adult tissues. For example, expression of *SRY* in adult tissues is not required for testis development in embryonic tissue.

Determining the basic genetic and physiological mechanisms behind sexually dimorphic traits is difficult because there are multiple genetic and environmental effects that can cause a sexually dimorphic pattern. Interplay between genetic sex, chromosomal sex, gonadal sex and phenotypic sex can cause similar phenotypic patterns for different reasons. As sexually dimorphic traits are studied, the goal is to explain the mechanisms and interactions that result in these patterns. For example, consider a trait that is only observed in males. Several different mechanisms can account for this pattern.

(1) Although it is very seldom correct, the first mechanism considered is that of holandric or Y-linked inheritance. In this mechanism the gene responsible for the trait is found on the Y chromosome and since only males have a Y chromosome only males have the phenotype. Only having males express the trait is not sufficient to demonstrate Y linkage, an analysis of the inheritance pattern or molecular localization is necessary.
(2) A second mechanism possible is that of X-linkage. If an X-linked recessive allele were seen in 1/1000 males then only 1/1,000,000 females would be predicted to express the trait. One reason a rare X-linked recessive allele would only be seen primarily in males is because for a female to be affected both parents must have the allele. For a rare allele this would only happen with inbreeding. In this explanation the male limited expression is a result of the statistical difference between having one

copy in males and two copies in females for expression of the phenotype rather than any real relationship to sex.

(3) Another mechanism is a male sex-limited trait, which would also only be seen in males. In this mechanism the linkage location of the gene is not the determining factor. A sex-limited trait can be from genes on either the autosomes or sex chromosomes; the determination is not related to gene location. Think of a gene controlled by high androgen levels, this gene would only be expressed in males since only males have the necessary androgen levels. The location of the gene does not matter since the control "switch" is the androgen level rather than the chromosomal location of the gene.

These examples are only first level mechanisms, where there is only one gene or mechanism affecting the trait; if there are multiple genes and mechanisms affecting a single trait the situation quickly becomes much more complicated.

3. Physiological effects of the sex chromosomes

In this section studies related to the regulation of blood pressure in the spontaneously hypertensive rat (SHR) will be used to illustrate how scientific questions arise and how experiments are devised. The SHR is the most commonly used experimental animal model of human essential hypertension. The hypertension in SHR is the result of multiple genes each adding an increase to the blood pressure (either systolic, diastolic, or mean). One of the primary genetic components in this model is a Y chromosome locus that increases blood pressure, thus demonstrating the importance of studying and understanding the sex chromosomes and their effects.

3.1. Y chromosome effects on blood pressure – animal and human studies

The fact that males have higher blood pressure than females in mammals during their reproductive years suggested that the Y chromosome could be involved in blood pressure regulation. Likewise, in the SHR, males have higher blood pressure than females and adult males develop a very high blood pressure (in the 200 mm Hg range for systolic pressure). In order to study borderline hypertension (140–160 mm Hg), which mimics beginning human hypertension, an SHR male can be crossed with a normotensive (Wistar Kyoto, WKY) female to study whether their offspring have borderline hypertension. In all borderline hypertensive rat studies the male was the hypertensive parent and the female normotensive. What would happen, then, if the reciprocal cross was performed with an SHR hypertensive female and a normotensive WKY male? The result of this reciprocal cross was that blood pressure in the sons was significantly lower than when an SHR male was crossed to a WKY female [6]. The comparison of blood pressure of sons from the reciprocal crosses was only consistent with a Y chromosome component (Fig. 2).

Therefore to further study this effect, a breeding protocol was designed to separate the Y chromosome hypertensive component from the autosomal hypertensive component

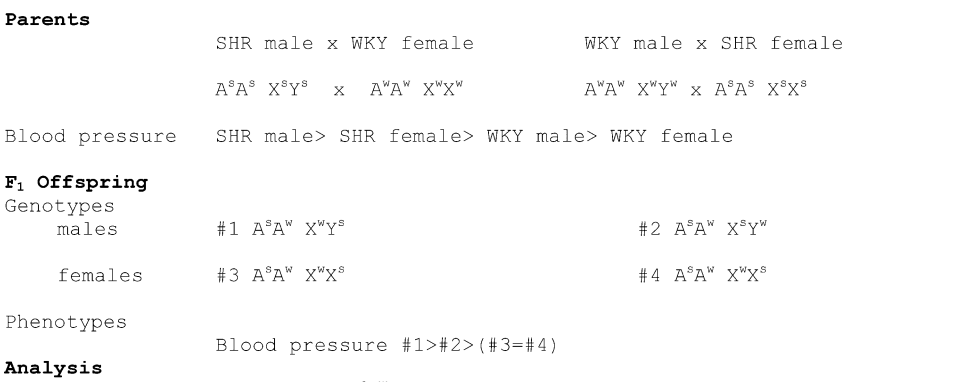

Fig. 2. Diagrammatic representation of the reciprocal crosses between SHR and WKY strains, demonstrating a Y chromosome effect on blood pressure. SHR autosomes and X and Y chromosomes are designated A^S, X^S, and Y^S. WKY autosomes and X and Y chromosomes are designated A^W, X^W and Y^W.

by producing two rat strains. One strain (designated SHR/a) had the autosomes and X chromosomes from the hypertensive female (SHR) and the Y chromosome from the normotensive male (WKY). The other strain (designated SHR/y) had the autosomes and X chromosomes from the normotensive female (WKY) and the Y chromosome from the hypertensive male (SHR). Both strains were developed using a backcross strategy to keep the Y chromosome constant while replacing the autosomes and X chromosome. These strains are designated as consomic strains because they have substituted or replaced an entire chromosome rather than congenic strains in which a gene or gene region is substituted or replaced. Blood pressure of the two consomic strains have been published [3,19], and in each the addition (or deletion) of the SHR Y chromosome adds (or subtracts) 15–20 mm Hg from the parental strain systolic blood pressure.

The consomic strains are maintained by backcrossing to the original female strain preventing random differentiation between the consomic strains and the parental strains. This ensures that in each generation the SHR and WKY strains are the genetic control strains. In addition to confirming the original observation of the hypertensive effect of the SHR Y chromosome, these strains have been used to determine the mechanisms of blood pressure elevation and the influence of the autosomal, X and Y chromosomes (for a review of Y chromosome hypertension, see Ref. [7]). These observations in SHRs are consistent with human pedigree studies that have implicated the Y chromosome in the determination of blood pressure and hypertension [1,12].

3.2. X chromosome effects on blood pressure

A genetic linkage map was constructed using microsatellite markers from data on the SHR stroke-prone (SHR-SP) crossed with WKY. This QTL mapping analysis demonstrated a significant blood pressure effect of a locus on the X chromosome (termed BP/SP-2) [9], and verified a hypertensive Y chromosome in SHR-SP rat. It is not surprising that an X chromosome may have a hypertensive locus since many genes are involved in blood pressure regulation.

The salt sensitive Sabra rat is another experimental animal model of hypertension. Using linkage techniques, 1–3 loci have been identified on the X chromosome that contribute to a salt-induced blood pressure rise in females, but not in males [21]. The two sexes regulate the hypertensive response to salt differently such that females have higher salt appetite than males [11].

3.3. Are sex hormones involved in Y chromosome hypertension?

Sex hormones are chemically classified as steroids. The ovary produces estrogens and the testis produces androgens. The major circulating androgens include dehydroepiandrosterone sulphate, dehydroepiandrosterone, androstenedione, testosterone and dihydrotestosterone in descending order of serum concentration. Dehydroepiandrosterone is mainly a product of the adrenal gland. It is regulated by adrenocorticotropic hormone (ACTH) and acts as a precursor for the peripheral synthesis of more potent androgens. Dehydroepiandrosterone is produced by both the ovary and adrenal gland, and is derived from circulating dehydroepiandrosterone sulphate. Testosterone circulates both in its free form and bound to proteins including albumin and sex steroid hormone-binding globulin. In terms of estrogens the major forms are estradiol, estrone and estriol in descending order of serum concentration. The most active form of estrogen is estradiol produced from testosterone in the theca interna cells of the ovary by p450 aromatase. In the ovarian granulosa cell androstendiones can produce estrone via p450 aromatase which can then convert to estradiol by 17 beta hydroxysteroid dehydrogenase. The details of steroid biochemistry will be covered in detail in subsequent chapters.

Mechanisms of hormone receptor activation are covered in detail in Chapters 3 and 4. However, for the purposes of this chapter, it is important to recall that the classic route of steroid action is via binding to a cytoplasmic receptor and translocation to the nucleus thereby affecting gene transcription. However, recent evidence also suggests that steroids have non-genomic effects. These effects are too rapid to be mediated by receptor binding and transcriptional activation and are usually associated with activation of signal transduction mechanisms like peptide hormones and ions [15]. With regard to non-genomic effects of testosterone, most studies show an increase in extracellular influx of calcium. However, estrogen has been reported to increase intracellular calcium via unloading from intracellular sites (see also Chapters 4, 8 and 9 for details).

3.4. Methods to determine hormonal effects

To study sex hormonal effects on phenotypes a variety of approaches have been used to manipulate hormone levels. These include castration (eliminate the source of the sex

hormones), castration and hormone replacement, hormone variations (androgens given to females and estrogens administered to males), steroid receptor blockade, and steroid receptor mutation. Studies in both normotensive and hypertensive male rats show that castration performed before puberty reduces adult blood pressure suggesting that testis or a product of the testis is required for complete penetrance of hypertensive loci in adult males. In adult females, ovariectomy in hypertensive rats elevates blood pressure suggesting a protective effect of the ovaries or estrogen. When testosterone is administered to ovariectomized females blood pressure increases and estrogen given to gonadally intact males lowers blood pressure. Blockage of androgen receptors with flutamide mimics castration and results in decreased blood pressure in adult males. The SHR Y chromosome when placed in a normotensive WKY background causes testosterone to rise at an earlier developmental age and blood pressure to increase, suggesting the presence of a Y chromosome locus through an unknown gene modulates testosterone production [4].

The testicular feminized male (Tfm) rat is another experimental animal extremely valuable in studying the influence of the androgen receptor on sex-based physiological differences. This mutation of the androgen receptor arose spontaneously in a King Holtzman breeding colony. The Tfm phenotype is not unique to this strain of rats as similar mutations and phenotypic effects occur in both mice and humans. The androgen receptor locus is on the X chromosome; thus half of the sons of carrier females express the phenotype. The affected males do have testes (retracted into the abdomen) since *SRY* is present on the Y chromosome, but because the defective androgen receptor cannot bind testosterone, result in female external genitalia. Serum testosterone levels in these animals are high, because negative feedback of testosterone production from the testes requires a functional androgen receptor. Therefore, in these animals, testosterone could cause non-receptor mediated effects, such as direct interaction with calcium channels since the effects of androgen receptors are removed.

Crossing a borderline hypertensive male (SHR/y) with a Tfm carrier female makes it possible to study the influence of the hypertensive Y locus in a Tfm genetic background. The Tfm male offspring had reduced blood pressure due to the lack of an androgen receptor blocking the effect of testosterone on the hypertensive phenotype [5]. There is a gene on the SHR Y chromosome which increases blood pressure. For this gene to express its phenotype both androgens and the androgen receptor are required. Thus the Y chromosome hypertension effect is both holandric and male sex-limited. These results with castration and Tfm crosses demonstrate some of the myriad of interactions possible with sex-related physiology.

3.5. Neuroendocrine interactions

Sex hormones can have important interactions with other hormones and regulatory systems. For instance, testosterone interacts with the sympathetic nervous system (SNS) and norepinephrine (NE) at the level of the alpha receptor. Gong et al. [8] showed that renal alpha 2-adrenergic receptor density was higher in males than females in both SHR and WKY rats. Castration of males reduced the renal alpha 2-adrenergic density by 50% while testosterone treatment returned the receptor density to control levels. The SHR Y

chromosome blood pressure effect is associated with indices of increased SNS activity including: increased adrenal gland NE and chromogranin A content, increased heart and renal NE turnover, increased plasma NE response to acute stress and there is a reduction in blood pressure after chemical sympathectomy or sympathetic blockade [2,20].

Other interactions of sex chromosome and hormones are manifested in specific behaviors such as aggression, mating, nest building and appetite. Detailed analyses of these behaviors are not covered in depth here. Hormonal effect on neuronal functions is covered in Chapters 17 and 18. However, related to the topic of this chapter, relationships among hypertension, aggression and salt appetite will be discussed briefly.

Behavioral research dealing with aggression has focused on two physiological systems, brain serotonin (5-hydroxytryptamine) and peripheral testosterone. Abnormal serotonergic function results in a number of neurological disorders, such as schizophrenia, depression, social phobias, obsessive compulsive disorder, post-traumatic stress disorder, Parkinson's disease and aggression. Testosterone can indirectly modulate serotonin expression, and may involve the Y chromosome as consomic strains of rats with different Y chromosomes have different aggressive behavior [18]. Future studies are focused on the genetic basis of the Y chromosome interaction on aggression.

Another behavioral phenotype associated with sex differences is salt intake. Free sodium intake (ingestion of sodium by animals in neutral or positive sodium balance) in most species of mammals is greater in females than males. Exogenous estradiol mediated the higher sodium intake in ovariectomized females during baseline and stress treatments [11]. In males, the SNS partially regulates sodium. Treatment with adrenergic blockers, NE depletors and blockers of central SNS outflow all reduced sodium consumption in SHR and WKY rats. Salt intake is also regulated by non-sex hormones such as ACTH and the renin–angiotensin system. However, sex-steroids influence production of these hormones and, therefore, potential exists for modulating behaviors through actions of steroids at organs other than brain.

For example, androgens regulate renin mRNA in both the adrenal glands and brain of mice. Renal and hepatic angiotensinogen mRNA levels in SHR are dependent on androgen in both sexes. Plasma testosterone and Ang II were measured simultaneously in male SHR and found to have significant positive correlation at 15 weeks of age. The combination of removing estrogen early in development and supplementing the ovariectomized females with testosterone can reveal strain differences in blood pressure response and in the renin–angiotensin system. In comparison of ovariectomized females supplemented with testosterone, SHR/y females, with the same chromosome content as WKY females, have significantly higher blood pressure and lower levels of renin and angiotensinogen mRNA in the kidney [13].

Sex influences expression of renal adrenergic receptors which will contribute to establishing a hypertensive phenotype. Renal fractional release of NE is increased due to the SHR Y chromosome [10]. Although NE in renal perfusate and renal tissue was significantly elevated by testosterone, the SHR Y chromosome produced a significant increase in renal NE content and release compared to the WKY Y chromosome. In kidneys expressing the androgen receptor mutant (Tfm) or in the presence of an androgen receptor blocker, release of renal NE was reduced [7]. Since release of NE is an index of

sympathetic neural activity, these observations have direct implication to the regulation of renal blood flow and electrolyte balance (see also Chapter 12).

4. Summary

In this chapter, discussion focused on how sex can be analyzed at several different levels: genetic chromosomal, gonadal, hormonal or phenotypic. The genes involved in sex-based differences are not all on the X or Y chromosomes.

Therefore, sex-based physiological differences can then be the result of a complex array of factors – sex chromosome differences, specific gene differences, expression variation, interaction of genes and gene–environment interaction. Sex hormones can influence many non-reproductive phenotypes such as the cardiovascular system, other endocrine organs, renal function and behavior.

The Y chromosome has a locus that elevates blood pressure in experimental animals and humans. Genetic modification of experimental animal models can be used to answer unique sex-based physiological questions such as the Tfm rat to study the role of the androgen receptor and Y chromosome consomic rat strains to explore the Y chromosome and physiological phenotypes. Abnormalities in sex chromosome in experimental animals have been used to better understand clinical disorders and basic biology of sex-based differences in humans.

5. Future directions

Much remains to be learned about genes located on sex chromosomes. In the future, it will be important to determine

- the gene on the Y chromosome that is responsible for increasing blood pressure;
- the mechanism by which the gene on the Y chromosome influences blood pressure; and
- the androgen dependent mechanisms of the Y chromosome locus.

This information from experimental studies can be applied to investigation of sex-related hypertension in humans.

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Endocrine control of sexual differentiation: effects of the maternal–fetal environment and endocrine disrupting chemicals

Susan C. Nagel and Frederick S. vom Saal

1. Introduction

The focus of this chapter is on the effects of gonadal steroids on differentiation of the indifferent embryo into male or female after gonadal determination. While no discussion of sexual differentiation would be complete without the role of androgens (testosterone, dihydrotestosterone, etc.), this chapter will focus particularly on the role of estrogens that have been less well described. In long gestation mammals, such as humans, most of these processes occur during fetal life. In short-gestation species, such as rats and mice, these processes begin during the last third of gestation and continue for one or two weeks in postnatal life. However, events that comprise the process of sexual differentiation are controlled by similar hormonal signals in all vertebrates.

Vertebrates respond to hormonal signals throughout their life, both endocrine messengers released into the bloodstream and paracrine messengers affecting neighboring cells. Developmentally important hormonal signals are constantly being altered by the environment. The "environment" here refers to the micro-environment of cells, the entire internal environment of a fetus, the intrauterine environment (which is particularly important when there are multiple fetuses), the internal environment (physiological state) of the mother, the external environment inhabited by the mother, and the chemicals (natural or man-made) that enter the mothers body via food, water or air.

Vertebrate development is referred to as being "epigenetic", since the genes that regulate development are regulated by the micro-environment of differentiating cells. For example, it is the position of an epithelial cell relative to the underlying mesenchyme within organs in the male reproductive tract that determines the genes that are permanently turned on or off (rendered active or inactive), based on the specific hormonal (paracrine) signals that pass from the underlying mesenchyme to the adjacent epithelial cells during "critical periods" in the differentiation process [9,35]. The hormonal regulators of developmental processes in vertebrates evolved to be highly responsive to environmental disturbances. As a result, the expression of developmentally important genes can be

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altered by the environment. Hence, expression of genes during development is not identical in any two individuals. This is true even in genetically identical individuals carried within the same uterus at the same time, because the fetal micro-environment will differ; one example, covered in more detail below, is the intrauterine position (IUP) phenomenon in litter-bearing mammals [54,69].

The processes involved in this turning on and off of genes is referred to as "epigenetic imprinting"; this term was initially applied only to silencing of genes on the chromosomes provided by the mother versus the father. Potential mechanisms for imprinting are DNA methylation, which refers to the modification of a specific base (cytosine) within a gene by addition of a methyl group, and acetylation of histone proteins associated with nucleosomes. For example, addition of a methyl group to a cytosine at a critical position within the promoter region of a gene can permanently "silence" the gene [51]. Thus, identical twins or clones may possess genes with the identical DNA sequences, but show differences in the expression of these genes and thus differences in morphology, physiology and behavior. During gamete formation, prior to fertilization, these epigenetic modifications of genes had been thought to be globally deleted, such that the embryo begins life without the "programmed" gene expression pattern of the parents. However, recent evidence suggests that epigenetic programming that occurs during fetal development in response to maternal exposure to estrogenic drugs can be transmitted to offspring by both males and females exposed as fetuses to the drug [45].

While it has been known that the physiological state of a woman during pregnancy (age, weight, presence of pathogens, or other disorders, etc.) impacts fetal development, it has only been recently (as the processes of epigenetic modification of genes have been revealed) that a less obvious factor has been recognized to influence the development of a fetus and newborn who is nursed. There is now consensus that maternal physiological processes that directly impact all aspects of development of her fetus and newborn during nursing, are influenced by the mother's entire life history, not only her immediate condition. A mother's physiology reflects her unique epigenetic program in addition to reflecting the presence or absence of specific alleles; importantly, the epigenetic program cannot be discerned by just examining the base sequence of genes. For example, the new field of medicine termed: "Fetal Origins of Adult Disease" focuses on the long-term impact of developmental events, such as nutritional imbalances due to maternal nutrition, that can predispose her offspring to a range of pathological conditions throughout life. The affected offspring can, in turn, produce offspring with disrupted development.

The second field of science that has this same general issue as its focus involves the study of "endocriine disrupting chemicals". This refers to the many chemicals used in plastics, pesticides, building materials, cosmetics and other common household products that either mimic or antagonize the action of endogenous hormones or in other ways disrupt the endogenous hormonal signals that regulate the differentiation and subsequent functioning of organs. Over the past decade, much has been learned about the impact on fetal development and subsequent effects on organ function and behavior due to exposure to endocrine disrupting chemicals [7,47]. In addition, with the realization that many chemicals used in household products have estrogenic or anti-estrogenic activity and that exposure to these chemicals can adversely affect fetal development, there has been a surge of interest in the normal role that estrogen plays in fetal development.

2. Estrogen action

Estrogens are a group of compounds that have a common mechanism of action: compounds that bind to and activate the estrogen receptor (ER) are considered to be estrogens. Although detailed discussion of the mechanisms of steroid receptors are presented in Chapters 3 and 4 for the purpose of this chapter, some specific information is required.

There are two broad categories of estrogens. Steroidal estrogens are endogenously produced hormones often referred to as the "female sex hormones". The most important steroidal estrogen in terms of potency is estradiol. The potency of a hormone is described by its affinity for its receptor and measured by the dissociation constant or K_d. The K_d is a mathematical constant and describes the concentration of estradiol required to occupy 50% of available ERs (for estradiol the $K_d = 0.05$ nM); the lower the K_d the higher the affinity, since less hormone is required to bind 50% of the available receptors. By comparison, the K_d of estrone, another steroidal estrogen, is 2 nM, 40-times less than estradiol. The other category of estrogens, xenoestrogens, are produced outside of the body or exogenously and most, but not all, have a lower affinity for ER than estradiol. Some xenoestrogens are naturally occurring, such as phytoestrogens produced by plants, but most are synthetic compounds produced by man. Some xenoestrogens were designed to be estrogenic, such as ethinyl estradiol, a component in oral contraceptives, but many other xenoestrogens were designed for other purposes and only coincidentally have estrogenic activity due to having a structure that allows them to interact with the hormone-binding domain of the ER.

Estrogens function within target tissues by binding to specific ERs, alpha and beta , located within target cell nuclei. Upon binding ligand, ER undergoes an activating conformational change that facilitates the interaction of ER with regulatory regions of target genes, known as estrogen response elements (EREs). However, there are other potentially important ER-mediated actions that may function through the interaction of the estrogen–ER complex with other signaling pathways, e.g. interactions with AP-1 or membrane-bound ER. Importantly, different ER ligands induce different conformational changes in the receptor, which is believed to regulate the interaction of ER with specific comodulator proteins [48]. Thus the ability of ligand bound ER to activate or suppress target gene transcription is also determined by the proteins that impact the ER-signaling pathway downstream of DNA binding [37] (see also Chapter 3).

2.1. Biosynthesis

Steroidal estrogens are small (MW $\approx$ 270), lipid soluble hormones synthesized by the gonads either from cholesterol taken up from blood or directly from acetate. In adult men and women, the bulk of circulating estrogen is derived from the testes and ovaries; however, there is also the conversion of androgen to estrogen by the enzyme aromatase in a number of other tissues, such as the brain, fat, muscle, liver, and accessory reproductive organs [33]. When the ovaries are quiescent (postnatally before menarche and after menopause) the bulk of the circulating estrogen is derived from aromatization of adrenal androgens, e.g. in adipose or muscle tissue. In the human female fetus, the ovaries secrete

estrogens, while the ovaries in rodents do not secrete estrogen during sexual differentiation. Estradiol is also synthesized in human placental trophoblast cells by the aromatization of androgens, which are produced primarily in the maternal and fetal adrenals [68]. In the fetal testis, aromatase (and thus estrogen synthesis) has been localized in Leydig and Sertoli cells [25].

2.2. *Serum binding proteins regulate estrogen uptake into cells*

Estradiol circulates in women between 0.3 nM during the follicular phase of the menstrual cycle and 0.7 nM during the luteal phase and around 0.08 nM in men. There often is considerable confusion about which fraction of circulating steroid hormone in blood is biologically and clinically relevant. Steroids circulate in blood in three forms: (1) as conjugates (for example, estradiol bound covalently to sulfate); (2) reversibly bound by ionic bonds to both low affinity (albumin) and high affinity (sex hormone binding globulin, SHBG) plasma binding proteins; and (3) free (unbound and unconjugated). It is this small free fraction in blood that is the bioactive fraction, i.e. the fraction that is able to pass from serum, dissolve in and pass through cell membranes, and bind to intracellular receptors where the hormone receptor complex functions as a transcription factor in association with other coregulator proteins and the general transcription machinery. There are no transport mechanisms to actively move steroids into cells, although plasma binding proteins can be actively transported into some cells [74].

Since the rate of dissociation of steroids from albumin is more rapid than for binding proteins with higher affinity for specific hormones, such as SHBG, there is some controversy concerning the degree to which a steroid bound to albumin can dissociate and become part of the free pool of steroid, particularly if capillary transit time is slow. However, strong arguments have been made that for most tissues, capillary transit time is too fast for the albumin-bound steroids to be a significant factor [13]. The concept that the free fraction of estradiol is the bioactive fraction is referred to as the free hormone hypothesis, and is illustrated in Fig. 1.

Steroidal estrogens have a low solubility in blood, and their association with serum proteins reduces hepatic metabolism and clearance and tightly regulates the bioactive fraction. The concentrations of serum steroid binding proteins vary depending on life stage, sex and species. In adult women and men, approximately 1.8 and 2.3% of the total estradiol is free, respectively [11]. In adult (10 weeks old) rats, approximately 2.7 and 2.8% of the total estradiol is free in diestrus females and males, respectively [39]. The glycoproteins, alphafetoprotein (AFP) in mice and rats and SHBG in humans are high-affinity estrogen binding proteins. Whereas rodent (but not human) AFP only binds estradiol, SHBG binds both testosterone and estradiol with high affinity ($K_d \approx 0.63$ and 1.5 nM, respectively) and circulates at approximately 28 nM in men and 37 nM in women [11]. SHBG thus has a higher affinity for testosterone than for estradiol. An interesting consequence of this is that as men age and SHBG levels increase, the proportion of total testosterone that is free decreases while the proportion of total estradiol that is free increases (reviewed in Ref. [75]). Although albumin binds estradiol with low affinity ($K_d \approx 17$ μM), it binds with high capacity (circulating around 560 μM in blood), and thus

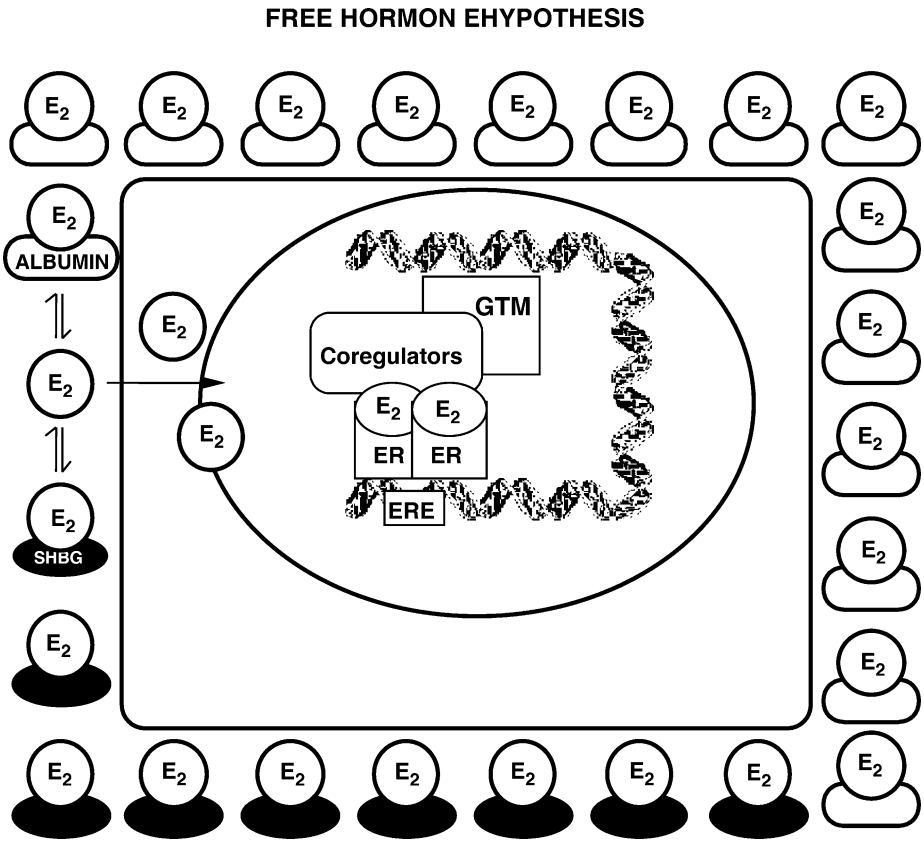

Fig. 1. In human circulation, the majority of estradiol is bound to the serum proteins sex hormone binding globulin (SHBG) and albumin. According to the free hormone hypothesis, only the free or unbound fraction of estradiol is available to diffuse into cells to occupy intracellular estrogen receptors (ER). Abbrev.: Estrogen response element (ERE), Estradiol (E2), general transcription machinery (GTM).

binds more estradiol than SHBG. In women, of the total circulating estradiol, approximately 37% is associated with SHBG, and approximately 61% is associated with albumin. In men, approximately 20% is associated with SHBG and 78% with albumin [11].

In pregnancy, AFP is produced in the liver of fetal rats, and the concentration of AFP is higher in fetuses than in the pregnant mother. The concentration of AFP reaches approximately 30 μM during the initial period of sexual differentiation (on gestation day 19) in fetal rats, and only 0.35% of total circulating estradiol is free. In contrast, after postnatal day 30 in rats, AFP is <0.7 nM in serum, and about 3% of total estradiol is free. What is clear is that while the total circulating estradiol in rat fetuses is approximately 10-fold higher than during diestrus in adult females, the reduction in the free fraction of estradiol by AFP maintains the bioavailable free concentration of estradiol at essentially

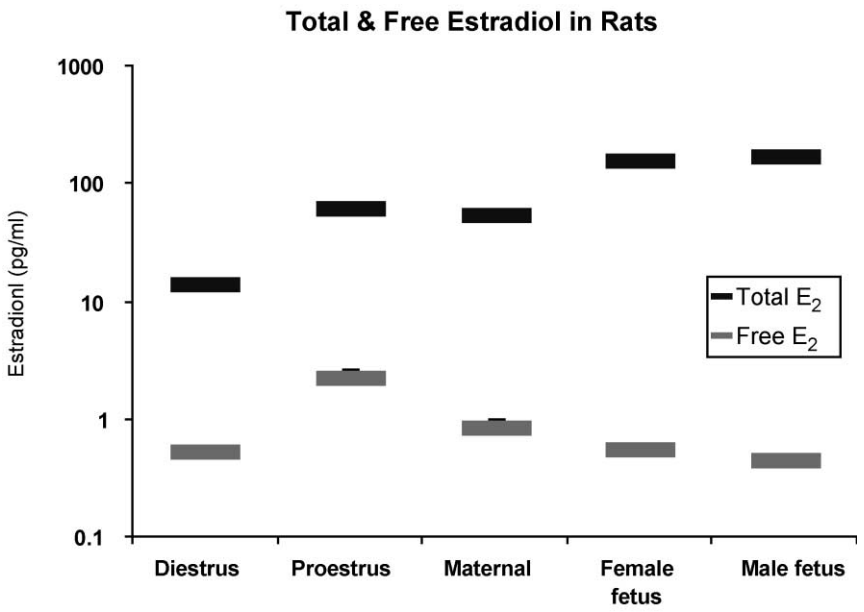

Fig. 2. Total and free estradiol in rats. Total estradiol determined by radioimmunoassay and free (unbound) estradiol determined by ultrafiltration dialysis were measured in adult rats and in fetal day 19 rats (vom Saal, unpublished data), see Ref. [40] for more details. While total estradiol changes depending on life-stage, free estradiol is kept within a narrow physiological range.

constant levels (Fig. 2). Thus, fetal rats are not exposed to supraphysiological levels of estradiol.

In contrast to fetal rats that produce AFP, human fetuses do not produce a high affinity estrogen binding protein. SHBG (the high affinity estrogen binding protein in humans) is synthesized in the maternal liver, and the free fraction of estradiol is lower in the mother than the fetus in humans. During the third trimester of human pregnancy when total maternal estradiol levels are $\approx$55 nM, there is a 10-fold increase in the SHBG concentration (from 37 to 400 nM), and less than 0.5% of circulating estradiol is free [11]. However, in umbilical cord blood from term fetuses, SHBG circulates at approximately 44 nM, near nonpregnant adult levels, and the reported free fraction of estradiol is 3.5%, while total estradiol is approximately 25 nM [59]. At parturition, it thus appears that human fetuses are exposed to relatively high levels of estradiol [78]. How the free concentration of estrogen in human fetuses is regulated by components of blood, particularly during the period of sexual differentiation during the first and second trimesters, is, as yet, not entirely clear.

Another critical factor in regulating bioavailability of steroids is conjugation, a covalent modification by specific enzymes. During pregnancy in humans and other primates, estrogens rise in maternal and fetal circulation primarily due to placental aromatization of maternal and fetal adrenal androgens. The concentration of estriol and

estradiol in the umbilical vein is 7-fold and 5-fold higher, respectively, than in the umbilical artery, indicating placental secretion into the umbilical vein. However, steroid sulfates are higher in the umbilical artery than in the umbilical vein, indicating fetal conjugation of placental estrogens [32]. For example, during human pregnancy both the maternal and fetal adrenals secrete high levels of a weak androgen, dehydroepiandrosterone (DHEA). However, over 90% of DHEA that is secreted by the fetal adrenal is dehydroepiandrosterone-sulfate (DHEA-S) [68]. Conjugation to a sulfate renders the compound hydrophilic, thus increasing solubility in blood but also decreasing metabolic clearance [16]. The conjugate has to be enzymatically removed in order for the steroid to have biological activity in a target cell.

In contrast to humans, in rats estrogen in maternal and fetal plasma does not involve an interaction between the maternal and fetal adrenals and the placenta, since the rat placenta does not contain the enzyme aromatase and thus cannot synthesize estrogen. The regulation of the free fraction of estrogen in human fetuses by the interaction of sulfating and desulfating enzymes in the placenta and maternal and fetal tissues, as well as by plasma binding proteins, both SHBG and albumin, in humans is thus different from rats and mice.

2.3. Estrogen action in adults

Steroidal estrogens circulate and act in both males and females. However, estrogens have been best characterized with regard to regulating a variety of functions in females, such as stimulation of growth and activity of the mammary gland and endometrium, preparation of the female reproductive tract for sperm transport, and stimulation and maintenance of female secondary sexual characteristics. The actions typically seen in the adult are referred to as activational effects and are reversible, i.e. once the estrogen is removed the response diminishes. However, there also can be irreversible effects of estrogens after the initial period of sexual differentiation in early development. At puberty in humans in both males and females, epiphyseal fusion is estrogen dependent and irreversible [58]. In mice and rats during pregnancy, mammary gland differentiation requires estrogen, progesterone, and prolactin, and some changes persist in the post partum mammary gland relative to the pre-pregnant state. However, most other described actions for adults are reversible [73].

Only recently has the importance of estrogen in the functioning of reproductive organs in adult males been identified. For example, the resorption of the fluid bathing spermatocytes as they exit the testis is critical for normal epididymal function; this is where the final phase of sperm maturation and the acquisition of motility occurs. If fluid resorption is impaired, which occurs in ER alpha knockout (ERKO) mice, the result is severe back-pressure on the seminiferous tubules, which causes testicular swelling and necrosis, and culminates in the complete loss of testicular function in adulthood [18].

2.4. Estrogen action in fetuses

In the fetus, estrogens have developmental and organizational effects in both males and females; these effects are typically irreversible and permanent. A considerable amount of

work has demonstrated that the perinatal mouse between gestation day 16 and postnatal day 7 (a period that corresponds to prenatal sexual differentiation in the human fetus between approximately gestation week 7 and 20) is sensitive to the permanent organizational effects of both endogenous and exogenous estrogen exposure, prompting the term "fragile fetus" to describe this phenomenon. Evidence from studies of the IUP phenomenon and administration of exogenous estrogens to developing fetuses that shows the rodent fetus to be very sensitive to small changes in endogenous and exogenous estrogens [2] is summarized in subsequent sections.

The human fetus is capable of responding to estrogens, as ER expression has been detected as early as the 10th week of gestation [63]. By mid-gestation, expression of ERs has been described in a wide variety of tissues: ER alpha in the uterus, ovary, testis, skin and gut, and ER beta in the fetal ovary, testis, adrenal and spleen [4]. The human fetus also appears to be capable of differential responses to the level of estrogen during development. For example, conditions associated with high estrogen levels, as seen in neonatal jaundice, severe prematurity and dizygotic twins, have been correlated with increased risk of breast and testicular cancer in adulthood [12]. Likewise, conditions associated with low levels of estrogen during pregnancy, such as pregnancy toxemia, are correlated with decreased risk of breast cancer in adulthood [12]. While the role of estrogen and its interaction with other steroids during human fetal life remains to be fully described, there is considerable information about steroid hormone action (particularly testosterone and estradiol) in rodent development, which is discussed below.

3. Effects of sex steroids on sexual differentiation

3.1. Testosterone and its metabolites mediate masculinization of male fetuses

Testosterone is the primary androgen secreted by the testes during fetal, neonatal and adult life in mammals. Beginning at about the sixth week of gestation in humans and gestation day 14–15 in mice and rats, the fetal testes begin secreting high levels of testosterone. In humans, by the end of the sixth month of pregnancy testosterone levels in male fetuses fall to levels similar to those in females, but during the first 6 months after birth, there is again a marked elevation in testosterone in males, which is not seen in females [34]. Testosterone levels increase again in males with puberty. Testosterone levels are also elevated in females during fetal life in humans, rodents and other mammals [52, 69]; there are effects of testosterone on female development (covered below), but they are less obvious than in males.

Pituitary leutenizing hormone (LH) is not required for testicular testosterone secretion in human male fetuses, and as in many other mammals, anencephalic male fetuses undergo normal sexual differentiation. Secretion of the placental glycoprotein, human chorionic gonadotropin (HCG), which has activity similar to LH, precedes secretion of testosterone by the fetal testes and subsequent masculinization. The accessory reproductive organs in males that differentiate (are masculinized) in response to testosterone derive from two different embryonic tissues, the Wolffian (mesonephric) ducts of mesodermal origin and the urogenital sinus (UGS) of endodermal origin (Fig. 3). The secretion of Mullerian inhibiting hormone by fetal Sertoli cells induces the regression of the Mullerian ducts,

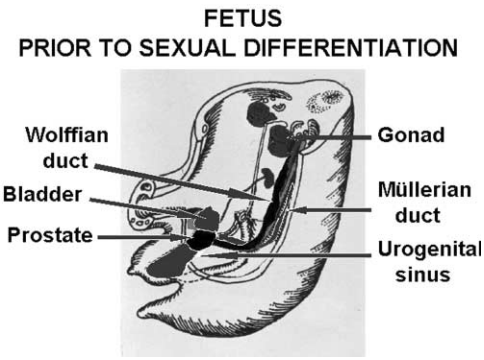

Fig. 3. Schematic diagram of the undifferentiated urogenital system at the transition from embryonic to fetal life. In humans, this would be at about 6 weeks after conception. Shown here are the differentiating gonads, the undifferentiated Mullerian ducts, which develop in females into the fallopian tubes, uterus and upper portion of the vagina, the Wolffian ducts, which develop in males into the epididymis, vas deferens and seminal vesicles, and the urogenital sinus (UGS). The prostate differentiates from the portion of the UGS just below the bladder.

which, along with the Wolffian ducts, are initially present in both males and females. Both males and females thus have the potential (depending on the levels of specific hormones, enzymes and hormone receptors) to form the reproductive organs characteristic of the opposite genetic sex.

Testosterone acts as both a primary hormone and as a prohormone, e.g. it is converted by intracellular enzymes to a more potent hormone. In tissues that contain 5α-reductase (cells in the brain, UGS, liver and many other tissues), testosterone is metabolized to 5α-dihydrotestosterone (5α-DHT), and in tissues that contain aromatase (brain, liver and many other tissues), testosterone is converted to estradiol. Both 5α-reductase and aromatase thus act to produce hormones that are more potent than testosterone within selected target tissues (these enzymes thus amplify the effects of the prohormone).

Expression of 5α-reductase in the UGS mesenchyme is required during the initial phase of differentiation, while this is not the case in the Wolffian ducts. In the Wolffian ducts (epididymis, vas deferens and seminal vesicles) the initial phase of differentiation is mediated by testosterone binding to androgen receptors, but after birth, the postnatal phase of ductal branching, and subsequent adult organ function requires expression of 5α-reductase and 5α-DHT binding to androgen receptors. Thus, inhibition of 5α-reductase or a mutation in the 5α-reductase gene does not interfere with the normal development of the Wolffian ducts in humans and other mammals that have been studied, whereas marked disruption of the differentiation of the external genitals and prostate occur, demonstrating the importance of the formation of 5α-DHT in these tissues during fetal life. One possible explanation for the lack of requirement for the formation of 5α-DHT in Wolffian duct differentiation is that there are higher levels of testosterone around the duct due to diffusion from the ipsilateral testis (this is also true for Mullerian inhibiting hormone levels). The absence of hormone secretion from one testis dramatically impacts differentiation of the ipsilateral (but not contralateral) Wolffian and Mullerian duct, while differentiation of the UGS still occurs, since the remaining testis compensates and

produces more testosterone into the systemic circulation (but not enough to influence the contralateral Wolffian duct). Thus, while female fetuses have substantial levels of circulating testosterone, the absence of locally high levels of testosterone, which in males would be secreted by each testis, results in the absence of development of the Wolffian ducts; females also have a lower level of 5α-reductase expressed in their embryonic Wolffian ducts relative to males [74].

3.2. Influence of gonadal steroids in female sexual differentiation

Development of fetuses with XX genotype into females is generally assumed to not require gonadal hormones to induce tissues to differentiate in a feminine direction. However, normal differentiation of oocytes and follicles may require high levels of gonadotropins (of pituitary and/or placental origin) as well as estradiol [73]. In mice carrying mutations in both ER genes ER alpha and ER beta, the ovaries in genetic females go through a redifferentiation process after puberty and contain testicular tissue [8]. This is consistent with the hypothesis that while estrogen does not appear to regulate the expression of genes involved in ovarian differentiation, estrogen may play an inhibitory role with regard to suppression of genes involved in testicular differentiation [27].

Differentiation of the female reproductive tract occurs prenatally in humans and both prenatally and postnatally in rodents. The cranial portion of the Mullerian ducts becomes the fallopian tubes, and the distal end of the fallopian tubes forms the ciliated fimbria. The caudal end of the Mullerian duct forms the uterus and upper part of the vagina. In the undifferentiated embryo, contact of the Mullerian duct with UGS induces the formation of the uterovaginal plate. The central cells of the plate break down to form the lumen of the upper vagina. The UGS differentiates into the lower one-half to two-thirds of the vagina. Wolffian ducts do not persist due to testosterone levels in females being too low in the immediate vicinity of Wolffian ducts to provide the required stimulation; there is also a lower level of 5α-reductase activity in the Wolffian duct in females resulting in a reduced potential to respond to testosterone, due to the difference in the ratio of testosterone to estradiol in males and females [73]. The labioscrotal swellings differentiate into the labia majora. The urogenital folds form into the labia minora that merge to form a hood over the clitoris, which develops from the genital tubercle. In males, the two genital swellings fuse with the genital tubercle and form the scrotum and penis under the action of 5α-DHT within these target cells.

During fetal life, female fetuses have high circulating levels of testosterone relative to adulthood in many species, such as humans, monkeys and mice. However, due to the absence of the much higher levels of testosterone (both systemic and in the immediate vicinity of the testes) seen in males, the active processes of masculinization and defeminization do not occur. There is obviously more to sexual differentiation than just sex differences in serum testosterone, since in rats, a small subset of female fetuses appear to have levels of circulating testosterone within the range of those detected in males, yet these females are not as masculinized as one would predict just based on examination of the testosterone. In rats and mice, unlike primates, testosterone is a major secreted steroid

by the placenta, since the rodent placenta converts progesterone to androgen but lacks aromatase and thus the capacity to convert androgen to estrogen. This emphasizes that it is the dose of a hormone, drug or environmental chemical, available to bind to hormone receptors and recruit receptor coregulators, and thus regulate transcription of specific genes that determines an animal's final sexual phenotype (i.e. all traits that distinguish males and females).

Hox genes occur in clusters in all multicellular animals and are expressed in defined anterior–posterior patterns. These highly conserved genes play a critical role in development of the major axes of the body. Sequential expression of a set of Hox genes is involved in differentiation of the reproductive tract in females. In the distal portion of the Mullerian duct that forms the oviduct, Hoxa-9 is expressed, in the uterine and vaginal plate region Hoxa-10 is expressed, and in the portion of the duct proximal to the UGS, Hoxa-13 is expressed. Developmental exposure to the estrogenic drug diethylstilbestrol (DES) leads to disruption of the normal sequence of expression of these developmentally important genes [3,23]. The finding that these genes are responsive to changes in estrogen suggests that estrogens play a normal role in the expression of these genes, and thus Mullerian duct differentiation. The WNT-7a gene is expressed in the Mullerian duct epithelium and regulates expression of the receptor for Mullerian inhibiting hormone in the adjacent Mullerian duct mesenchyme [50], where estrogen and androgen antagonize and facilitate, respectively, the action of Mullerian inhibiting hormone [10]. This provides an interesting example of how cells communicate with each other to control differentiation and how the sex hormones interact with other developmentally important signals, thus accounting for differences between males and females in the development of embryonic tissues.

Variation in levels of testosterone and estradiol in female mouse and rat fetuses (due to being positioned in utero between male or between female fetuses) leads to marked differences in a wide range of traits throughout postnatal life. These findings provide evidence that gonadal steroids have effects on the normal course of sexual differentiation in females. If variation in these reproductive traits is related to gonadal steroid levels in female rodents, then it is likely that variation in these hormones (due to a variety of factors) also results in differences in phenotype in other female mammals, such as humans, which show marked individual variation in gonadal steroid levels during the fetal period of sexual differentiation. For example, there are differences in maternal/fetal gonadal steroid levels related to maternal age, parity, number of fetuses, race and diet [62].

This is a markedly different view of the normal process of sexual differentiation in female mammals than the view that endogenous gonadal steroids do not play an active role in the differentiation of the female phenotype. An important aspect of these findings is that variation in endogenous steroid concentrations within a "normal" range leads to marked variation in reproductive traits in female rodents without rendering any of the animals infertile. The implications of these findings is that events that impact fetal hormone levels, such as maternal exposure to environmental chemicals that alter the levels of estrogenic or androgenic activity during sexual differentiation, could alter the course of differentiation of the reproductive system in females as well as males. Evidence for these concepts is presented in Section 4.

4. Environmental factors influence sexual differentiation in males and females

4.1. Serum testosterone and estradiol levels differ due to intrauterine position in male and female fetuses

In litter-bearing mammals such as mice, rats, gerbils, pigs and even humans carrying more than one fetus, there is either direct or indirect evidence that hormones produced by fetuses of one sex pass into adjacent fetuses. In litter-bearing species, animals have been classified based on the sex of adjacent fetuses: 2M fetus is between two males, 1MF fetus is between a male and a female fetus, and 2F fetus is between two female fetuses (Fig. 4). During sexual differentiation the testes in male fetuses produce high (adult) levels of testosterone, with the result that fetuses located next to a male receive supplemental testosterone relative to fetuses located next to a female (2M > 1MF > 2F). In mice and gerbils, female fetuses have higher serum estradiol levels than males (this is not true in humans and other primates that have been examined), and a mouse or gerbil fetus located next to a female receives a supplement of estradiol (2F > 1MF > 2M). The transport of steroids between fetuses is by diffusion through the amniotic fluid and fetal membranes surrounding each fetus [71].

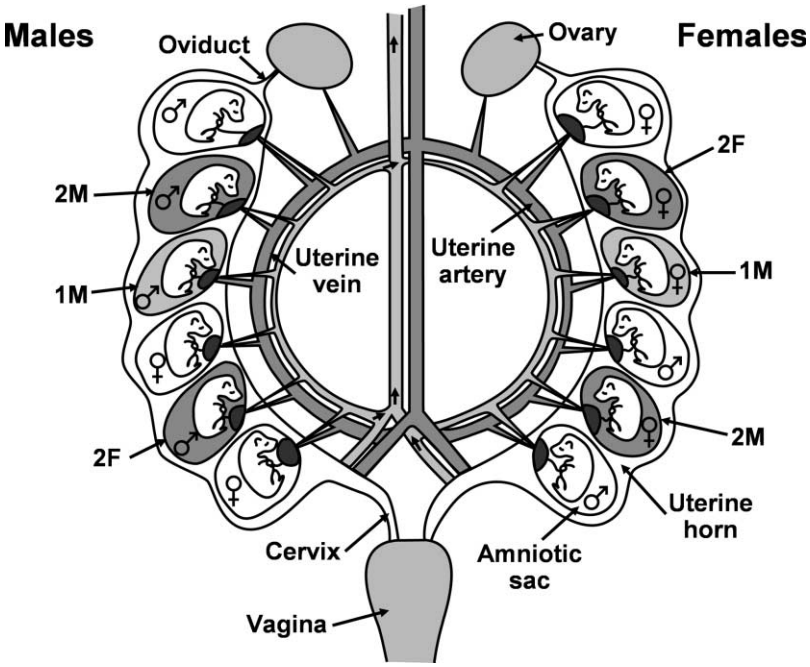

Fig. 4. Schematic diagram of the pregnant mouse or rat uterus showing male and female fetuses in each uterine horn. The intrauterine placement of males and females is random, and the probability of a male or a female fetus having two adjacent male fetuses (referred to as a 2M), an adjacent male and a female (1M) or 2 adjacent female fetuses (2F) occurs at a frequency of 1/6, 3/6 and1/6, respectively, for both males and females for litters of 12 pups, which is typical of many laboratory stocks of mice and rats [70].

4.2. Postnatal consequences of intrauterine position in females

With regard to sexual differentiation in females and the potential for effects of IUP on development, it is well documented from studies in rodents, dogs, monkeys and humans that during fetal life androgen influences postnatal childhood play behavior (part of what is referred to as one's gender role) in genetic females. A fascinating observation is that the effects of elevated androgen on play behavior in females are virtually identical in different species that have been studied, such as rats, monkeys and humans. For example, human females with elevated fetal androgen due to an adrenal enzyme defect that results in abnormal adrenal androgen secretion show as children more "rough and tumble" type play behavior, which is characteristic of males [1]. These studies have provided convincing evidence that during fetal life, androgen (mainly testosterone) can exert a permanent (organizational) effect on an individual's behavior expressed at various times after birth.

A female's IUP relative to other male and female fetuses influences many traits in litter-bearing species. These findings are consistent with many findings showing permanent effects on female fetuses of manipulations of sex steroid levels (via maternal exposure to drugs or chemicals with sex hormone activity). For example, developmental exposure to elevated testosterone in 2M female mice is associated with an enlarged anogenital separation (this tissue becomes part of the scrotum in males), delayed puberty, and increased aggressiveness (increased "male-like" infant play and subsequently in adulthood a higher dominance status of the animal) [69]. In contrast, 2F female mice show early puberty, consistent with the effects on female offspring of administering pregnant mice low doses of estrogenic drugs or chemicals [20]. 2F females also have very regular estrous cycles, while 2M females show irregular cycles (greater variability) and, overall, significantly longer estrous cycles. 2F females show a higher level of sexual behavior (receptivity or lordoses) relative to 2M females when paired with a male. Finally, during aging, 2M females cease producing litters at an earlier age than 2F females when allowed to repeatedly produce litters throughout life [54,71].

One of the most interesting findings in comparisons of 2F and 2M female mice and gerbils is that the prior IUP of a female, which is a random developmental event, influences the sex ratio of the litters she produces as an adult. Specifically, in both mice and gerbils, 2M females produce male biased litters ($>60\%$ males) while 2F females produce female biased litters ($>60\%$ females). There is thus an increased probability that 2M females will produce more 2M male and 2M female offspring (since there are more males available to be positioned next to) relative to 2F females [54]. The mechanism for this phenomenon is unknown, but there is also evidence that human exposure during early development to environmental chemicals, such as dioxin and pesticides, can alter the sex ratio of the next generation; based on reports of exposed populations in Italy and Minnesota [26].

4.3. Postnatal consequences of intrauterine position in males

As in females, IUP in males also clearly influences development. There is a correlation between variation in testosterone and morphological, physiological and behavioral traits in male mice. It is also well known that there is altered masculinization due to disruption of

androgen synthesis, metabolism or response potential. Specifically, in cases where there is a deficit in testosterone synthesis, a genetic mutation resulting in a reduced capacity to convert testosterone to the more potent androgen, 5α-dihydrotesterone, in target organs, or a mutation in the androgen receptor gene resulting in a reduced ability to bind androgen, there are clear consequences seen in genetic males (see Chapter 1 for effects on blood pressure). However, what has been quite surprising is that during fetal life, the difference between male mouse fetuses in serum estradiol (2F > 1MF > 2M males) is also related to marked differences in phenotype in males. Since 2M, 1MF and 2F males also differ in testosterone levels (2M > 1MF > 2F), only by independently manipulating one hormone without changing the other can causal relationships be established between a particular trait and the hormone that mediates development of the trait.

We initially found that a difference in total serum estradiol between 2F and 2M male mouse fetuses of 23 pg (23 trillionths of a gram) per mL of serum was related to a permanent increase in the size of the prostate (that develops from the UGS) and an increase in prostatic androgen receptors, but a decrease in the size of the seminal vesicles (that develops from the Wolffian duct and a decrease in seminal vesicle 5α-reductase activity (but no change in seminal vesicle androgen receptors) [46,71]. Fetal IUP also influences prostate size in rats, which was found using computer-assisted 3D reconstruction of the fetal reproductive organs [66,67,71]. We were particularly interested in the finding that the small difference in total serum estradiol between 2F and 2M male mice appeared to be sufficient to induce a cascade of developmental events, which, as indicated below, have profound consequences on prostate development and on subsequent prostate function throughout the remainder of life. Because only 0.2% of total serum estradiol is free during fetal life in mice, the difference in free (biologically active) estradiol in the blood of 2F and 2M male mouse fetuses is only 0.05 pg/mL. The fact that this difference is sufficient to produce a change in organ development demonstrates the exquisite sensitivity of fetal organs to compounds with estrogenic activity.

We manipulated serum estradiol levels during sexual differentiation in male mouse fetuses by implanting silastic capsules containing estradiol in pregnant female mice. There was an increase in free estradiol in the 1MF male fetuses of 0.1 pg/mL (the increase was from 0.2 to 0.3 pg/mL associated with an increase in total serum estradiol from 94 to 1146 pg/mL). Importantly, this did not result in a difference in serum testosterone, so effects of differences in only estradiol could be studied (which is not possible when comparing 2M and 2F males). The very small increase in serum estradiol resulted in a permanent increase in androgen receptor protein (that lasts throughout life), and an increase in the number of prostate glands and hyperplasia of the glandular epithelium; these effects were detected at the end of the first day of prostate differentiation by computer-assisted 3D reconstruction [75].

Mechanisms of these effects were examined in cultured prostate mesenchyme cells as these are the “master” tissue that induces differentiation of the adjacent epithelium to form the prostate glands. Very small increases in estradiol (0.1 pg/mL and above) caused a significant increase in androgen receptor mRNA in primary cultures of fetal prostate mesenchyme, demonstrating that androgen receptor gene transcription is initiated by estradiol. Taken together, these findings show that estrogen alone does not regulate the differentiation of the prostate, but instead, modulates the sensitivity of the tissues in

the prostate to the stimulating effect of androgen by modulating the number of androgen receptors.

Stimulatory effects of a very small increase in estradiol on the prostate had not previously been recognized, since all studies of the effects of estrogen on the developing reproductive system had involved very high doses, which have the opposite effect of low doses and result in the inhibition of prostate development (as well as interfering with development of the rest of the male reproductive system). It is well known in endocrinology that low, physiologically relevant doses of hormones can stimulate responses which are inhibited at high doses; these are referred to as inverted-U or biphasic dose-response relationships [75,76].

An interesting aspect of comparisons of 2M and 2F males is that elevated estradiol in 2F male fetuses is also associated with an increase in adult sexual behavior (frequency of mounting and intromitting). This is consistent with the well-documented finding that testosterone does not directly stimulate the differentiation of neurons in the brain that mediate male sexual behavior in rats and mice. Instead, these neurons contain the estrogen-synthesizing enzyme, aromatase, and for "masculinization" of these neurons to occur, testosterone must be converted to estradiol by aromatase; differentiation of these neurons is thus mediated by estradiol binding to ERs. This has been demonstrated by studies involving inhibition of aromatase and blocking of estrogen versus androgen receptors. In summary, the presumed "female" hormone, estradiol, plays a role in the normal development and subsequent functioning of male reproductive organs as well areas of the brain involved in masculine sexual behavior. This developmental effect of estrogen on masculine sexual behavior appears to be unique to rodents, whereas in primates, the conversion of androgen to estrogen in the brain does not appear to mediate the development of masculine sexual behavior.

There are many aspects of male sexual differentiation that are not mediated by or otherwise modulated by estrogen, unless pharmacological doses are administered, which can disrupt many aspects of development [62]. The development of neural systems that mediate aggression between male rodents, unlike male sexual behavior, is not regulated by the metabolism of testosterone to estradiol by aromatase, and instead, is mediated by the direct action of testosterone on neurons that contain androgen receptors. This is also true for the seminal vesicles, which differentiate from the Wolffian ducts during fetal life under the direct action of testosterone. Not surprising, therefore, is that in comparisons of 2M and 2F male mice, 2M males, which as fetuses have highest testosterone, have the largest seminal vesicles and are the most aggressive males within a litter. In contrast, the 2F males have enlarged prostates and are the most sexually active.

The studies of the effects of IUP have revealed that the process of masculinization differs depending on the endocrine response system present in a tissue during differentiation. It thus is not possible to characterize a male with low versus high plasma testosterone or low versus high plasma estradiol as being more or less masculinized; only different. However, this is only true if the variation in these steroids is within a "normal" physiological range. Concentrations of testosterone and estradiol that are either above or below this "normal" range can disrupt organ development and subsequent function.

The IUP phenomenon also was important in revealing the exquisite sensitivity of fetuses to very small perturbations in gonadal steroid concentrations. This has profound

implications for all species, including humans. There are many sources of variation in fetal hormone levels: maternal age, parity, race, diet and stress all are related in differences in maternal sex hormone levels. There is no barrier to movement of steroids between mother and fetus, although at one time there was a common misconception that there was a "placental barrier". The placenta does not act as a barrier to the movement of steroids or other small lipophilic molecules from the mother to the fetus [47].

4.4. Endocrine disrupting chemicals

Endocrine disrupting chemicals mimic or antagonize the action of hormones (such as estrogen, androgen and thyroid hormone) or otherwise disrupt endocrine function. These chemicals include pesticides, plastic monomers and additives, and industrial products and by-products [7]. There are a large number of low molecular weight, lipophilic endocrine disrupting environmental chemicals that can pass freely into the fetus, where the fetus has a very limited capacity to metabolize these foreign (xenobiotic) chemicals [2,7]. The primary focus of endocrine disruptor research is on development rather than in adulthood, since during development hormones have permanent "organizational" effects in tissues [7]. Of great concern is that the impact of hormonal disruption during fetal life may not be expressed in terms of clinical symptoms until adulthood. Long-latency outcomes of fetal exposure to drugs or environmental chemicals that are not easily detected at birth are extremely difficult to uncover in epidemiological studies [47]. The tragic story described below demonstrates how difficult it is to find a relationship between fetal exposure to chemicals and adverse outcomes later in life, even when the causal agent is a drug prescribed by physicians.

4.5. Adverse effects on offspring due to administration of diethylstilbestrol to pregnant women

The best example of a long-latency but profoundly adverse outcome due to fetal exposure to an endocrine disrupting chemical is the potent estrogenic drug DES. DES was prescribed to pregnant women thought to be at risk for spontaneous abortion, but DES was also marketed as being good for a developing fetus and was administered to women because in the 1940s–1960s (prior to the thalidamide disaster), the medical community was, in general, unaware of the potential harm to fetuses of maternal drug and chemical exposure [37]. In fact, there was a mistaken belief that the placenta provided a protective barrier for the fetus. There is now extensive evidence that DES and other low molecular weight lipophilic molecules readily cross the placenta into the fetus, where rates of clearance are very slow, resulting in prolonged exposure. Over about 25 years, millions of women were prescribed DES and, based on observing the babies at birth, DES was thought by physicians to be completely safe. In the early 1970s a physician saw eight girls in a short period of time who had a rare cancer (vaginal adenocarcinoma) [17]. This eventually led to the discovery that they were daughters of women who had taken DES, and that DES daughters had a myriad of abnormalities, including severely deformed uteri, immune disorders and behavioral problems [44]. There were also abnormalities in male offspring

as well, but relatively little research has been conducted on DES sons [62]. The vaginal cancer findings led to DES being banned in 1972. This iatrogenic event also led to a huge amount of research, particularly in mice, on the effects of developmental exposure to DES.

Of great importance is that the effects of DES were not detected by highly trained physicians and nurses at the time of birth. The absence of abnormalities that can be detected on external examination at birth often leads to the assumption that exposure to other drugs and endocrine disrupting chemicals are safe. An interesting current example is that a large number of women become pregnant every year in the USA and Europe while taking ethinyl estradiol (the estrogen used in oral contraceptives), primarily due to missed pills (three missed pills per month is common, and the unplanned and unexpected pregnancy rate is approximately 3% for women taking oral contraceptives). Even though abnormal development of the male reproductive system has been reported in male mice whose mothers were exposed to doses of ethinyl estradiol 250-times lower than doses in oral contraceptives, due to the absence of detectable adverse effects at birth, physicians still consider fetal exposure to ethinyl estradiol to be of no concern [65]. There have been no studies of the long-term health effects of exposure to oral contraceptives during the fetal period of sexual differentiation.

Consequences of developmental DES exposure are almost identical in mice and humans [44]. While once this might have seemed remarkable, the last decades have revealed an unexpected conservation at the molecular level of the genes and intercellular signaling systems that regulate development not only in all vertebrates, but in all multicellular animals [14]. The significance of these findings is that while there was evidence long before DES was banned that developmental exposure to DES produced abnormalities in mice, this was ignored as irrelevant to human health. An understanding that during the early period of organogenesis the mechanisms mediating development have changed little, particularly in mammals, should now result in a greater degree of caution regarding the potential for human health effects when animal studies reveal adverse effects. This is particularly important when the effects require a long latency to be observed, as was the case with DES, making detecting such effects in humans at birth highly unlikely. Importantly, few attempts have been made to link fetal exposure to drugs and chemicals to disorders that become apparent in adulthood [47].

There is now convincing evidence from animal studies, and an initial confirming study of DES daughters, that the offspring of both males and females exposed as fetuses to DES produce offspring with abnormalities, even though these DES grandchildren were not directly exposed to the drug [45]. The mechanisms that mediate this "epigenetic" inheritance are, as yet, unknown. Taken together, these findings demonstrate that events during a mother's fetal life, as well as her life history up to and through her own pregnancy, can directly impact the development of her fetus. This also appears to be true for males, suggesting permanent effects of chemical exposure during fetal life on spermatocytes [62].

4.6. Environmental endocrine disrupting chemicals alter fetal development

There is now evidence from invertebrates and every class of vertebrate that exposure during critical periods in development to endocrine disrupting chemicals can profoundly

alter the course of sexual development in both males and females [7,47]. Bisphenol A is an ER ligand and is an example of an endocrine disrupting chemical [44]. Bisphenol A is released from polycarbonate products, such as, baby bottles, cages and water bottles used for laboratory animals, and the resin lining of metal food cans, and interestingly bisphenol A was recently reported to make up 84% of the estrogenic activity in landfill leachate [5,6,21,22,28,29,41,61]. Importantly, bisphenol A has been found in human maternal and fetal serum at 0.2–10 μg/L [24,31,56]. Of particular concern, we and others, have shown that bisphenol A alters normal development at or below these current exposure levels. For example, developmental exposure to 2–100 μg/kg Bisphenol A results in accelerated puberty in females, permanent alterations in the mammary gland and the vagina, and increased adult prostate weight [15,19,20,22,30,36,43,49,55,59,60,64,72].

In some cases, the outcome of these experiments is unexpected. For example, bisphenol A is a very potent estrogenic chemical in terms of stimulating prostate growth in rat and mouse fetuses, with the potency of bisphenol A being approximately 100-fold lower than the very potent estrogenic drug DES. In contrast, in the mouse uterus, bisphenol A is approximately 100,000-fold less potent in terms of stimulating uterine growth relative to DES in adulthood [42,43]. A current hypothesis is that both the prostate and uterus have similar ERs that bind bisphenol A and DES. However, it is likely that the binding of bisphenol A to ERs does not result in the recruitment of coregulators required for the transcription of estrogen-regulated genes in the uterus, since bisphenol A does not interact with the ER in exactly the same manner as does estradiol or DES [53].

Because of variability in the endogenous levels of sex steroids, effects of exposure to endocrine disrupting chemicals are likely to vary based on the background endogenous levels of the hormone whose activity is being supplemented by chemicals with agonistic activity or opposed by chemicals with antagonistic activity. Recently, direct evidence for this was provided in a study where bisphenol A was administered to pregnant mice using a dose that resulted in blood levels of bioactive bisphenol A in mouse fetuses that were within the range of bisphenol A detected in human fetal cord blood [56,77]. Overall, female offspring exposed prenatally to a very low dose of bisphenol A (2 μg/kg/day fed to pregnant females) showed an increased rate of growth and early puberty. However, females with the lowest endogenous levels of estradiol (due to being positioned between male fetuses in utero, 2M females) showed no effect of bisphenol A, while females with the highest endogenous levels of estradiol (due to being positioned in utero between female fetuses, 2F females) were most dramatically affected [20]. Variation in the endogenous levels of testosterone and estradiol also are related to differences in the inhibitory effects of dioxin (a by-product of the production of chlorinated chemicals) on fetal prostate development in rats, with dioxin significantly reducing serum estradiol and prostate size in 2F males, while dioxin did not have this effect in 2M males [67].

5. Future directions

Understanding the molecular mechanisms that lead to tissue-specific and life-stage-specific effects of hormones and endocrine disrupting chemicals is a major focus of current research. Now that the genome of humans and a number of experimental animals,

including mice, has been sequenced, experiments are being conducted to identify the genetic pathways mediating responses, such as the increase in growth of the prostatic glandular epithelium in male fetuses or the stimulation of uterine growth in females in response to estrogen. In addition, the signaling molecules that control the transcription of various genes, particularly those that are critical for normal development, are being actively investigated, as are the components of the transcriptional machinery that control gene expression. Although the major emphasis of this chapter has been effects of hormone disruption on sexual development, how these compounds affect other physiological systems remains to be explored.

At one time, it was thought that all actions of steroids were via intracellular receptors that acted as transcription factors (responses are thus slow), while protein hormones were unable to enter cells and exerted rapid effects on cellular enzyme systems through receptors located in the extracellular region of the cell membrane. Evidence now supports the interaction of steroid hormones and endocrine disrupting chemicals with rapid-response signaling systems rather than "classical" genomic pathways that regulate gene transcription. These nongenomic response pathways need to be characterized during development. These pathways may differentially affect phenotype of adult males and females. Finally, the creation of animals with altered genomes (transgenics and knockouts) has provided important new information, and new model animals are being developed at a rapid rate [42]. The next decade should be an exciting period as new information about mechanisms mediating sexual development is reported.

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General mechanisms of steroid receptors and receptor coregulator action

David G. Monroe and Thomas C. Spelsberg

1. Introduction

Steroid hormones exert their pleiotropic biological effects by signaling through a group of intracellular molecules termed "steroid hormone receptors". A large superfamily of these receptors exists which mediate the biological effects of numerous hormones including estrogens, androgens, progesterone, glucocorticoids and mineralcorticoids. This chapter reviews the generalized structure and function of this important group of signaling molecules. Due to the focus of this textbook, the discussion will be limited to the receptors involved in estrogen and androgen signaling.

2. Biosynthesis and transportation of the sex steroids

The synthesis of the sex steroids (estrogens and androgens) occurs in response to numerous neuroendocrine signals. The central nervous system signals the hypothalamus to stimulate the pituitary, which releases hormones involved in targeting the reproductive organs to produce the sex steroids. The ovary produces and secretes estrogens (mainly 17β-estradiol and estrone) and the testes secrete androgen (testosterone). Numerous reviews are available describing these processes [11].

The main sex steroids are produced from cholesterol in a complex series of enzymatic reactions involving over 10 enzymatic activities. Fig. 1 represents a general outline of the biosynthesis of the sex steroids. Estrogens are produced from androstenedione by the aromatase enzyme, a cytochrome P450 enzyme primarily expressed in the ovaries. Estrone and 17β-estradiol are in reversible equilibrium. Testosterone is also derived from androstenedione through the actions of 17β-hydroxysteroid dehydrogenase, primarily expressed in the testis. The conversion of testosterone to the more active androgen metabolites occurs in target tissues. The 5α-reductase enzyme irreversibly converts testosterone to dihydroxytestosterone (DHT), whereas the aromatase enzyme irreversibly converts testosterone into 17β-estradiol.

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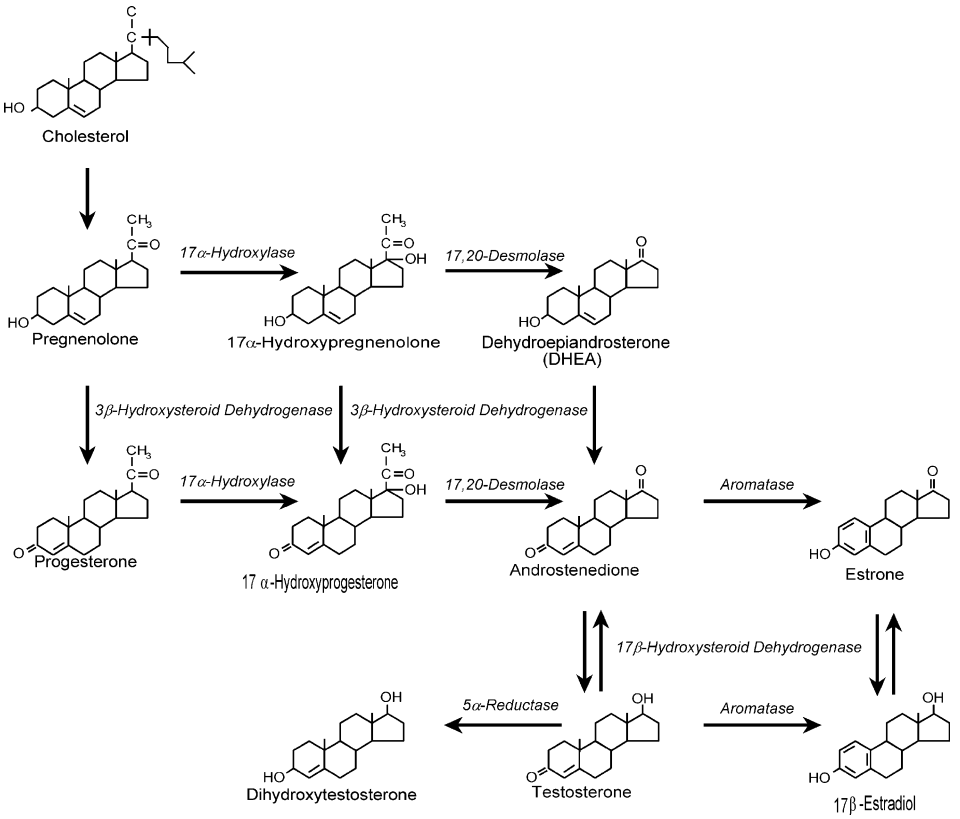

Fig. 1. Biosynthesis of estrogens and androgens. The synthesis of the sex steroid hormones involves the conversion of cholesterol esters through a complex set of enzymatic reactions involving numerous enzymes. Testosterone can be converted to dihydroxytestosterone, (DHT) through the actions of 5α-reductase whereas aromatase action converts testosterone to 17β-estradiol. This represents an important bifurcation of the sex steroid biosynthetic pathway culminating in either an active androgen or estrogen, respectively. (Adapted from Ref. [13] with permission.)

Androgens and estrogens circulate via the bloodstream. Because their chemical composition is hydrophobic, most are bound to carrier proteins (i.e. albumin) that facilitate their transportation to various target tissues. In fact, only 1–3% of the total circulating sex steroids exists free in solution. It is this free steroid and the albumin-bound steroid (approximately 35–55% of the total steroid) that is available to enter target tissues and exert their specific biological effect. Since the sex steroids are hydrophobic in nature, they are able to enter target cells through simple diffusion.

Once inside the cell, the steroid hormone encounters the steroid hormone receptor. These receptors are soluble proteins that function to bind the steroid hormone and also transmit the signal to the cell nucleus culminating in a transcriptional response. The events that follow are described in Fig. 2. In the absence of hormone (also called the ligand),

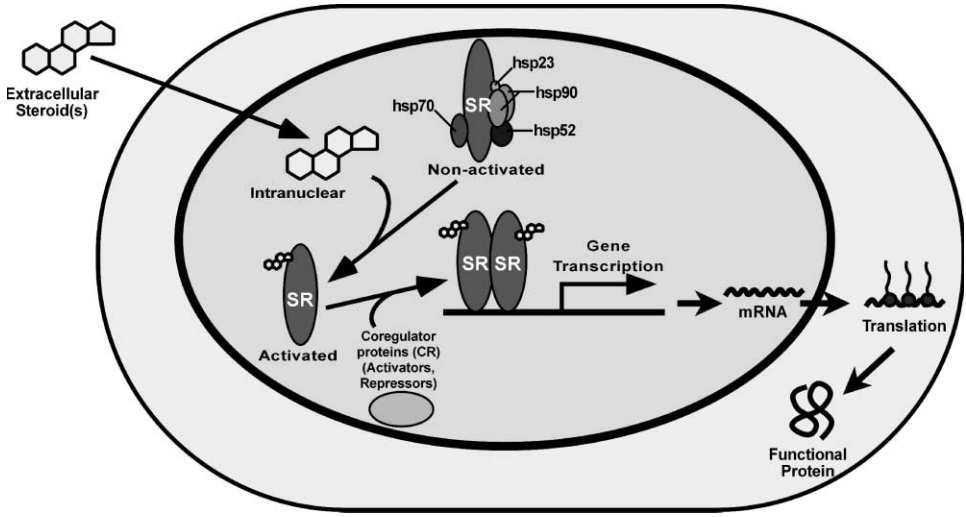

Fig. 2. The steroid hormone response pathway. The steroid enters the cell encounters the inactive steroid receptor (SR) bound with numerous HSP chaperones. Steroid binding induces dissociation of the SR from the HSPs. The ligand-bound SR dimerizes and binds specific HRE in the regulatory regions of certain genes. Coregulator proteins (including coactivators and corepressors) associate with the active SR inducing (or repressing) gene transcription. (Adapted from Ref. [13] with permission.)

the monomeric steroid hormone receptor is bound by numerous chaperone proteins termed "heat shock proteins" (HSPs) and resides primarily in the cell nucleus. Once the ligand binds the steroid hormone receptor, the HSPs dissociate allowing the receptor to homodimerize (two identical receptors bound together). How does the ligand-bound steroid receptor then function to regulate transcription? This property of the steroid hormone receptor is largely based on the primary sequence of the receptor and is discussed in Sections 3–6.

3. Generalized structure of steroid hormone receptors

Estrogens (i.e. 17β-estradiol) utilize the estrogen receptor. Two highly related isoforms of the estrogen receptor exist, which are encoded by two separate genes on separate chromosomes. In humans, estrogen receptor α (ERα) or estrogen receptor 1 is on somatochromosome 6 (q24–q27) and estrogen receptor beta (ERβ) or estrogen receptor 2 is on somatochromosome 14 (q21–q22). Both estrogen receptor isoforms bind 17β-estradiol with similar affinities, and as discussed later, both utilize similar DNA response elements. Androgens (i.e. DHT) utilize the androgen receptor encoded on the X chromosome. Interestingly, while almost all other ligand-dependent hormone receptors have two isoforms of the receptor, only one known androgen receptor isoform exists.

The ligand-dependent steroid hormone receptors, including the estrogen and androgen receptors, are classified as Type I nuclear hormone receptors. Type I receptors are soluble and recognize their respective DNA binding elements only when activated by ligand. Type

II receptors include receptors that bind non-steroidal compounds such as thyroid hormone (thyroid receptor; TR), retinoic acid (RAR, RXR) and vitamin D (VDR). These receptors are bound to DNA in the absence of ligand where they function to repress gene transcription. Activation of transcription is elicited through binding of their cognate ligand. In this chapter, we will concentrate on the Type I receptors (specifically estrogen and androgen receptors).

All Type I receptors share a similar domain structure [10]. Fig. 3 is a schematic diagram describing the various domains in both estrogen receptors and the androgen receptor. The N-terminal region (A, D) of the receptor shows less conservation of sequence among the steroid hormone receptors and is involved in transcriptional activation functions. The central (C, D) and C-terminal (E, F) regions of the receptor exhibit a much higher level of sequence similarity and have a number of different functional domains.

The C-terminal region of steroid hormone receptors (E, F) is involved in binding the steroid molecule and is termed the ligand-binding domain (LBD). The LBDs for the various receptors regulate the activity of the protein. For example, its amino acid sequence enables the estrogen receptor to bind 17β-estradiol with high affinity. Similarly, the androgen receptor LBD binds DHT with high affinity. The specific amino acid residues lining the ligand-binding pocket in the LBD provide the necessary chemistry to facilitate binding to a specific steroid. Thus, the estrogen receptor cannot bind androgens with high affinity and vice versa. In the case of the estrogen receptor, deletion of the LBD results in a receptor that can still bind to a DNA-binding domain (DBD), however, is non-functional.

The crystal structures of various LBDs in the absence of ligand (apo-receptor) and in the presence of ligand (holo-receptor) have been solved. These studies have revealed that

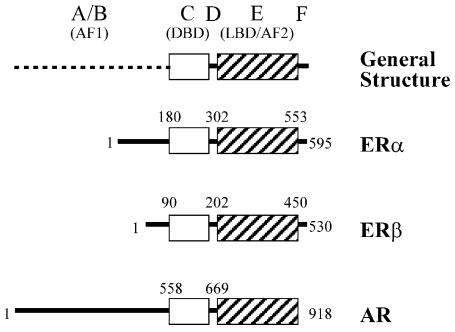

Fig. 3. Generalized structure of the estrogen (ER) and androgen (AR) receptors. The schematic diagrams of the steroid receptors proceed from the amino- to the carboxy-terminus and the numbers represent the specific amino acid residues. The ERα and ERβ protein isoforms are transcribed from different genes and represent protein products of 595 and 530 amino acids, respectively. All the receptors shown contain a highly divergent A/B domain (N-terminal region) that contains the activation function-1 (AF-1) domain involved in transactivating transcription in a cell-specific manner. The central, highly conserved C region contains the DND involved in recognizing and binding specific HRE in the regulatory region of hormone-responsive genes. The E region contains the LBD and a second overlapping AF-2 domain. These regions are involved in binding their cognate steroid ligand and activating transcription in a ligand-dependent manner, respectively. The C and E domains are linked through a "hinge" region (D). The carboxy-terminal F domain is highly divergent among steroid receptors and is involved in modulating receptor function. (Adapted from Ref. [13] with permission.)

nearly all steroid hormone receptors share a significant conservation in the overall secondary structure of the LBD, involving 12 α helices in the recognition and binding of the steroid specific to the steroid receptor in question [1]. In the case of estrogen receptor, the binding of 17β-estradiol induces a conformational change in the orientation of helix 12 that allows for the transmission of the transcriptional signal by recruiting transcriptional coregulators (described in detail later). This repositioning of helix 12 contributes to creating a unique interaction surface called "activation function" or AF-2. Some steroid hormone receptors, including the androgen receptor and estrogen receptor-α, contain an amino-terminal AF-1 domain that functions independent of ligand, and may be involved in cell- and promoter-specific responses. However, the AF-1 domain is less well characterized than the AF-2 domain. The AF-1 and AF-2 functions of the androgen receptor, for example, have been shown to act synergistically to activate DHT-dependent gene transcription [2], uncovering the importance of overall tertiary structure and intra-protein interactions in transcriptional activation. The AF-1 and AF-2 functions of the estrogen receptor-α isoform do not appear to act synergistically.

4. Steroid hormone receptor binding to DNA elements

The DBD of steroid hormone receptors is located centrally in the receptor. The secondary protein structure of the DBD is composed of two zinc-finger motifs involved in binding to specific elements on the DNA (described in detail later). The core DBD contains two α helices extending from the base of each zinc finger. The N-terminal α helix, also called the recognition helix, is involved in recognizing and binding specific DNA sequences through hydrogen bonding. The recognition helix lies along the major groove of DNA, while the C-terminal α helix lies above and perpendicular to the recognition helix. This α helical domain does not make specific contacts with DNA. Numerous experiments have demonstrated that steroid receptors are monomeric in solution. Upon addition of their cognate ligand, the receptors bind as dimers in a cooperative manner to their respective DNA-binding site. However, what are these binding sites and how are they structured?

The DBD binds specific DNA sequences involved in transcriptional activation of hormone-responsive genes. These DNA sequences are called hormone response elements (HRE), and in the case of estrogen and androgen receptors, they are termed estrogen response element (EREs) and androgen response element (AREs), respectively. HREs generally consist of two 6-basepair (bp) halfsites consisting of a specific sequence(s) separated by a 3-bp spacer of inconsequential sequence. A generalized "consensus sequence" has been determined for each steroid hormone receptor, however, numerous examples of deviation from this consensus are available [11]. The consensus sequence for the androgen receptor is "TGTTCT" whereas estrogen receptors generally utilize "AGGTCA". The two halfsites, consisting of an entire HRE, are oriented in an inverted repeat fashion on the DNA, with each halfsite able to bind one molecule of the activated steroid receptor dimer. In conclusion, the HRE sequence, orientation, and spacing all provide the necessary determinants for binding of the proper steroid hormone receptor.

Some steroid hormone receptors can elicit transcriptional effects through non-ERE DNA binding elements. For example, the estrogen receptor can bind cooperatively with SP1 (another transcription factor) to DNA elements composed of an ERE-halfsite and an SP1 site, or in some cases, just an SP1 site. The estrogen receptor can also bind to DNA elements indirectly via protein–protein interactions. For example, the estrogen receptor binds the AP1 transcription factor protein (composed of fos-jun heterodimers) bound to its cognate AP1 DNA binding element, and elicits estrogen-dependent transcriptional effects. These examples highlight the diversity of mechanisms in which steroid hormone receptors can signal through non-canonical DNA binding elements.

Each domain of a nuclear hormone receptor can function independently of the whole protein. For example, replacement of the estrogen receptor LBD in the androgen receptor molecule would result in a protein that binds 17β-estradiol, since the replaced LBD originated from the estrogen receptor. However, this chimeric molecule would bind to DNA and activate transcription in a manner consistent with the androgen receptor.

5. Coregulators – diversity and function

Steroid hormone receptor coactivators are involved in enhancing the transcriptional signal of the steroid hormone receptors following ligand binding. Steroid receptor coactivator-1 (SRC1), the founding member of the SRC family (also called the p160 family, based on their similar sizes of 160 kDa), was originally identified as an interacting protein with the progesterone receptor (PR) LBD, another member of the steroid hormone receptor family [9]. Further analysis demonstrated that SRC1 exhibits transcriptional coactivation properties not only with the PR but also with numerous members of the steroid receptor family, including both estrogen receptors and the androgen receptor. Two additional members of the p160 family of coactivators, SRC2 and SRC3, were also identified which interact with and coactivate numerous steroid hormone receptors [6,12]. A schematic of the structure of the SRC family of coactivators and the relevant protein domains is provided in Fig. 4.

The p160 family of proteins mediate the interactions with steroid hormone receptors through a centrally located receptor interaction domain (RID), which contains three α helical LXXLL motifs (where X = any amino acid) necessary for interaction with steroid hormone receptors [3]. Binding of ligand to the receptor induces conformational changes, which allows association of the coactivator's RID domain with the LBD/AF-2 function of the receptor. Mutation or deletion of the SRC RID domains inhibits physical interaction with the steroid receptor LBD and thus abolishes the transcriptional coactivation potential of the SRC molecule.

The p160 family of coactivators contains intrinsic transcriptional activation domains that contribute to the overall transcriptional activation elicited by the ligand-bound steroid hormone receptor. These domains function to recruit other molecules involved in activating transcription such as CBP/p300. One interesting domain found in both SRC1 and SRC3 is the histone acetyltransferase (HAT) domain. This domain functions to modulate chromatin structure into a conformation permissive to transcriptional activation. HAT activity serves to transfer acetyl groups to specific lysines in histones. This is thought

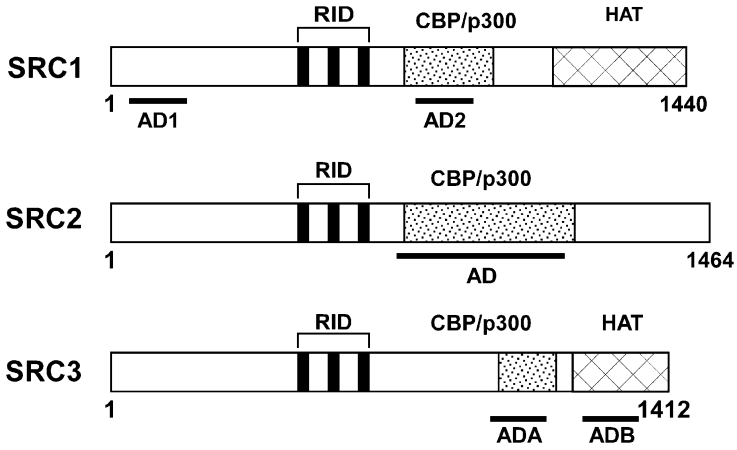

Fig. 4. Generalized structure of the p160 family of transcriptional coactivators. The schematic diagrams of these coactivators proceed from the amino- to the carboxy-terminus and the numbers represent specific amino acid residues. These coactivators typically contain three centrally located LXXLL motifs (where X = any amino acid), termed the RID, that are involved in binding the LBD/AF-2 domain of the steroid receptor. Solid lines underneath each SRC molecule indicate the locations of intrinsic activation functions (AD) that assist in recruiting general transcription factors. Both SRC1 and SR3 contain a histone acetyltransferase (HAT) catalytic domain and all three SRCs contain a domain that interacts with CBP/p300.

to "loosen" the histone's grip on DNA and thus facilitate the entry of other transcription factors, or the basal transcriptional machinery itself, to activate transcription. Thus, the various coactivators serve numerous functions in the processes of transcriptional activation.

Transcriptional repression by steroid hormone receptors is mediated by the recruitment of corepressors. These molecules can function by competing with coactivators for the LBD on steroid receptors. This is the mechanism for the selective estrogen receptor corepressor REA (repressor of estrogen action), which directly competes with SRC1 [7]. Other transcriptional corepressors, however, bind the LBD either in the absence of ligand or in the presence of antagonists.

The structure of corepressors in many ways mirrors that of coactivators. For example, many corepressors contain histone deactylation activities that appear to antagonize transcriptional activation by recruiting factors involved in histone deactylation, often called HDACs [4,8]. Thus, the activities of corepressors and coactivators often target similar processes in opposing manners (e.g. HAT activity versus HDAC activity). This careful balancing act of the functions of coactivators and corepressors is a major component in determining whether a gene is activated or repressed.

6. Chronology of gene responses following steroid receptor binding

Thus far, events are described that occur following ligand binding to the steroid hormone receptor that ultimately lead to the activation or repression of a gene promoter. Fig. 5 summarizes the chronology of steroid responses following binding of the steroid

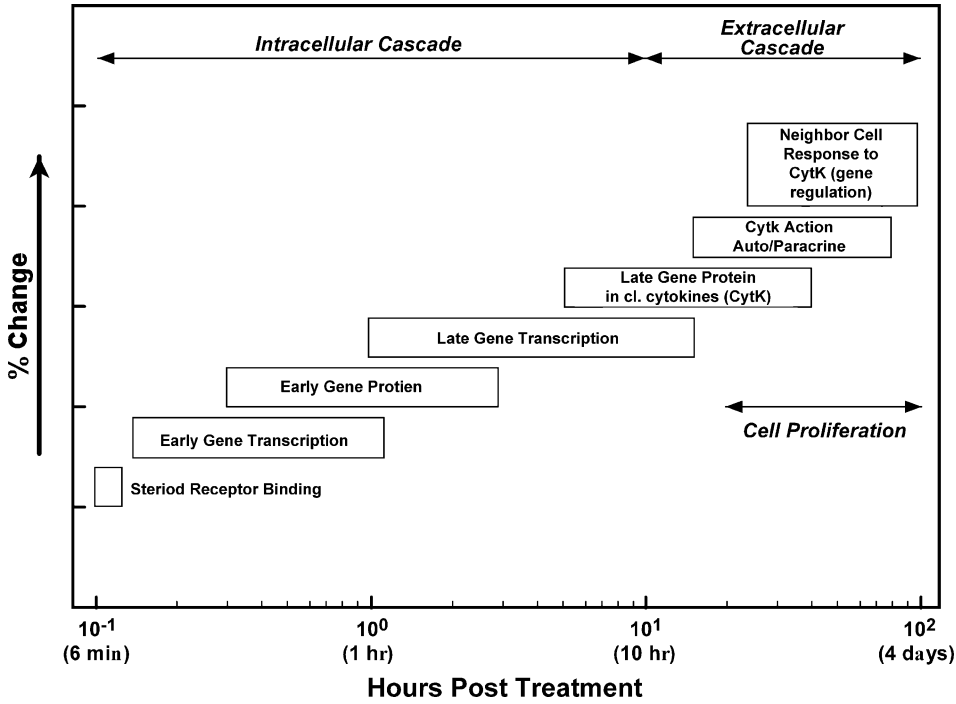

Fig. 5. Chronology of steroid hormone responses. This schematic diagram outlines the potential sequence of events that occur following ligand binding to the steroid receptor. Genes which contain functional HREs are activated within one hour of hormone treatment and are termed "primary response" genes. The protein products of the primary response genes may encode transcription factors that activate late gene transcription, occurring anywhere from 1 to 24 h post-hormone treatment. These late responding genes are termed "secondary response" genes and are dependent on de novo protein synthesis. Some secondary response genes may encode cytokines, which are involved in stimulating autocrine and paracrine responses in an extracellular cascade. (Adapted from Ref. [13] with permission.)

hormone receptor to its DNA binding element. The genes that directly bind steroid hormone receptors are termed "primary response" genes. Primary response genes are characterized by a rapid response to hormone treatment (ranging from a few minutes to a few hours) and their activation is independent of de novo protein synthesis. In this model, often referred to as the "cascade model" [5], some primary response genes encode transcription factors that can directly influence the transcriptional activity of "secondary response" genes. Since the activation of secondary response genes is dependent on de novo protein synthesis, a characteristic lag period extending from a few hours to a few days following hormone treatment is observed in their synthesis. Some of the secondary response genes may encode cytokines, peptide hormones involved in numerous processes including cell signaling, often resulting in subsequent, steroid-induced neighbor cell responses to the cytokine (often termed the "extracellular cascade"). Withdrawal of hormone results in cessation of the hormonal response and results in a tissue/organ phenotype consistent with the unstimulated state. However, the addition of hormone can

restimulate the primary and secondary response cascade as described above. This in fact occurs during the female menstrual cycle where estrogen levels fluctuate resulting in specific hormonal responses dependent on the circulating estrogen concentration.

7. Summary

Sex steroid hormones (e.g. estrogens and androgens) are synthesized from cholesterol in a complex set of reactions and that the intracellular action of these hormones is transmitted through their cognate steroid hormone receptor (e.g. estrogen receptor and androgen receptor, respectively). These receptors contain domains involved in ligand binding, DNA binding and transcriptional activation (AF-1 and AF-2). The transcriptional effects of the steroid hormone receptors are elicited through the recruitment and actions of steroid hormone receptor coactivators and corepressors. It is the actions and interactions of the coregulators that determine the transcriptional outcome of the gene promoter. Steroid responsive genes can be classified as either "primary" or "secondary", depending on the requirement for de novo protein synthesis and the rapidity of the response.

8. Future directions

One of the major questions involving the function of steroid hormone receptors is the identification of the specific coregulator complexes involved in the connection of the ligand-bound steroid hormone receptor to the transcriptional machinery resulting in the regulation of hormone-responsive genes. Although both ERα and ERβ bind 17β-estradiol with similar affinity and appear to bind similar DNA elements (i.e. EREs), there are many genes regulated by only one estrogen receptor isoform. It has been postulated that these differences in the activation potential of ERα and ERβ lie in the differential recruitment of coregulator complexes. The identification of specific coregulator complexes involved in the regulation of particular hormone-responsive genes is a major focus of endocrinologists and biochemists. It is the identity of these coregulator complexes that may explain how the same hormone receptors acquire very different functions in distinct cell types and may explain sex-based differences in hormone action.

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Non-genomic actions of hormones

Richard H. Karas

1. Introduction

As described in Chapter 3, steroid hormones are classically thought to signal by diffusing passively into the intracellular space where they bind to and transcriptionally activate their cognate receptors. It has been clear for many years, however, that this transcription-dependent pathway cannot account for all of the observed effects of steroid sex hormones. For example, there are reports from over 60 years ago demonstrating that steroid hormones can induce physiologic responses within minutes of exposure to the hormone. These rapid responses to steroid hormones occur too quickly to be mediated by transcriptional activation of the classical nuclear hormone receptors. Furthermore, a variety of rapid effects of steroid hormones have now been clearly shown to occur in the absence of alterations in gene expression. Hence, these effects are referred to as non-genomic actions of hormones. This chapter will explore the molecular mechanisms that mediate the non-genomic actions of steroid sex hormones.

2. Rapid physiologic effects of hormones

One of the earliest reports of a rapidly occurring steroid sex hormone effect was of estrogen-induced arterial dilation. Indeed in 1940 it was reported that estrogen could acutely dilate the artery in the ear of a rabbit. This phenomenon has now been replicated in a variety of vascular beds by many investigators. The endothelial cells of blood vessels control vasomotion predominantly through regulated production and release of nitric oxide. The aspects of vasomotor regulation that require the endothelium are known as endothelium-dependent responses and the study of these responses has evolved into a topic of intense research interest for a number of reasons. First, impaired endothelial function develops early in the atherosclerotic process, even before the lumen of the vessel has been significantly decreased, and the loss of normal production of nitric oxide is now believed to contribute to the initiation and progression of atherosclerosis. Individuals with poor endothelial function are at increased risk for developing cardiovascular diseases such as heart attacks and stroke, and medical and lifestyle interventions that reduce cardiovascular risk also improve endothelial function. Based on these considerations, there has been

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considerable interest in further understanding effects of hormones like estrogen on vasomotor regulation.

Williams et al. initiated the current era of investigation regarding the rapid vasodilatory effects of estrogen by examining the effect of estrogen on endothelial function in a group of monkeys who had been fed a highly atherogenic diet. They reported that short-term (20 min) exposure to a single intravenous dose of ethinyl-estradiol markedly improved the impaired endothelial function observed in these ovariectomized female monkeys [25]. This landmark study led to additional studies in women that also documented improvements in endothelium-dependent vasorelaxation acutely after a single dose of estrogen [18]. Importantly, this effect of estrogen can be observed with physiologically relevant concentrations of the hormone. Some studies have shown similar acute vasodilatory effects in men as well as women, suggesting that the cellular mechanisms mediating this response operate independent of genetic sex, i.e., the XX or XY genome of the individual. The potential clinical relevance of these findings is supported by the observation that acute administration of sublingual 17β-estradiol significantly increases exercise duration in postmenopausal women with known cardiac ischemia [19]. It should be noted that long-term exposure to estrogen also enhances arterial dilation by altering expression levels of a number of vasoactive substances, but these effects likely are mediated by the classical estrogen receptor-dependent transcriptional pathway. The molecular mechanisms that mediate the rapid, non-genomic effects of estrogen on endothelium-dependent vasorelaxation will be discussed in detail below.

Rapid, non-genomic effects of steroid sex hormones have also been reported in other non-vascular tissues, and for other hormones. For example, progesterone has been known for some time to induce an important maturation phase in the development of the xenopus oocyte. This response has been shown clearly to be a non-genomic one in that progesterone-induced oocyte maturation occurs despite the presence of Actinomycin D, an inhibitor of gene transcription. Furthermore, progesterone also induces maturation of enucleated oocytes. Progesterone has also been shown to non-genomically induce the acrosome reaction in human sperm. Rapid, non-genomic effects of steroid sex hormones have also been reported in the central nervous system, in inflammatory cells, and in pancreatic islet cells [22]. Rapid, non-genomic effects of testosterone have also been reported. For example, testosterone has been shown to rapidly increase intracellular calcium concentration in osteoblasts [11]. One additional manner in which steroid hormones can alter physiologic functions in a transcription-independent way is by altering levels of circulating oxidants. Several estrogens, for example, have been found to possess various levels of anti-oxidant activity.

3. Mediators of non-genomic effects

Taken together, these observations reviewed above demonstrate that steroid hormones can exert a variety of physiologic effects with a rapidity that precludes their being mediated by transcriptional activation of steroid hormone receptors. These findings, thus, present something of a mechanistic conundrum. Broadly speaking, two hypotheses have been put forward to explain how these effects are transduced in cells. The first, and

seemingly more likely hypothesis is that proteins other than the known steroid hormone receptors mediate the non-genomic effects, and thus these pathways have been referred to as "non-receptor mediated pathways". This terminology is somewhat misleading as these effects could, of course, be mediated by proteins that would still be considered to be receptors though they are distinct from the classical ligand-activated transcription factors that characterize this family of receptors. The second hypothesis to be considered is that non-genomic actions of hormones are indeed mediated by the classical receptors, but the receptors are signaling by alternative, non-transcriptional pathways. Support for the non-receptor mediated pathway has come from a variety of sources demonstrating that rapid, non-genomic effects could also be mediated by hormone isoforms that do not activate the classical receptors. For example, both 17β-estradiol (the stereoisomer that binds to and transcriptionally activates the classical estrogen receptor) and 17α-estradiol (the stereoisomer that does not transcriptionally activate the classical estrogen receptor) have both been shown to rapidly block currents through L-type calcium channels. Further support for the "non-receptor mediated" hypothesis was derived from studies demonstrating that pharmacologic inhibitors of the classical steroid hormone receptors do not block some non-genomic effects. However, as additional studies have been reported, it became clear that the studies supporting the "non-receptor mediated" hypothesis were performed with supra-physiologic concentrations of steroid hormone (i.e. micromolar concentrations as opposed to nanomolar concentrations of hormone; reviewed in Ref. [13]). Thus, these findings have little or no relevance to the physiologic effects of steroid hormones that are observed at concentrations 1000-fold less and, therefore, they will not be discussed further in this chapter.

In contrast to the situation with the proposed "non-receptor mediated" pathway, support for the alternative hypothesis, that non-genomic actions of steroid hormones are mediated by classical receptors signaling via alternative, non-transcriptional mechanisms, has been both strong and convincing. Early support for the idea that classical steroid hormone receptors can participate in alternative signaling pathways came from the work of Watson and her colleagues, who showed many years ago that cell surface membrane associated-proteins can be identified by antibodies specific for the classical estrogen receptor [16]. Membrane-associated steroid hormone receptors have now been reported by a number of investigators in a number of different cell types (reviewed in Ref. [14]). One technique frequently employed in attempts to identify cell surface steroid hormone receptors is the use of steroid hormones covalently bound to a polymer or a large protein such as albumen that cannot cross the plasma membrane. The rationale behind the use of such agents is 2-fold. First, when these agents are also linked to a fluorescent marker, they can readily be detected bound to the cell surface. Second, if a downstream signaling event is initiated by exposure to albumen-linked hormone, then it can be argued that binding to a cell surface receptor triggered the ensuing event. Both of these aspects are highly questionable, however, as both the albumen and the hormone can bind non-specifically to the cell membrane, and perhaps more importantly, it has clearly been shown that these reagents also contain a significant amount of free hormone non-covalently associated with the complex that can also activate intracellular receptors [23]. Thus, experiments using these reagents must be interpreted with caution. Having

said that, a variety of other approaches have been used that have definitively identified estrogen receptors in membrane fractions of vascular endothelial cells, endometrial cells, and pituitary cells.

Using cDNA transfection approaches, several groups have demonstrated that overexpression of wild-type estrogen receptors results in localization of a subpopulation of the receptors to the plasma membrane. As will be described below, more recent work has convincingly demonstrated that it is this membrane-associated population of estrogen receptors that mediate estrogen-induced, non-genomic activation of nitric oxide production by vascular endothelial cells. An N-terminally truncated estrogen receptor approximately 46 kDa in size, has been cloned from MCF-7 cells [6]. This isoform of estrogen receptor alpha also localizes to the cell membrane and transduces rapid effects of estrogen [20]. The distribution of estrogen receptors between the cell membrane and the nucleus is tightly regulated by the cell in response to various physiologically relevant stimuli [12].

Somewhat less progress has been made in understanding how the rapid non-genomic effects of progesterone are mediated. There is evidence that the non-genomic effects of progesterone also are mediated via cell surface receptors. For example, the xenopus oocyte maturation process described above can be activated by treatment of the oocytes with polymer linked, cell-impermeant progesterone, whereas injection of progesterone directly into the cytoplasmic space within the oocyte does not produce this response (reviewed in Ref. [24]). Cell surface progesterone binding proteins have been reported and these have been isolated and partially characterized using biochemical techniques. In addition, two groups have isolated at least partial clones for putative membrane-associated progesterone receptors [4,9]. In each of these instances the candidate membrane progesterone receptors bear little homology to the classical progesterone receptors suggesting that the non-genomic effects of progesterone are mediated by receptors distinct from the classical progesterone receptor. Importantly, however, neither of these putative membrane receptors have definitively been shown to transduce non-genomic effects of progesterone. Further complicating the situation are reports that reconstitution experiments using transient transfection approaches have shown that forced expression of a classical progesterone receptor can confer rapid, non-genomic responses in cells that otherwise are not progesterone responsive. For example, the xenopus oocyte maturation described above can be enhanced and accelerated by injection of the xenopus homologue of the classical progesterone receptor B isoform, and injection of anti-sense oligonucleotides against this receptor renders the cells unresponsive to progesterone treatment [24]. Thus, additional work will be required to determine which proteins are or are not responsible for mediating the non-genomic effects of progesterone.

Much less is known regarding any non-genomic effects of testosterone. As noted above [11], there are some reports in the literature regarding alterations in specific physiologic parameters that occur rapidly after exposure to physiologically relevant concentrations of testosterone. However, elucidation of the receptors that initiate these responses, and identification of the signaling pathways that mediate them are unknown at the present time.

4. Downstream signaling activated by non-genomic pathways

Several cellular signaling pathways have now been shown to be activated by non-genomic actions of steroid hormones. These studies have provided evidence of the diversity of cellular control systems regulated non-genomically by steroid hormones. In addition, significant progress has also been made in elucidating the role of these downstream signaling pathways in mediating some of the rapid physiologic effects described above. To date, progress has been made in the identification of the molecular pathways that mediate acute estrogen-induced nitric oxide release by the endothelium that is responsible for estrogen-mediated vasodilation. Therefore, this pathway will be described in detail in this section. This body of literature is also important because it confirms earlier observations suggesting that classical estrogen receptors can mediate non-genomic actions, and further it identifies the membrane associated estrogen receptors as those responsible for transducing these signals.

Nitric oxide is produced in vascular endothelial cells by endothelial nitric oxide synthase (eNOS), a specific isoform of nitric oxide synthase. In vitro, estrogen induces eNOS-dependent production of nitric oxide in cultured vascular endothelial cells within minutes of exposure [6,10]. Inhibitors of gene transcription do not block this rapid release of nitric oxide, nor is new protein synthesis required. Transient transfection experiments using Cos1 cells that do not express either endogenous estrogen receptors or eNOS, demonstrate that co-transfection of both estrogen receptor α and eNOS can reconstitute estrogen-induced release of nitric oxide [3]. These findings definitively implicate the classical estrogen receptor α in mediating this non-genomic effect of estrogen. A subpopulation of estrogen receptors localized in caveolae, cell membrane-based structures that contain the key components of many signaling pathways including eNOS, are responsible for initiating this non-genomic effect [1]. Estrogen receptor β also can mediate activation of eNOS by estrogen [2].

A critical component to developing an adequate understanding of how non-genomic actions of hormones are mediated includes identification of the molecular signaling pathways that transduce these signals from the initiating receptor to the downstream effector that ultimately produces a physiologically relevant response. Some progress has also been made toward this goal regarding estrogen-mediated activation of eNOS. Experiments using a variety of pharmacologic inhibitors have demonstrated that blockade of several signaling pathways disrupts estrogen's effect on eNOS activity. Reports to date indicate that genestein (an inhibitor of tyrosine kinases), pertussis toxin (an inhibitor of the heterotrimeric G protein subunit Gαi), PD98059 (an inhibitor of MEK1-mediated activation of MAP kinase), and wortmannin and LY294002 (both inhibitors of PI3 kinase-mediated activation of Akt), each block estrogen-mediated activation of eNOS (Fig. 1; reviewed in Ref. [14]). These observations have two important implications. First, the demonstration that blockade of each of a number of different signaling pathways alone can inhibit estrogen-mediated activation of eNOS supports that the molecular pathway linking estrogen-activated estrogen receptors to activation of eNOS likely includes a complex interplay between several signaling pathways. Second, these observations also suggest that through non-genomic actions, estrogen can activate diverse downstream signaling pathways that themselves likely regulate several other effector proteins, in addition to

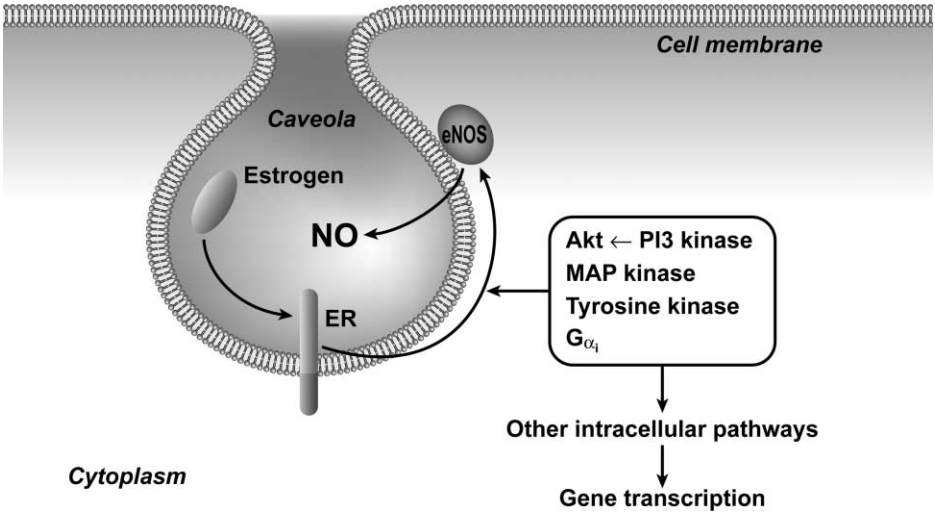

Fig. 1. Schematic of pathways identified to be involved with estrogen receptor activation of endothelial nitric oxide synthase (eNOS) needed for the production of nitric oxide (NO) in vascular endothelial cells. Pharmacologic blockers of each of these pathways disrupt estrogen-induced activation of eNOS. In addition, these pathways also can initiate a cascade of intracellular events some of which result in altered gene transcription. Abbreviations: Akt, a serine–threonine kinase important for the regulation of several crucial cellular processes; G_{α_i}, guanine nucleotide regulator protein subunit which inhibits guanylate cyclase; MAP, mitogen-activated protein; PI3, phosphotidyinositol trisphosphate (adapted from Ref. [14]).

eNOS. This second point continues to be an area of intensive investigation by a number of laboratories. Indeed, in a variety of cells, estrogen has been shown to activate each of the four pathways noted above. For example, in MCF-7 breast cancer cells, estrogen treatment rapidly leads to tyrosine phosphorylation of the oncogene src. Relevant to activation of the oncogene src is the demonstrated interaction between estrogen receptor α and the tyrosine kinase receptor for insulin-like growth factor 1 [7]. Short-term treatment of Cos7 and HEK293 cells with estrogen induced increased phosphorylation of this receptor in a manner consistent with the activation of its intrinsic tyrosine kinase activity [7]. This is noteworthy because activation of receptor tyrosine kinases such as the insulin-like growth factor receptor is one of the most important pathways that activate src. Work from several laboratories has shown that estrogen can rapidly activate Gα [17,26]. Estrogen has also been shown to activate both MAP kinase [15] and Akt [21], two serine/threonine kinases that have been directly implicated in short-term regulation of eNOS activity. Other kinases previously shown to be rapidly activated by estrogen include both JNK and p38. Phospholipase C_β has also been shown to be activated rapidly by estrogen.

In addition to these enzymatic pathways, estrogen can activate ion channels in a non-genomic manner (see also Chapters 8 and 9). Estrogen rapidly alters the function of the potassium channel BK_{Ca} which changes membrane potential of excitable cells affecting subsequent activation. One common pathway that may relate to activation of many of these signaling systems is that physiologic levels of estrogen can induce rapid spikes in intracellular calcium, likely due to the release of calcium from the endoplasmic reticulum.

Estrogen-induced calcium spikes have been reported in a variety of cell types under a number of different experimental conditions.

Activation of estrogen receptors rapidly increases cellular levels of cGMP and cAMP resulting in the activation of cyclic nucleotide regulated kinases. However, it is unclear whether changes in cellular cyclic nucleotides are directly activated by hormones or result from changes in other enzyme activity like NOS or cyclooxygenase.

Non-genomic activation of specific signaling pathways has also been reported in response to progesterone treatment. For example, progesterone decreases intracellular cAMP and activates MAP kinase in the xenopus oocyte. Progesterone also alters both chloride and calcium fluxes during induction of the acrosome reaction in sperm. How activation of these additional pathways relates to downstream physiologic effects of the steroid sex hormones remains unclear.

5. Summary and future directions

Experimental evidence supports the concept that steroid sex hormones can alter cell function in the absence of changes in gene expression. These non-genomic actions have been observed in cells from a variety of organ systems, including bone, the central nervous system, the inflammatory system, the reproductive system, and the cardiovascular system. Non-genomic actions of steroid hormones can alter the physiologic function of these organ systems. Examples include progesterone-induced acrosomal reactions in sperm and estrogen-induced arterial vasodilation. Somewhat surprisingly, it is also clear that the classical steroid hormone receptors can mediate non-genomic actions of steroid hormones, though it remains to be determined whether there are other receptors that also can mediate these non-genomic effects. This question is particularly relevant to the non-genomic signaling pathway for progesterone as the existence of putative non-genomic, membrane-associated progesterone receptors distinct from the classical receptors have been proposed. Short-term exposure to steroid sex hormones can lead to rapid activation of a variety of intracellular signaling pathways, and some of these have been specifically implicated in mediating the downstream physiologic effects of these hormones. Identification of these non-genomic signaling pathways has important implications in several regards. For example, these observations support that steroid hormone receptors evolved to transduce signals within the cell utilizing pathways that are independent from the classical effector pathway related to the regulation of gene transcription. This suggests that there are opportunities for developing therapeutic agents that may act via the non-genomic pathway without affecting those aspects of receptor signaling that are dependent on altered gene expression. One can thus envision the development of pharmacologic agents that act as potent vasodilators by activating eNOS via non-genomic activation of estrogen receptors. If such an agent were able to activate eNOS, but not activate the genomic program required for stimulation of cellular proliferation, potential adverse effects such as the promotion of breast or uterine cancer, might be avoided. This represents one conceptual aspect of the potential for the development of a new class of compounds called selective estrogen receptor modulators, or SERMs. A number of SERMs are currently being developed with the long-term goal being the development of novel compounds that can

selectively recapitulate certain aspects of estrogen-mediated signaling, but within a specific and restricted distribution of responsive tissues. Some progress has already been made toward identifying compounds that can induce non-genomic responses without altering gene transcription. For example, a synthetic estrogen receptor ligand, 4-estren-3α, 17β-diol, can prevent programmed cell death in both osteoblasts and osteoclasts, apparently without activating estrogen receptor-dependent gene transcription [8].

Though considerable progress has clearly been made in understanding the mechanisms that mediate the non-genomic actions of steroid sex hormones, many important questions remain unanswered. Relevant to the discussion above, it remains unclear the extent to which the rapid activation of non-genomic signaling pathways also contributes to the longer-term, genomic pathways. The potential for an important link between these two signaling pathways is suggested by the observation that some of the rapid effects of these hormones are also known to ultimately activate factors that control gene expression. For example, estrogen rapidly activates MAP kinase, and MAP kinase is known to phosphorylate and activate a number of potentially relevant transcription factors. Indeed, one of the transcription factors that MAP kinase is known to phosphorylate and transcriptionally activate is the estrogen receptor itself. This raises the possibility that the rapid, estrogen-induced, non-genomic activation of MAP kinase might initiate a cascade of events that ultimately results in transcriptional activation of the estrogen receptor itself. There also remain important gaps in the understanding of the molecular signaling pathways that transduce the non-genomic actions of these hormones from their initiation point to the final effector proteins, and further experimentation will be required to fill these gaps. Similarly, hormones have been shown to non-genomically activate a number of signaling pathways, but the effect of activation of these pathways on cellular behavior has not yet been elucidated. Finally, though it is clear that physiologic responses can be elicited following acute exposure to steroid hormones, the true physiologic relevance of this must continue to be considered, given the lack of naturally occurring situations when the body would be exposed to rapidly changing concentrations of these hormones.

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Hormone receptor polymorphisms

Amanda M. Shearman

1. Introduction

Signaling by steroid hormone receptors is key to both normal development and a wide range of disease mechanisms. Understanding the functioning of hormone receptors and the impact of heritable variation in their genes will be crucial to the design and targeting of therapeutics to treat commonly occurring, genetically complex diseases associated with hormone action, including cardiovascular disease, breast cancer, prostate cancer and osteoporosis.

Much is already known of how steroid hormone receptors function, including what domains mediate ligand binding, DNA binding and transcriptional activation (see Chapter 3). Insight into physiological processes regulated by these receptors has come from individuals with very rare single gene (Mendelian) defects in the androgen and estrogen receptors. However, the occurrence and impact of common genetic variation in genes for these receptors is only beginning to be catalogued and studied in a comprehensive manner in large populations of men and women. This chapter will describe some general concepts about genetic polymorphisms and association studies, and then provide examples of how polymorphisms in the *estrogen* and *androgen receptor* genes relate to physiology. These illustrations will provide a review of the known classes of heritable genetic variation and models of inheritance that will be applicable to the actions of other hormone receptors.

2. The human genome contains many types of polymorphisms that may determine disease status or response to therapy

The current progress and methodology of identifying genotypes that underlie human phenotypes has been reviewed in detail [3]. It has been estimated that any two unrelated people have a difference in DNA sequence on average every 1000 base pairs (bp) [14]. The Human Genome Project has now completed sequencing over 99% of the human genome. Genes, other functional elements and polymorphisms in the 3×10^9 bp that make up the genome are being annotated and catalogued. There are a number of different types of polymorphisms, for example, tandem repetitive elements (minisatellites, or di- tri- and

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tetranucleotide repeats called microsatellites, and minisatellites which have larger repeat units), insertions, deletions and the more common single nucleotide polymorphisms (SNPs). For SNPs with a minimal allele frequency of 5% it is expected that there will be one every 450 bp, or an estimated 7.1 million in the human genome [21]. An ongoing component of the Human Genome Project is the SNP Consortium which is constructing a dense map of SNPs that will help us to locate and identify genes involved in human disease [32].

Given the vast quantity of genomic information now available, one challenge is to determine what characterizes the polymorphisms that are most important in terms of disease causation and prognosis. There are many genes for which some involvement in disease etiology is hypothesized and might thus be targets for drug discovery. The process of drug discovery and development is, however, very expensive, takes many years and rarely generates a product that reaches the patient. The targets that are among the most promising for pharmaco-genetic analysis (the study of how the actions of, and reactions to, drugs vary with the patient's genes) are those known to interact with drugs already in use. For example, the estrogen receptors are critical to the action of estrogen replacement therapy and a number of selective estrogen receptor modulators (SERMs), such as tamoxifen and raloxifene, have been successfully used to treat breast cancer and osteoporosis.

One of the most immediate benefits of studying the relationship between the genetics of hormone receptors and disease outcomes may be to differentiate between individuals who will respond to a particular drug and those who will not. A good example of this type of study was in the response of asthma to treatment with an inhibitor (ABT-761) of the *5 lipoxygenase* gene (*ALOX5*) [10]. *ALOX5* has a catalytic role in the synthesis of leukotrienes important in inflammation and immediate hypersensitivity. The *ALOX5* promoter contains between three and six tandem repeats of a zinc finger (Sp1/Egr-1) transcription factor binding site (GGGCGG). Wildtype individuals with five such repeats in one or both alleles improved when treated with the *ALOX5* inhibitor while variant individuals with 3, 4 or 6 (deletion of one or two or addition of one) Sp1 binding sites did not respond. *In vitro* assays with promoter–reporter constructs showed that all of the mutant alleles had significantly diminished transcriptional activity.

3. The location of a polymorphism within a gene determines what effect it may have

Although there is considerable variability, the average gene contains approximately 10 exons that are each 240 bp in length and are separated from one another by nine 5000 bp (5 kb) introns so that the entire gene is over 50 kb in length. The 5′ and 3′ untranslated regions average 200 and 800 bp, respectively [11]. In addition, there is a promoter region containing control elements that may precede the start of transcription and extend for some tens of kilobases. Overall, only about 3% of the average gene is coding and the rest is untranslated.

There may be hundreds of SNPs and other polymorphic elements in any gene. Association studies require hundreds or thousands of participants and genotyping each polymorphism is quite expensive in terms of reagent costs. Each additional statistical test that is carried out also incurs a penalty by raising the threshold required for statistical significance. It is not only necessary to select a promising candidate gene to examine in

association studies of common diseases, but also to take care in selecting only the most likely polymorphisms in that gene.

Polymorphisms occur at different frequencies in different parts of the genome. Exons, particularly those that code for crucial protein domains, rarely contain polymorphisms. Rare exonic SNPs that cause non-synonymous changes and result in altered protein structure are promising candidates for disease association. Similarly, insertions or deletions of one or more base pairs or alterations that generate premature stop codons may all have a significant effect on protein structure and are thus good targets for association studies.

Polymorphisms that affect transcription or gene splicing may have a critical role in gene expression and be worth studying. In the promoter, in the vicinity of the transcription start site, are a number of well-characterized motifs including the TATA and CAAT boxes, and transcription factor binding sites (including estrogen receptor binding sites). Other components of the promoter, including enhancer elements, may also occur throughout the gene but are less well characterized. Around half of all human genes are estimated to undergo alternative splicing, which is regulated by control elements that occur in the intronic (up to 200 nucleotides away from the splice junction) or exonic sequences (up to 125 nucleotides away from the splice junction) [24].

To date, the majority of polymorphisms known to cause disease phenotypes are missense/nonsense mutations (59%), followed by deletions (22%), aberrant splicing (10%), insertions/duplication (7%), complex rearrangements (2%), regulatory mutations (1%) and a small number of repeat variations (0.1%) [3]. Although the aim of this book is to bring together information about sex-based biological functions beyond reproduction, a review of molecular defects in the androgen receptor and male sexual development provides several general conclusions [25]. First, mutations in the open reading frame that cause premature termination, aberrant splicing or exonic deletions, and result in incomplete ligand or DNA binding domains are associated with complete resistance to the ligand. In contrast, amino acid substitutions are able to provide a range of phenotypic outcomes depending on the level of receptor function that remains in the target tissues.

4. SNPs with no direct functional effect can provide significant results in association studies due to linkage disequilibrium

There are synonymous coding SNPs (cSNPs) that result in silent changes with no alteration in amino acid and intronic SNPs that appear to have no direct functional effect. Some of these SNPs do, however, provide significant results in association studies. This may be due to the genotyped SNP being in Linkage Disequilibrium (LD) with another polymorphism that is the causal variant. LD is simply the non-random association of the alleles of two polymorphisms. It is somewhat correlated with genomic distance separating the markers, but is more closely correlated with the commonality in ancestral origins of the two polymorphisms. It is worth noting that polymorphisms in strong LD do not have to have similar allele frequencies, for example, if Marker *A* has two alleles: *A* (frequency 50%) and *a* (frequency 50%) and Marker *B* has two alleles: *B* (frequency 90%) and *b* (frequency 10%); then if the *b* allele only occurs on a chromosome with the *a* allele the two markers will be in total LD. Particularly where there is no functional rationale for

analyzing a specific polymorphism, genotype data from multiple linked markers can be used to construct haplotypes that delineate the chromosomal segments present in the population in that region and may be more informative than analyzing each of the markers separately. Haplotype analysis allows probable locations of historical crossovers to be identified and thus allows localization of disease mutations. The human Haplotype Map or "HapMap" project is characterizing the patterns of LD across the genome in the hope that this information will help in the identification of genes for complex traits [4]. LD-based analyses will be most fruitful where each disease locus has a single susceptibility allele, as opposed to many mutations arising in different, independent haplotypes.

The "common disease common variant" hypothesis is currently popular, and common disease alleles will be the easiest to find and most promising for directing medical interventions. Mendelian disorders are often caused by many different mutations in a single gene and there is no reason to believe that some complex traits will not have similar architecture with more common but less penetrant alleles at each of several genes [30].

5. The results of an association study should be interpreted with caution

All association studies have limitations, for example, a phenomenon called population stratification can generate false positive results [5]. In studies of unrelated individuals, there is an assumption that any observed differences in frequency between the case and control groups relate to the studied phenotype. This will not always be a valid assumption as it is well known that allele frequencies vary significantly in different populations, independent of disease prevalence. If both differences in allele frequency and differences in disease prevalence exist between the case and control groups then spurious positive results may be obtained. Family bases association studies (not described in this chapter) where comparisons are made between related individuals avoid this problem but are not always appropriate, for example, where late onset or rareness of a disease prevents collection of families containing multiple affected members.

In order to avoid undue increases in the p value required for significance it is best to use biologically based *a priori* hypothesis and minimize multiple testing. Significant results in one association study must be confirmed via one or more independent replication studies. Recently over 300 association studies of common variants in a variety of genes were included in a meta-analysis to examine the likelihood of replication and it was concluded that an initial significant finding, when followed by a second study with $p < 0.001$ or two studies with $p < 0.01$ are both is strongly predictive of future replication [23]. Even if a study has been successfully replicated there may be heterogeneity across different ethnic groups.

6. Polymorphisms occur in hormone receptor genes

Currently SNPs in every gene are being collated in a public SNP database (http://www.ncbi.nlm.nih.gov/SNP). To date there are around 300 *estrogen receptor alpha* SNPs; of these one is in the 5′ untranslated region, 12 are in the 3′ untranslated region, and four cause synonymous changes at the codons for amino acid positions 10, 87, 325 and 594. The remainder of the SNPs are intronic. The *estrogen receptor beta* gene is smaller and

only around 100 SNPs are so far listed for it, however, they are distributed in approximately the same manner with six in the untranslated regions, three that cause synonymous changes (at amino acid positions: 156, 328 and 392) and the remainder are intronic. Of around 100 SNPs listed for the *androgen receptor* two causes synonymous changes (at amino acids 213 and 839) while another SNP may cause a non-synonymous change from Alanine (Ala, A) to Aspartic Acid (Asp, D) at amino acid 646. This conversion of a non-polar to an acidic side chain might have functional consequences, however, this and many of the other SNPs have not yet been validated and are only suspected of being true polymorphisms. The SNPs are identified by the alignment of DNA sequence from a handful of individuals and may be due to a sequencing error; this is determined experimentally by genotyping the suspected SNP in a large sample of individuals (for example, 100 people) which, if the SNP exists, will provide an estimate of its heterozygosity (the frequency of heterozygous individuals for that marker).

7. Homozygosity for a premature stop codon in *estrogen receptor alpha* causes multiple abnormalities

One of the first descriptions of human phenotypic changes due to inherited abnormalities of an estrogen receptor was that of a man with estrogen resistance [37]. The linear growth of this individual continued into adulthood (he was 204 cm or approximately 6 ft 8 in. tall) and his bone mineral density (BMD) was several standard deviations below what would be expected for an adult of similar age. His blood lipid concentrations were also outside the normal range and a medical examination revealed that he suffered from atherosclerotic disease that was unusually advanced for his age. He had elevated levels of endogenous estrogens and administration of high doses of exogenous estrogen had no detectable effect on his abnormalities. DNA sequencing showed a cytosine-to-thymine transition at both alleles of codon 157, in exon two of *estrogen receptor alpha*, resulting in a change from CGA (Arg) to TGA (stop). It was discovered that his parents were first cousins and each was a heterozygous carrier of the mutation that created the premature stop codon. Such examples of homozygous null individuals, produced by rare consanguineous marriages can be equivalent to having a "human knockout" for the gene in question and provide a unique insight into the gene's functions, and help identify genes in which common variants may play a role in physiological variability in the general population. This individual provided evidence that suggested that estrogen and estrogen receptor alpha signaling is important in skeletal maturation and mineralization in men as well as women and also that variation in estrogen receptor function may relate to coronary artery disease risk in men. We have recently shown, using a sample of around 1700 unrelated individuals from the Framingham Heart Study, that there is in fact a significant association of genotype of an *Esr1* polymorphism (*PvuII* in intron 1, discussed in section 9 of this chapter) with a substantial increase in risk of Cardiovascular Disease [35].

Just before this man with estrogen resistance was identified the first *estrogen receptor alpha* knock-out mouse (*αERKO*) was generated (reviewed in Ref. [9]). The male *αERKO* mice had decreased BMD, similar to the man with estrogen insensitivity. These mice also

provided evidence to support a role for estrogen receptor alpha in determining bone lengthening, as the mice had bones that were significantly shorter and also smaller in diameter. This size difference was common to both male and female mice, but in contrast to the male mice with reduced BMD the female mice had normal BMD [16]. This example shows the insight that can be gained from experimental animals, but emphasizes how results may not be identical from one species to the next or one gender to the other.

8. Expansion of a protein coding CAG repeat in exon 1 of the *androgen receptor* causes spinal and bulbar muscular atrophy

Spinal and bulbar muscular atrophy (SBMA or Kennedy disease) is characterized by progressive neuromuscular weakness and wasting due to loss of motor neurons in the spinal cord and brain stem and is a representative of the inherited "triplet repeat diseases". SBMA is caused by expansion of a polymorphic CAG repeat in exon 1 of the *androgen receptor*, located on the X chromosome (at Xq11-q12), reviewed in Ref. [6]. In SBMA patients, there are between 40 and 66 tandem copies of the CAG while normally there are only between 7 and 31 repeats. As with other triplet repeat diseases (e.g. Huntington's disease, an autosomal dominant neurological disorder) age of onset correlates with the number of repeats, longer CAG tracts correlate with earlier age of onset and greater severity of the symptoms of the disease. The CAG repeats encode a stretch of polyglutamines that are not directly involved with DNA or ligand binding. The mutant protein misfolds, forms aggregates and interacts abnormally with other proteins. There is evidence of increased androgen receptor degradation, and altered post-translational modification, affected men show symptoms of androgen insensitivity.

As the androgen receptor is on the X chromosome, men with SBMA have a single allele with an expanded CAG repeat. In women, two expanded alleles are required for symptoms that may be absent altogether or limited to occasional muscle cramps. It has been suggested that the gender difference in severity is due to men having a higher level of androgen receptor stimulation that results in a higher impact of abnormal transcriptional regulation, or the effects of random X chromosome inactivation in women.

The expanded triplet repeat tracts are hypervariable, undergoing alteration in approximately a quarter of all meioses, with a larger mutation rate in male meioses than in female meioses. This frequency of mutation is extremely high when compared to SNPs which have a mutation rate of 10^{-6}, while the microsatellites in non-coding regions have mutation rates of 10^{-3} per locus per gamete per generation [40]. The triplet repeat diseases are believed to be rare and the repeat expansions that cause them should not be confused with the polymorphic microsatellites (di-, tri- or tetranucleotide repeats) that occur extensively throughout the non-protein coding regions of the genome.

9. Intronic polymorphisms in the *estrogen receptors* may be associated with bone mineral density

In contrast to the rare Mendelian disorders such as SBMA where there is a direct causal relationship between a single genetic defect and a disease, the majority of commonly

occurring diseases have a complex etiology that includes both genetic and environmental components. The current difficulty with trying to comment on sex differences with respect to hormone receptor polymorphisms in complex traits is that this area of study is at a very early stage. In many instances it has not yet been determined which variants are significant in association studies in one sex, let alone compare that data with the other sex. A good example is BMD which has a heritability estimated at between 0.5 and 0.6 [18], larger than many other complex traits, and is a risk factor for osteoporotic fracture. There is evidence that in both men and women BMD shares common hormonal determinants [13,20]. There have been multiple studies of several *estrogen receptor alpha* variants with BMD [1,2,19, 29,33,34]. Most of these studies have examined two SNPs in intron 1 by Restriction Fragment Length Polymorphism (RFLP) analysis, one of the first techniques used to detect polymorphisms, with the restriction enzymes *PvuII* and *XbaI*. There was inconsistency amongst the studies, most of which were carried out on 100 or 200 women. Recently a meta-analysis that combined the results from 30 study groups, with a total of almost 6000 women found that the *XbaI* variant remained significantly associated with BMD [17]. There are relatively few studies of these variants in men, however, in a study of approximately 700 women and 700 men from the prospective population-based offspring cohort of the Framingham Heart Study which is based in Massachusetts, no associations were found between *XbaI* and *PvuII* genotypes and several measures of BMD (unpublished observations from our group). While a similar European study of around 1000 men and 1000 women, carried out haplotype analysis that included the *PvuII* and *XbaI* polymorphisms along with a TA repeat, and obtained significant results in women but not in men [38]. Unpublished results from our group show that in men there is also significant interaction between smoking status, *ESR1*, *PvuII* and *XbaI* genotypes and bone mineral density. There is no known function that is altered by the *XbaI* variant, while the PvuII allele determines whether a putative binding site for the myb family of transcription factors is present or absent. We have recently shown that the *PvuII* SNP genotype is associated with risk of myocardial infarction in men; there is no equivalent data in women [35]. This interaction of intron 1 variation with multiple phenotypes and environmental constituents emphasizes the complexity of the genetics of complex traits, a subject area only starting to be investigated in any depth. Given the level of environmental variation that may not be accounted for, it may not be surprising that the results of association studies often vary from study to study.

Estrogen receptor alpha was originally thought to be the only estrogen receptor but more recently a second receptor, estrogen receptor beta was identified. Both receptors are expressed in osteoblast and osteoclast cells in the bone and may be important in determining BMD. In mice lacking *estrogen receptor beta*, decreased bone resorption and increased trabecular bone volume has been observed in females but not in males [39,42].

The first human study of the *estrogen receptor beta* gene and BMD identified a significant association of an allele of an intronic dinucleotide (CA) repeat polymorphism, D14S1026, with lumbar BMD in a sample of 204 healthy post-menopausal Japanese women [26]. A second study found significant association of a D14S1026 genotype with both lumbar spine and femoral BMD measures in 120 pre-menopausal but not in 205 post-menopausal women from China [22]. In older individuals, bone density is a function of

peak bone mass and also the rate of subsequent bone loss; thus the age of individuals studied from a single population may be a cause of variability in significance of such results. The relationship between the CA repeat and four other polymorphisms in *estrogen receptor beta* and measurements of hip (femoral neck, Ward's area, and trochanter) and lumbar spine (L2-L4) BMD in 1518 unrelated participants (723 men and 795 women) from the Framingham Study (Ref. [36] and unpublished observations from our group) found significant association of the CA repeat with measures of femoral but not spinal BMD, in both men and women. The large studies had differences in BMD of around 4 or 5%; thus it will be important in the future to study populations large enough to have power to detect the effects of gene–gene and gene–environment interactions which may have a significant impact that has not yet been identified.

10. A dinucleotide repeat polymorphism in the promoter of *estrogen receptor alpha* may have opposite effects on behavior in men and women

The TA repeat polymorphism in the promoter of *estrogen receptor alpha*, that was included in some of the studies of BMD outlined earlier has over 20 different alleles, each defined by the number of TA repeats (between 9 and 27). Unlike the CAG repeat that we described in the *androgen receptor* this polymorphism is in the promoter region of the gene encoding *estrogen receptor alpha* and, therefore, does not directly cause any variation or defect in the estrogen receptor alpha protein sequence. However, there is evidence to support the hypothesis that the length of the microsatellite allele affects some promoter function [7,31]. In this particular example, the microsatellite may be close to an enhancer element involved in regulation of estrogen receptor alpha expression [2] and thus the different alleles affect expression of estrogen receptor alpha and may alter the total number of intact receptors. In women, short TA repeat alleles have been associated with yet another phenotype – high anxiety scores, while in men the opposite result has been observed with longer alleles associated with high anxiety scores [8,41]. The possibility that specific variants in the *estrogen receptor alpha* gene have opposite effects in men and women is supported by evidence from experimental animals where males lacking *estrogen receptor alpha* are less aggressive while females are more aggressive than wild-type mice [27,28]. Although neither anxiety association results in men or women have yet been replicated in other studies, this type of sex difference is likely to generalize to other variants and other genes. It is thus important to carry out *steroid receptor* association studies in both men and women and to analyze the results separately in the two sexes.

11. *Estrogen receptor* genotype may be associated with response to hormone therapy

There is evidence from study of around 300 women, randomly assigned to hormone therapy or to a placebo that individuals with one specific *estrogen receptor alpha* genotype have a significantly greater increase in HDL-cholesterol level in response to treatment when compared to individuals with the other genotypes [15]. This response is most pronounced for HDL subfraction 3 (HDL_3) and may support a role for estrogen receptor

alpha in lipid metabolism, as was suggested by characterization of the individual described earlier with estrogen resistance. In a study of over 400 post-menopausal women there was evidence of an interaction between hormone therapy and a two locus genotype that included the same *PvuII* SNP in *estrogen receptor alpha* that was reported in the study of HDL-cholesterol [15] and another SNP in the vitamin D receptor [12]. The two-locus genotype, present with a frequency of 10%, appeared to be responsible for over 30% of the total increase in bone density in women on hormone therapy for more than 5 years. Although these studies of estrogen therapy were small and have not been replicated, it is exciting to think that this may just be the first of many studies that will ultimately define a set of genetic tests that may allow appropriate targeting of estrogen and other hormone related therapies.

12. Summary and future directions

1. The polymorphisms associated with a disease may cause an altered amino acid or premature stop codon, aberrent splicing, alteration of some promoter function, or simply be in LD with another variant that affects one of these things.
2. Currently there have been few association studies of steroid receptor polymorphisms that include both men and women. Of those that there have been, for example in the estrogen receptors, the results in men and women may be quite different, even opposite to one another.
3. Studies of rare Mendelian disorders may provide information of use in directing investigations of common diseases with complex modes of inheritance.
4. The architecture of complex traits remains to be studied; it is unclear whether many will result from one or two common polymorphisms that will be easiest to find and clinically most useful or whether there may be a large number of rarer alleles affecting a single outcome.
5. Identification of genotypes that determine responsiveness to treatment by currently approved drugs is a clinically important goal. The fact that many such drugs act via hormone receptors makes these important targets for genetic studies. In addition, such studies may provide information to help design therapeutics for treatment of many of the commonly occurring, genetically complex diseases.

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Sex, hormones and the endothelium

Stephanie J. Murphy, Marguerite T. Littleton-Kearney, Louise D. McCullough and Patricia D. Hurn

1. Introduction

The vascular endothelium contributes actively to regulation of vascular tone through release of vasoactive factors towards the vascular smooth muscle. In addition, endothelium-derived factors released into the blood affect platelet aggregation and activation of leukocytes. Expression of adhesion molecules on the endothelial surface is necessary for leukocyte interaction with the vascular wall. This chapter will review central concepts of how sex steroids impact endothelial functions.

2. Estrogen

17β-estradiol (E2) is the major circulating estrogen in mammals and the most potent mediator of estrogenic activity in both sexes. Although 17β-estradiol is the best studied of the natural estrogens, its catabolic products, estrone and estriol, also bind estrogen receptors (ER) and may influence vascular performance. This section will focus on key concepts in E2's actions on vascular endothelium. Detailed literature reviews are found elsewhere [5,8,16].

2.1. Effects on blood flow

Effects of endogenous cyclic variation in estrogens on blood flow have been evaluated in conducting arteries and arterioles to reproductive organs and non-reproductive organs such as skin and skeletal muscle. However, much of the E2's role in vascular physiology has been demonstrated through studies of estrogen deficiency in post-menopausal women and in surgically ovariectomized animals prior to and following estrogen therapy (ET). From all of these studies, several general tenets can be identified. First, E2 promotes vasorelaxation, the overall result being increased blood flow and decreased vascular resistance. Hence, E2 has been classified as an anti-ischemic hormone. Second, native estrogens and exogenous estrogens improve endothelial function, including vasomotion,

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when administered to healthy but E2-deprived vessels or to atherosclerotic vessels. For example, early studies showed that endothelium-dependent vasodilation is impaired in aortic and femoral arteries from ovariectomized animals and restored by exogenous E2 [3]. In classic studies of atherosclerotic monkey coronary arteries, E2 partially restores dilator responses to acetylcholine, while ovariectomy produces constriction [22]. Third, E2 modulates vasoconstrictor stimuli, altering the net balance of signaling in a regional circulation toward dilation. Lastly, while these generalized properties are useful, it must be emphasized that vascular sensitivity varies in response to native estrogens or ET. In order to predict sensitivity, one must consider differences among regional circulations, among vessel type (conducting vs. resistance vessels), pharmacological variables such as dose and exposure duration, and the potentially complicating factor of progestin co-administration. However, the vasodilatory effects of estrogen seem to be independent of genetic sex, that is, XX and XY genome.

To affect change in blood flow, estrogen affects functional characteristics of both smooth muscle and endothelium. Effects on the smooth muscle are discussed in Chapters 7 and 8. Here the focus will be on effects of the endothelium. Actions of estrogen on the endothelium can be classified as three equal functions. First, estrogen promotes release of endothelium-derived factors. Second, estrogen promotes an anti-inflammatory endothelial surface by reducing expression of adhesion molecules and suspension of apoptosis. Third, estrogen promotes proliferation and migration of endothelial cells to damaged blood vessels and stimulates tube formation which is essential for angiogenesis, although blood vessels of different anatomical origin respond differently to a given steroid concentration [9]. In general, acute application of estrogen to isolated blood vessels causes dose-dependent relaxations associated with release of nitric oxide, prostacyclin and inhibition of endothelin-1 (Table 1).

2.2. *Mechanisms of estradiol actions*

Because there are partial or full estrogen responsive elements present in the promoters of some vascular genes, a prevailing hypothesis is that E2 amplifies transcription of vascular mediators. Both known ER subtypes have been identified in a wide variety of vessel types and in vascular endothelium of both sexes and polymorphisms in estrogen receptor alpha are associated with vascular disease in men. Vascular ER expression is not static and can be altered by ER ligand availability and reduced by atherosclerosis. However, E2 also has acute non-genomic actions, in some cases ER-mediated; so it is likely that multiple signaling pathways work in integration to elicit cell responses. E2's nuclear transcriptional action can also be integrated with membrane-associated rapid signaling, i.e. these two properties are not necessarily distinct steroid mechanisms operating independently (for details, see Chapters 3–5).

Regulation of vascular genes. Genes important to endothelium-dependent vasodilation, cell growth, migration and proliferation, maintenance of structure and shape, atherogenesis and inflammation are responsive to estradiol. In some cases, the gene's promoter contains estrogen-responsive-elements. Representative genes include cyclooxygenase-1 (COX-1), inducible COX-2, prostacyclin synthase, endothelial and inducible isoforms of

Table 1
Sex and estradiol effects on endothelium in vitro

Source/type	Dose/concentration	Mechanism
Reproductive tissue vessels		
Guinea pig, uterine artery	Pregnant vs. non-pregnant	Endothelium dependent; NO mediated
Human, uterine artery	Menstrual cycle	Endothelium dependent; multiple mediators
Ovine, uterine artery	Chronic 100 μg/day for 3 days	Mediated via NO
Coronary vessels		
Rat, coronary artery	Female, randomly cycling vs. male	Endothelium dependent; regulated by endothelial Ca^{2+} and NO
Porcine, coronary artery	Acute 10^{-9} M (2 h) and chronic (18–22 h)	Endothelium dependent; ER and NO mediated
Cerebral vessels		
Rat, middle cerebral artery	Females vs. male, castrates with replacement	Endothelium dependent; NO and COX mechanisms
Mouse, middle cerebral	Females vs. male, castrates with replacement	Endothelium dependent; NO and COX mechanisms
Rat, middle cerebral artery	Male vs. female, castrates with replacement	Reduced EDHF-mediated dilation by mechanism involving $K^{+}_{(CA)}$ channels
Conducting vessels		
Rabbit, aorta	Female vs. male	Endothelium dependent; increased basal release of NO
Rat, aorta	Pregnant vs. non-pregnant; plasma values not reported	Increased NO release and eNOS gene expression
Rat, aorta	Water soluble formulation, cyclodextrin estradiol	Requires activation of eNOS through Hsp 90/Akt mechanisms
Rabbit, femoral artery	Chronic: 100 μg daily, 4 days in vivo	Endothelium mediated; not through prostacyclin
Rat, tail artery	Acute 10^{-7}–10^{-5} M	Endothelium dependent; non-genomic
Resistance vessels		
Rat, mesenteric artery	(1) Chronic 10μg/day (2) Cycling female	Enhances endothelium dependent relaxation
Rat, mesenteric artery	Acute 10^{-7} M	Endothelium dependent
Rat, mesenteric (180–260 μM)	Acute 10^{-7}–10^{-5} M	ER independent; inhibition of calcium release
Rat, mesenteric artery	Acute10^{-6} M	ER and NO independent; plasma membrane mechanism
Rat, mesenteric artery	Acute 10^{-5} M	Endothelium independent relaxation
Microcirculation		
Rat, gracilis muscle	Chronic (50 μg/kg) for 3 weeks	NO mechanism
Rat, gracilis muscle	Chronic (50μg/kg)	EDHF dependent
Rat, muscle arterioles	Acute 10^{-10}–10^{-4}M	Endothelium dependent, NO mechanism

(*continued*)

Table 1
continued

Source/type	Dose/concentration	Mechanism
Veins		
Porcine, femoral vein	Acute 10^{-9}–10^{-5} M	Endothelium dependent; NO and K^+ channel mechanisms, ER independent

Abbreviations: Ach, acetylcholine; K^{2+}_{Ca}, calcium dependent K^+ channel; COX, cyclooxygenase; EDHF, endothelium dependent hyperpolarizing factor; eNOS, endothelial nitric oxide synthase; ER, estrogen receptor; Hsp, heat shock protein; NO, nitric oxide.

nitric oxide synthase (NOS), caveolin-1, pre-proendothelin-1, matrix metalloproteinases 1 and 2, vascular endothelial growth factor, heat shock proteins 25/27 and 70, angiotensin II type 1 receptor and NADPH oxidase. The importance of E2-induced vascular gene transcription (both increases and decreases) to vascular behavior has been well documented for the NOS and COX isoforms and to some degree for endothelin-1. In addition to classical steroid actions at the nucleus, rapid E2 signaling via intracellular kinases can also lead to gene transcription, achieving a net cell-specific effect [5].

Non-genomic vasomotor signaling. Rapid signaling mechanisms are particularly relevant to how E2 impacts endothelial cell function. Exposure to E2 rapidly activates calcium flux, cAMP, phospholipase C, inositol triphosphate (IP3), G proteins and at least three mitogen-activated protein kinases (MAPK) pathways. These include the serine/-threonine kinase ERK (extracellular-regulated kinase), phosphoinositol-3 hydroxy kinase (PI3K)-Akt kinase and c-Jun N-terminal kinase (JNK). These signaling pathways modulate migration and proliferation of endothelial cells, functions important in vascular repair.

Rapid signaling may be mediated by known ERs or by novel ERs with pharmacological profiles that differ from ERα or ERβ. Characterization of such novel receptors is an intensely active area of investigation at present. A detailed description of genomic and non-genomic actions of steroids can be found in Chapters 3 and 4.

Signaling pathway estradiol–ER–eNO. The best characterization of estrogen's effects on endothelial cells is modulation of endothelium-derived nitric oxide (NO). NO, as a free radical and a gas, is the endogenous physiological nitrovasodilator. Not only does the molecule cause dilation of vascular smooth muscle and inhibit proliferation of vascular smooth muscle, but it also inhibits platelet aggregation. This pathway will be discussed in detail as an example of how other steroids may affect endothelial function. E2 infusion causes rapid release of endothelial NO (eNO). Furthermore, physiological concentrations of E2 increase eNOS activity within 5 min in cultured endothelium. The interactions between E2 and endothelial NOS are complex and multi-factorial. A combination of mechanisms may work together to enhance NO bioavailability within vascular cells, i.e. E2 can increase eNOS transcription directly, limit inactivation of NO through biochemical pathways and/or utilize non-genomic signaling to increase enzyme activity. Recent work has begun to elucidate this latter signaling pathway. Numerous studies demonstrate that ERα is important in E2's rapid activation of eNOS. ERα may partner with $G\alpha_i$ protein

and/or one or more intracellular kinase cascades to then subsequently activate eNOS. ERα–eNOS signaling is localized to endothelial cell plasma membrane domains known as caveolae where a subpopulation of ERα co-localizes with structural coat protein caveolin-1. Chambliss et al. [1] have proposed the existence of a "steroid receptor fast-action complex" within caveolae, where ERα and eNOS are co-localized along with G proteins that mediate downstream events (see Fig. 1 of Chapter 4).

The identity of the downstream kinases involved in this pathway is under active investigation. Membrane impermeant forms of E2 activate endothelial cell MAP kinase via the ER, then stimulate cGMP production and NO release [13]. A second kinase transduction mechanism has been identified, where E2 signals through PI3 kinase and downstream serine–threonine protein kinase Akt to phosphorylate and activate eNOS. ERα, not ERβ, physically couples with the p85α regulatory subunit of PI3 kinase, triggering the production of intracellular phosphoinositides, activation of Akt and production of eNO [15]. In E2 treated endothelial cells, this signaling process requires approximately 20 min for maximum effect. Whether these events occur in vivo is not clear. The E2–eNOS rapid signaling pathway continues to be intensely studied, but remains controversial in several aspects. One of the key unknowns is the precise identity of the membrane-associated ER that is necessary and sufficient for E2's non-genomic release of NO.

Signaling pathway estradiol–ER–Prostacyclin. E2 also acts, at least in part, by non-genomic mechanisms to increase prostanoid products of arachidonic acid metabolism by the cyclooxygenases. One such product is prostacyclin which reduces platelet aggregation. E2 increases prostacyclin (6-keto-prostaglandin F1α) in human and animal endothelial cells by non-transcriptional mechanisms. In pulmonary artery endothelial cells, E2 activates prostacyclin synthesis at a threshold of 10^{-10} M within 5 min, an effect that is ERβ dependent but not accompanied by increased COX1 or COX2 protein [14]. Further work will be required to understand the functional significance of this pathway.

Non-cell type specific actions. Many estrogens have well established, lipid antioxidant activity, particularly the catechol estrogens (2-hydroxy estrone and 2-hydroxy estradiol), estrone and 17β estradiol. Lipid peroxidation is one of the initiating reactions causing endothelial damage concomitant with development of atherosclerosis. E2 reduces lipid peroxidation by inactivating the hydrogen peroxide, superoxide and hydroperoxyl radicals that oxidize lipoproteins. The action of estrogens may be important to limit oxidative stress in endothelial cells associated in early development of atherosclerosis. Because this action of estrogen involves a chemical interaction, effects would most likely occur in both men and women.

3. Progestins

Progesterone, a natural progestational hormone produced cyclically by the ovarian corpus luteum, is increasingly recognized as a modulator of vascular function and susceptibility to vascular disease. Many synthetic progestins are of interest because of their use in hormone therapy or as contraceptives. These exogenous progestins can have variable and even opposing vascular endothelial effects to that of progesterone.

3.1. Effects on blood flow

Progesterone appears to have a wide range of effects on systemic and local vascular flow and resistance (for review, see Ref. [8]). There are large regional differences as well as species differences in the steroid's ability to act as a vasodilator in vascular beds. For example, acute administration decreases forearm blood flow and increases local vascular resistance in women. In female pigs, intravenous progesterone dilates the mesenteric, renal and coronary beds without changing systemic blood pressure. In contrast, the steroid does not alter coronary blood flow in rabbit. Variances in the progestin type utilized in experimental studies cloud interpretation of sex-linked vascular effects. While progesterone increases forearm blood flow in women, treatment with medroxyprogesterone acetate (MPA) produces no such effect in men. This statement may imply a sex difference in response to progestin or simply an effect of formulation. Lastly, some investigations have focused on the importance of progestins only in relation to their actions when given in combination with estrogen. For example, in some but not all series, MPA attenuates estrogen's capacity to enhance endothelium-dependent vasodilation in postmenopausal women (without altering estrogen's atheroprotective and antioxidant effects). In many animal studies, progesterone antagonizes estrogen-induced increases in regional blood flow (e.g. uterine and coronary circulation).

These observations in vascular beds are in contrast to observations obtained from isolated arteries where acute application causes relaxation. However, there is poor agreement as to whether this dilator action is endothelium-dependent. Similarly, there is little agreement as to progesterone's robust ability to enhance endothelium-dependent responsivity to dilator agonists such as acetylcholine. An early report suggests that progesterone administered to dogs does not enhance endothelium-dependent relaxation in subsequent studies of isolated coronary vascular rings [6]. Subsequent investigations of isolated vessels treated acutely with progesterone described the steroid's ability to augment endothelium-dependent and independent vasodilation, once again with regional vessel and species-related differences. For example, progesterone induces endothelium-dependent relaxation in rat mesenteric artery, but endothelium-independent relaxation in human placental arteries and veins [11]. Synthetic progestins such as chlormadinone acetate (CMA) and norethisterone acetate (NETA) produce reversible endothelium-independent vasorelaxation in aortic vessels. However, progestin action on the endothelium is readily evident in diseased vessels. For instance, oral MPA in ovariectomized monkeys improves impaired endothelium-dependent dilation of atherosclerotic coronary arteries [23]. Studies of progestin/estrogen co-administration suggest that progestins such as MPA diminish estrogen's ability to restore endothelium-dependent vasodilation in atherosclerotic arteries. However, undesirable interactions between the hormones are not universal and appear linked to the type of progestin formulation.

3.2. Other effects on endothelium

Study of endothelial cells in culture allows for detailed analysis of progesterone's effect that help to explain apparent disparate vascular actions in vivo. In vitro exposure of human endothelial cells to progesterone over several days suppresses endothelial cell

proliferation, inhibits migration and alters secretion of extracellular matrix proteins. The steroid also inhibits progression through the cell cycle, an effect requiring a PR-mediated mechanism. These data suggest that chronic PR activation could have deleterious effects on growth and maintenance of endothelial cell cultures. In cultured endothelial cells, progesterone (but not MPA) represses expression of tumor necrosis factor (TNF)-α-activated vascular cell adhesion molecule-1 (VCAM-1) expression. This is important because VCAM-1 mediates mononuclear leukocyte-selective adhesion to vascular endothelium, an important step in the pathogenesis of atherosclerosis.

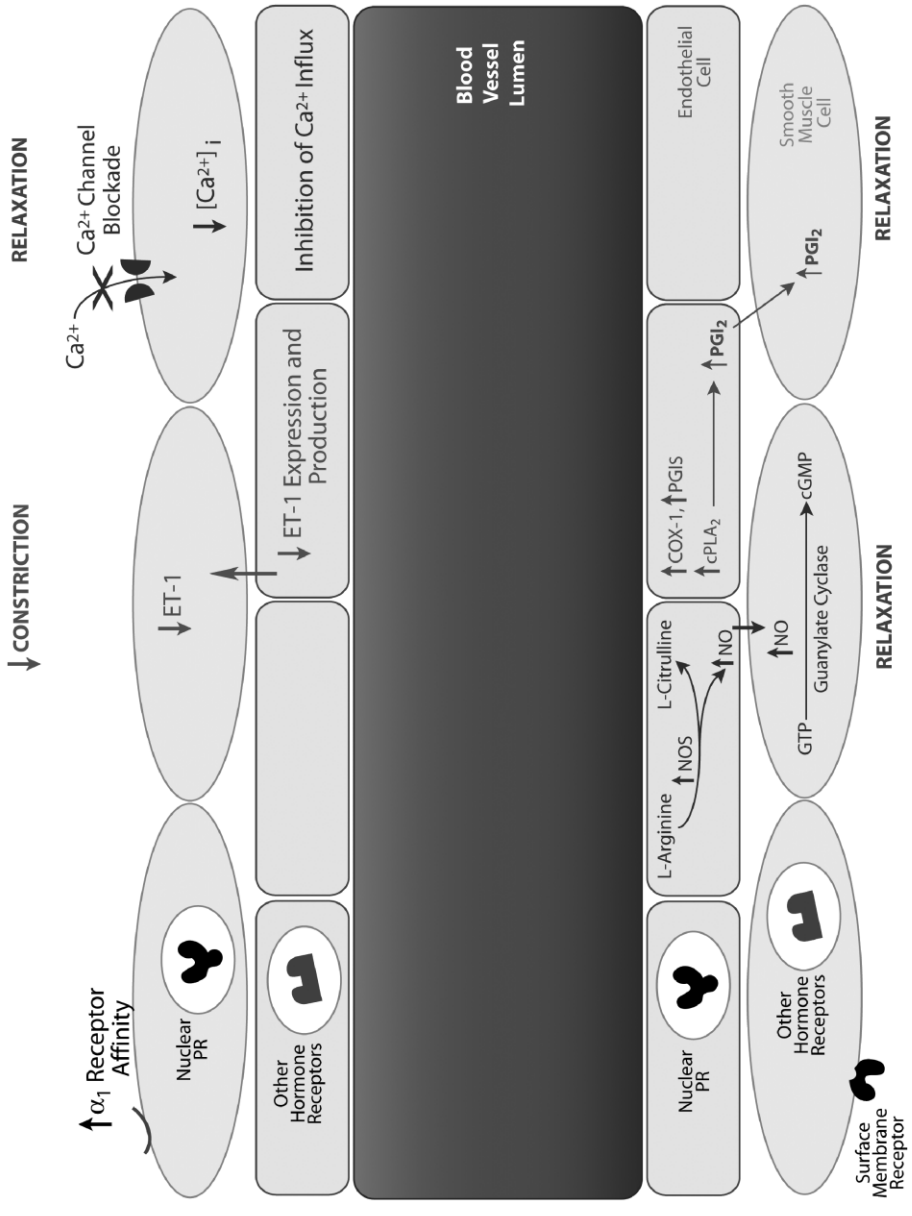
Another process implicated in the development of atherosclerosis is adherence of platelets to the vascular wall. Two products of arachidonic acid metabolism, thromboxane and prostacyclin (PGI_2), promote and inhibit platelet aggregation, respectively. Progesterone significantly reduces thromboxane A_2 receptor ($Tx A_2 R$) density in endothelium of non-human primate coronary arteries. Alternatively, progesterone increases expression of several enzymes involved with PGI_2 synthesis in uterine and systemic arteries. Whether this leads to enhanced PGI_2 is not clear, and the sex specificity of these responses has not been identified. These observations suggest that progesterones may interact with thromboxane and/or PGI_2 mechanisms in protecting vascular endothelium.

3.3. Mechanisms of progesterone signaling

A number of signaling pathways have been implicated in progesterone's actions in endothelium (Fig. 1), suggesting that both rapid signaling and transcriptional mechanisms could be important.

Progesterone receptors (PRs). Progesterone may mediate its vascular endothelial effects via intracellular receptors. Currently, there are two recognized isoforms (A and B) of the human PR. Expression of vascular endothelial PRs has been controversial, primarily due to variability of vessel types examined, species investigated and techniques employed. Nevertheless, endothelial PRs have been found in both arteries and veins. Arterial PR levels are responsive to pregnancy and changing hormone cycles in uterine artery in females, although it is unclear if fluctuations occur in vessels not associated with reproductive tissue or in males. For example, PR concentrations in uterine artery are highest during the follicular phase of the menses (low serum progesterone, high estradiol) and decline during the early luteal phase (rising progesterone). During menopause, PRs decrease. Estradiol is well recognized for its induction of PR by ER-mediated transcriptional action, whereas progesterone decreases PR expression. The presence of PRs in blood vessels and the sensitivity of PR level to stimuli offers some support for the hypothesis that progesterone, alone or in combination with estrogen, modulates blood flow through a PR-mediated pathway.

Other hormone receptors. Many of the synthetic progestins bind to non-PR endothelial hormone receptors thus allowing these compounds to express additional non-progestogenic effects. For instance, CMA and cyproterone acetate competitively bind and have a high affinity for androgen receptors thus exhibiting potent anti-androgenic activity.


↓CONSTRICTION
RELAXATION
↑α1 Receptor Affinity
Nuclear PR
Other Hormone Receptors
Surface Membrane Receptor
↓ET-1
Ca2+
Ca2+ Channel Blockade
↓[Ca2+]i
↓ET-1 Expression and Production
Inhibition of Ca2+ Influx
Blood Vessel Lumen
L-Arginine
↑NOS
L-Citrulline
↑NO
↑COX-1, ↑PGIS
↑cPLA2
↑PGI2
Endothelial Cell
↑NO
GTP
Guanylate Cyclase
cGMP
↑PGI2
Smooth Muscle Cell
RELAXATION
RELAXATION

Drospirenone is a unique progestin in that it is a spironolactone analogue, an aldosterone antagonist, with demonstrated anti-mineralocorticoid activity.

Vascular mediators. A number of putative mediators are under current study as endothelial targets of progesterone. There is evidence that progesterone can mediate a balance between endothelium-derived vasodilators and vasoconstrictors in hormone sensitive vessels (Table 2, Fig. 1).

A majority of evidence implicates NO in progesterone's ability to produce or augment endothelium-dependent relaxation. Progesterone treatment increases systemic plasma levels of NO in ovariectomized animals and enhances endothelial NOS protein expression in a regionally dependent manner, for example, upregulating NOS in uterine endothelium but not in systemic arterial endothelium [12]. In contrast, the steroid increases NOS activity in many vascular preparations, including aortic rings and mesenteric, renal and coronary vessels. Acute relaxation of isolated rabbit pulmonary arteries by progesterone is also NO and cGMP dependent. These studies would suggest an NO-mediated non-genomic mechanism for the more rapid vascular effects of progesterone.

Like estrogen, progesterone also modulates intracellular calcium. Calcium homeostasis is crucial to endothelial cell function and, therefore, alterations in calcium flux and modulation of calcium channels may serve as possible non-genomic vascular mechanisms for progesterone's effects in endothelium. For instance, progesterone inhibits both thapsigargin- and endothelin 1-induced increases in cytosolic calcium concentration in aortic endothelial cells in situ, suggesting an action involving inhibition of calcium influx [20].

Vascular endothelial cells produce and release endothelin-1 (ET-1), a potent vasoconstrictor and mitogen for vascular smooth muscle cells. In cultured bovine aortic endothelial cells, progesterone inhibits ET-1 through a negative transcriptional regulatory mechanism [7]. Some work in animal models have examined sex differences in response to ET-1 and the effects of sex steroids on ET-1 production and release. Previously, sex differences in activation of the ET-1 pathway have been demonstrated in deoxycorticosterone (DOCA)-salt rats. Ovariectomy in female DOCA-salt rats increases mesenteric arterial mRNA expression of ET-1 and endothelin receptor β subtype relative to intact

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Fig. 1. Candidate mechanisms of progesterone signaling. Progesterone's overall vasodilatory effect on vasculature may be mediated through both genomic and non-genomic signaling mechanisms. Genomic mechanisms involve direct interaction of progesterone with its receptor, as well as synthetic progestins with both the PR and other hormone receptors. Candidate target genes that are regulated by genomic progesterone pathways are yet to be clearly identified. However, progesterone is known to increase expression and activity of several enzymes involved with PGI_2 synthesis (COX-1, $cPLA_2$, PGIS) and NO production (NOS), factors which contribute to vasodilatation. Progesterone also decreases expression and production of some endothelial-derived factors like the vasoconstrictor, ET-1, thereby promoting vessel relaxation. Non-genomic progesterone signaling pathways can involve cell surface receptors such as a putative surface membrane receptor for progesterone. These pathways can also include Ca^{2+} ion channel interactions and effects on intracellular Ca^{2+} itself as a second messenger, leading to relaxation of blood vessels. Progesterone is known to increase α_1-adrenergic receptor binding affinity. Calcium (Ca^{2+}), Cyclooxygenase 1 (COX-1), Endothelin 1 (ET-1), Cyclic guanosine monophosphate (cGMP), Guanosine triphosphate (GTP), Nitric oxide (NO), Nitric oxide synthase (NOS), Phospholipase A_2 ($cPLA_2$), Progesterone receptor (PR), Prostacyclin (PGI_2), Prostacyclin synthetase (PGIS). With permission [8].

Table 2
Vascular mediators as endothelial targets of progesterone

Source	Vascular effect	Mediator	Progesterone signaling
Endothelium derived factors or receptors	Vasodilation	NO	8NO production and release, 8NOS/eNOS activity and expression
Receptors		PGI_2	8production of key enzymes COX-1, $cPLA_2$, PGIS) for PGI_2 production; 8PGI2 production
	Vasoconstriction	ET-1	9ET-1 expression and production
		TXA_2	No effect on TXA_2 production
Intracellular factors	Contraction when 8$[Ca^{2+}]_i$ or relaxation when 9$[Ca^{2+}]_i$	Intracellular calcium $[Ca^{2+}]_i$	Calcium channel blockade and/or calcium influx leading to relaxation

Abbreviations: COX-1, Cyclooxygenase 1; eNOS, Endothelial nitric oxide synthase; ET-1, Endothelin-1; $[Ca^{2+}]_i$, Intracellular calcium; NO, Nitric oxide; NOS, Nitric oxide synthase; $cPLA_2$, Phospholipase A_2; PGI_2, Prostacyclin; PGIS, Prostacyclin synthetase; TXA_2, Thromboxane A_2.

females, whereas hormone replacement with estradiol, with or without progesterone, normalized these responses [2]. Progesterone may not be critical in regulation of endothelin-1 in males because of low circulating levels of progesterone. However, this remains to be determined.

4. Testosterone

Compared to the female sex hormones, the effects of androgens on the endothelium have been much less studied. Therefore, relatively little is known about testosterone's role in modulating vascular function at the cellular or molecular level. Nevertheless, testosterone is clearly vasoactive in reproductive tissues and other regional circulations, and vascular endothelium may be an androgenic target.

One of the challenges associated in evaluating vascular actions of testosterone is the steroid's lability and sensitivity to environmental factors. In men, normal circulating levels of testosterone fluctuate 3-fold over the course of the day (10–30 nM), with highest levels in the morning. While most androgens originate from testicular Leydig cells, adrenal sources contribute to intravascular levels as well. Generally regulated by luteinizing hormone (LH) via LH receptors located on Leydig cell surfaces, circulating plasma levels are also greatly influenced by prolactin, cortisol, insulin, estradiol and other biochemical actors. In women, normal testosterone levels are considerably lower than men at 0.6–2.5 nM. Roughly half of the plasma steroids arise from adrenal and ovarian thecal cell sources while the remaining half originate from skin, liver and adipose tissue. An additional challenge in evaluating testosterone's role in vascular physiology arises from the fact that the androgens and ovarian hormones share a common derivative, pregnenolone, as well as several synthetic pathways and enzymes. Testosterone is

synthesized by two pathways: through progesterone metabolism to androstenedione or through conversion of 17α-hydroxypregnanolone to dihydroepiandrosterone (DHES). Androstenedione is a fundamental synthetic step for both testosterone and estrogen. Further complicating the picture, testosterone is readily converted to 17β-estradiol by a P450 aromatase or to a more potent intracellular metabolite, dihydrotestosterone or DHT by 5α-reductase. As discussed later, while many studies provide convincing evidence for testosterone as a vasodilator, others suggest that in some instances testosterone can depress vasomotor responses. No doubt these inconsistencies result from differential effects on target organs, however, local conversion of testosterone to estrogen or to another androgen must be considered.

4.1. Effects on blood flow and vascular diameter

In humans, most data arise from studies of coronary artery disease, rather than under physiological conditions. Available evidence suggests that acute testososterone infusion produces coronary vasodilation and improves perfusion. However, these data also emphasize that vasoreactivity to testosterone is highly controlled by dose or exposure duration. For example, flow-mediated brachial artery dilation is differentially responsive to testosterone. Men with established coronary artery disease demonstrate a rapid and robust endothelium-dependent vasodilation to injected steroid; however, chronic oral treatment triggers an endothelium-independent effect as well [4]. Furthermore, high-dose androgens depress endothelium-dependent dilation. There are also large regional differences in the steroid's ability to act as a vasodilator in vascular beds, as well as species differences [8].

There is convincing evidence for testosterone's sexually dimorphic action on vascular reactivity. Under most conditions, males or vessels from males are more sensitive to testosterone than are their female counterparts. For example, pulmonary and coronary arteries relax to testosterone to a greater degree in males vs. females. Furthermore, vessels from males with high androgen background relative to females are less responsive to contractile agents such as vasopressin than are females [17]. However, inherent sex-linked sensitivity to some contractile agonists (EC_{50}) can be altered by manipulating background sex steroid levels. For example, rings of the thoracic aorta are more sensitive to vasopressin-induced contraction in female rats vs. intact, testicular-feminized males (X-linked recessive defect in androgen receptor function) [18].

In isolated vessel preparations from several species and in humans, testosterone incubation triggers concentration-dependent relaxation. Testosterone concentrations as low as 100 pM elicit vasorelaxation, an effect that is not reversed by P450 aromatase inhibition [19]. Nevertheless, most of these studies have employed supra-physiological concentrations of steroid. Other studies document testosterone-induced attenuation of vasorelaxation to dilators or vasoconstriction. Nanomolar concentrations can attenuate maximal relaxation to endothelium-dependent dilators including bradykinin and A23187 [10]. Although most studies demonstrate that testosterone produces vasorelaxation or amplifies responsiveness to vasodilators, there is little agreement that an endothelial site of action is necessary or sufficient to produce vasodilation. Removal of the endothelium

partially attenuates testosterone-induced vasodilation in aortic and mesenteric vessels. However, equally important are endothelium-independent mechanisms involving activation of large conductance (BK_{ca}), voltage-activated (K_v) and ATP-dependent (K_{ATP}) potassium channels within vascular smooth muscle (see Chapter 7).

4.2. Mechanisms of testosterone signaling

The specific effectors of testosterone's vasomotor activity have not been extensively studied and remain controversial. One indirect mechanism by which testosterone could induce vasodilation is via aromatization to estradiol, but this is an incomplete explanation since several reports demonstrate that enzymatic inhibition of the P450 aromatase fails to block testosterone-induced vasodilation.

Most studies emphasize the rapidity at which vessel responses occur and consequently implicate the presence of membrane-associated non-transcriptional processes. However, the role of the androgen receptor in rapid signaling remains understudied at present. Androgen receptors are present in vascular endothelium and smooth muscle and limited evidence suggest that expression may be greater in males vs. females.

Nitric oxide may also be a mediator of testosterone's endothelial actions. Testosterone enhances dilation to endothelium and NO-dependent dilators, particularly in pathological conditions such as hypertension. Further, these rapid dilatory properties in coronary vessels can be blocked by NOS antagonists, suggesting that NO mediates the effect. Whether conversion from testosterone to estradiol is an intermediate step preceding NO activation is unclear.

5. Future directions

The role of primary sex differences in endothelial signaling independent of sex steroids has received little attention. In contrast, interest in harnessing the potential of sex steroids in preventing or ameliorating vascular disease has led to a complex clinical and experimental literature that now requires unifying hypotheses. Estradiol has been extensively examined for effects on endothelial vasomotor signaling and an integrative model is needed to account for parallel or serial genomic and non-genomic mechanisms of action in endothelium. The elegant signaling pathways that are steadily being disclosed through in vitro investigation will need validation in the intact tissue and animal. The precise identity of endothelial plasma membrane ERs will require elucidation. Progesterone has been less well studied and interactions between progesterone and estradiol require further study and clarification. Many progestins, including progesterone, antagonize E2's action in the vasculature. It is clear that progestin type, formulation and administration conditions are important factors when dissecting effects on vascular endothelium. Understanding these factors is particularly vital because progestins can have variable or even opposing vascular effects relative to the vasodilator progesterone. The presence of endothelial PRs suggests that receptor-mediated transcription could be physiologically important and potential gene targets such as ET-1 remain to be identified. Lastly, the vascular properties of androgens have

been greatly understudied and available data are replete with inconsistencies. Fundamental studies are needed to evaluate if there are basic differences in responsivity to testosterone amongst regional circulations, vessel types and vascular cell targets. Genomic sex may modulate effects of testosterone, and these limited observations require better understanding because androgens have physiological roles in both sexes. Few data are available to establish if androgen receptors are involved in testosterone's rapid vasomotor actions, and if there is signaling analogous to ERα activation of eNOS in a rapid kinase cascade.

Clarification of how steroid receptors are regulated, identification of common intracellular pathways of activation and dissection of genomic and non-genomic actions are necessary for development of novel vascular steroid-like pharmacological agents that would benefit cardiovascular function in men and women.

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Sex hormones and the vascular smooth muscle

Julia M. Orshal and Raouf A. Khalil

Abbreviations: BK_{Ca}: large conductance potassium channel; $[Ca^{2+}]_i$: intracellular free Ca^{2+} concentration; ER: estrogen receptor; HRT: hormone replacement therapy; MAP kinase: mitogen-activated protein kinase; MLC: myosin light chain; OVX: ovariectomized; $PGF_{2\alpha}$: prostaglandin $F_{2\alpha}$; Phe: phenylephrine; PKC: protein kinase C; SHR: spontaneously hypertensive rat; VSM: vascular smooth muscle; WKY: Wistar–Kyoto rat.

1. Introduction

Cardiovascular diseases such as hypertension and coronary artery disease represent some of the most common and costly diseases in the Western world. The incidence of cardiovascular diseases in men aged 30–50 is greater than women of similar age, suggesting gender-related vascular protective effects [4]. Also, the incidence of cardiovascular diseases increases after menopause in women, suggesting that female hormones may provide some protection against development of this disease. This concept is further supported by data from observational studies that hormone therapy for symptoms of menopause may also provide vascular protection [14].

Sex hormones are known to interact with cytosolic and nuclear receptors and to induce a host of genomic effects in various vascular cells. Sex hormones may also interact with specific plasmalemmal receptors and induce additional non-genomic vascular effects (Fig. 1) [9,14]. Although several reviews have described the role of genetic sex, sex hormones and sex hormone receptors in modifying the incidence of cardiovascular disease [4,9,14], little information is available regarding the changes in vascular functions with sex and gonadal hormones. Sex differences in endothelial cell function and sex hormone-induced endothelium-dependent vascular relaxation have been discussed in Chapter 6 of this book. The purpose of this chapter is to provide an insight into how genetic sex and sex hormones influence regulation of vascular smooth muscle (VSM). In this chapter, the sex differences in vascular tone and how sex hormones and their receptors contribute to these differences will be outlined first. Effects of sex hormones on VSM cell growth and proliferation will then be described and finally, a detailed description of the effects of sex

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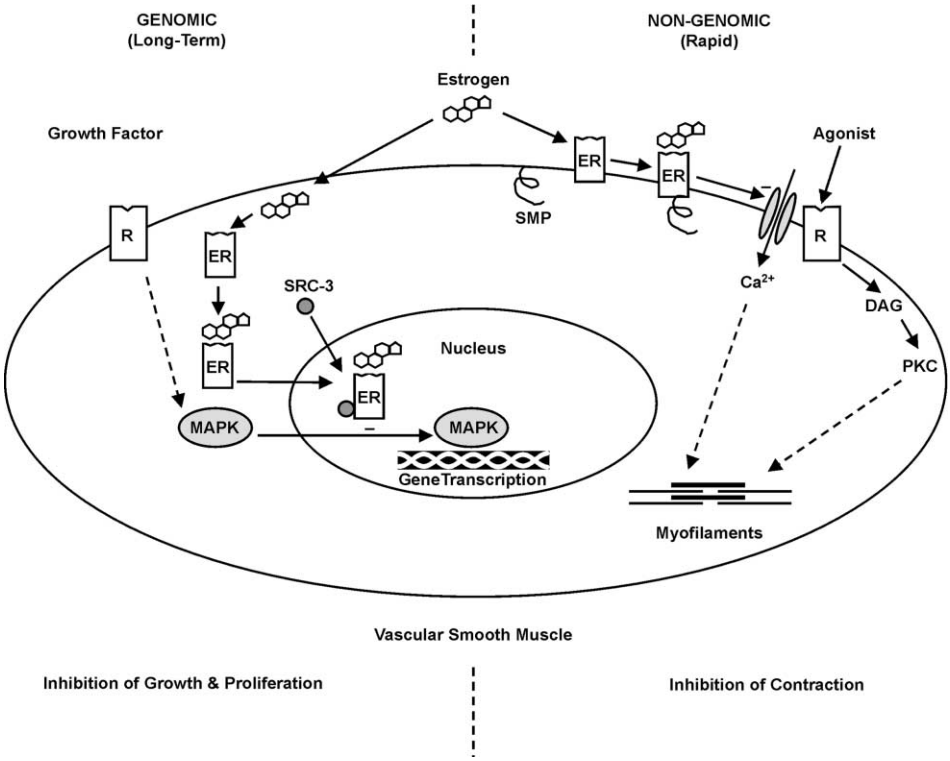

Fig. 1. Genomic and non-genomic effects of estrogen in VSM. In the genomic pathway, estrogen binds to cytosolic/nuclear ER, leading to inhibition of growth factor-activated MAP kinase and gene transcription, and thereby inhibition of growth and proliferation of VSM. In the non-genomic pathway, estrogen binds to plasma membrane ER, leading to inhibition of agonist-activated mechanisms of VSM contraction such as Ca^{2+} and PKC. SRC-3, steroid receptor coactivator-3; SMP, signal-modulating protein.

hormones on the signaling mechanisms of VSM contraction will be provided. The chapter will end with a perspective on potential areas for future investigations to better understand the mechanisms underlying sex differences in the functioning of VSM.

2. Sex differences in vascular tone

Sex differences in VSM tone have been reported [12]. For example, vasoconstrictor agonists such as norepinephrine cause less forearm vasoconstriction in women than in men [14]. Also, the contraction to the α-adrenergic agonist norepinephrine or phenylephrine (Phe) is greater in the aorta of sexually mature male than female rats [12]. Vasopressin-induced contraction of isolated rat aorta also exhibits sexual dimorphism, but the contraction in females is almost twice that in males [12]. However, the pressor response to vasopressin infusion in vivo is greater in male than female rats. The difference could be related to the possibility that vasopressin may exhibit a tachyphylactic response in isolated vessels which is different from its effects in vivo.

Sex differences in coronary arterial tone have also been demonstrated. Oxidized low-density lipoprotein enhances 5-hydroxytryptamine-induced contraction to a greater extent in isolated coronary arteries from male than female pigs. These differences in the coronary vasomotor effects of oxidized low-density lipoproteins may contribute to the greater incidence of coronary artery disease in males than premenopausal females.

Sex differences in vascular tone may be due to direct vascular effects of sex hormones, possibly through interaction with specific hormone receptors in the vasculature rather than influenced by genetic sex alone. This conclusion is supported by observations that contraction of isolated aorta is not different between castrated and intact male rats, but significantly enhanced in ovariectomized (OVX) females compared with intact females. These results also suggest that the differences in vascular tone are less likely related to androgens and more likely related to estrogens [6].

3. Sex hormone receptors in VSM

Receptors for estrogen, progesterone and testosterone are found in various types of smooth muscle. The sex hormone receptors appear to have different subtypes, tissue distribution, and can be modulated by various agonists and antagonists (Table 1).

Two estrogen receptor (ER) subtypes have been identified, ER-α and ER-β [1,9]. Some studies suggest that ER-α mediates the protective effects of estrogen in response to vascular injury. However, ER-β is more widely distributed in the body than ER-α. Also, ER-β is the receptor form that is predominantly expressed in VSM of human coronary artery, iliac artery, aorta and saphenous vein, particularly in women [9]. Other studies have also demonstrated the induction of ER-β mRNA expression after balloon vascular injury to male rat aorta. Furthermore, experiments on transfected HeLa cells have shown that in response to 17β-estradiol, ER-α is a stronger genetic activator than ER-β at low receptor concentrations. However, at higher receptor concentrations, ER-α activity self-squelches, and ER-β becomes the stronger activator. These data support a role for ER-β in the direct vascular effects of estrogen and in the regulation of vascular function and blood pressure [5].

ERs are transcription factors that require coactivators to exert transcriptional activity. The steroid receptor coactivator-3 (SRC-3) is highly expressed in VSM cells. SRC-3 interacts with estrogen-bound ERs and strongly coactivates the transcription of target genes in cultured VSM cells (Fig. 1), suggesting that SRC-3 may facilitate ER-dependent vasoprotective effects against vascular disease [16].

ERs have been localized in the nucleus, and continuous shuttling of the receptor between the cytoplasm and the nucleus has been suggested. Sex steroids diffuse through the plasma membrane and form complexes with specific cytosolic and/or nuclear receptors, which then bind to chromatin and stimulate the expression of a set of genes with a specific sex steroid-responsive regulatory element (Fig. 1) [14]. However, estrogen can also bind to the plasma membrane of various vascular cells and induce rapid cellular events within seconds or minutes of application, suggesting additional non-genomic action triggered by a signal-generating receptor on the cell surface [4]. Recent studies also suggest possible interactions of ER with signal-modulating proteins or co-activators in the plasma membrane (Fig. 1) [11] (see also Chapters 4 and 6).

Table 1
Sex hormone receptor distribution, agonists and antagonists in VSM

	Estrogen	Progesterone	Testosterone
Tissue distribution	Mouse aorta	Rat aorta	Rat aorta
	Rat aorta, carotid, uterine and tail artery [1]	Rabbit uterine artery	Primate coronary artery [14]
	Rabbit uterine artery	Primate aorta and coronary artery	
	Primate coronary and carotid artery		
	Human coronary and uterine artery [14]	Human thoracic aorta, internal carotid, coronary and uterine artery [7,14]	
Agonists	17β-Estradiol	Progestins, progesterone	Testosterone
	Estradiol valerate, cypionate, benzoate	Medroxyprogesterone acetate	Dihydrotestosterone
	Ethinyl estradiol, diethylstilbestrol	3-keto-desogestrel	Dehyroepiandrosterone
	Phytoestrogens: genistein, daidzein, coumestrol, α-zearalanol, zearalenone, naringenin, taxifolin, biochanin A	Levonorgestrel	Androstenedione
		Gestodene	
		Norethisterone, norgestimate R5020	
	Organochlorine pesticides		
Antagonists	ICI 182,780 (Fulvestrant)	RU 486 (Mifepristone)	Flutamide
	ICI 164,384	ZK 98,299	Hydroxyflutamide
	Cytochrome-P450 inhibitors: 3-methylcholanthrene phenobarbital, 1-aminobenzotriazole	ZK 98,734	Casodex
		Onapristone	
	Catechol-*O*-methyltransferase inhibitors: quercetin, OR486		
Agonists/ antagonists	Tamoxifen		
	Raloxifene		
	4-Hydroxytamoxifen		
	6-Carboxymethyl genistein		
	Toremifene, raloxifene		
	Enterodiol, enterolactone		

Another hormone receptor that has been located in VSM is the progesterone receptor. Both progesterone receptor-α and -β have been identified. Progesterone receptors appear to have a direct role in the regulation of gene transcription and VSM cell proliferation, mainly through activation of progesterone receptor-β [14]. Whether the distribution of progesterone receptors is different in VSM of males and females is unclear and should be examined in future studies.

Testosterone or androgen receptors have also been identified in VSM cells. The expression of androgen receptors in VSM appears to vary depending on the sex and the status of the gonads. The androgen receptor protein, as detected by Western blot in rat aortic smooth muscle cells, is less in the cells of females than those of males. In uterine smooth muscle of monkey, androgen receptor mRNA levels are up-regulated by combined estradiol plus testosterone treatment while estradiol treatment alone had little or no effect, suggesting that a collaborative action of estradiol and testosterone enhances androgen receptor expression [14].

4. Vascular effects of sex hormones

The interaction of sex hormones with their receptors could lead to various genomic and non-genomic vascular effects. Although the vascular effects of estrogen are not completely understood, some general and specific effects have been suggested (Table 2). Some of the prominent vascular effects of estrogen include antiproliferative effects on VSM [3] and direct modulation of vascular reactivity [2,14].

The vascular effects of progestins are less clear. Although estrogen may have favorable cardiovascular effects in most circumstances, progestins may oppose or reverse estrogen's atheroprotective effects. Also, direct effects of progestins on vascular tone may modify the

Table 2
Beneficial vascular effects and possible clinical applications of sex hormones

	Estrogen	Progesterone	Testosterone
Vascular effects	Inhibition of VSM proliferation /migration [3] VSM relaxation and vasodilation Decrease low density lipoproteins, increase high density lipoproteins Inhibition of lipoprotein oxidation Attenuation of atherosclerotic lesions Favorable modulation of homocysteine Increased antiplatelet aggregation factors, decreased platelet adhesion Inhibition of intravascular accumulation of collagen [14]	Inhibition of VSM proliferation/ migration Facilitate the vascular inhibitory effects of estrogen Acute vascular relaxation [14]	Inhibit VSM proliferation Acute vascular relaxation Coronary vasodilation Anti-atherosclerotic effects [14]
Clinical applications	Coronary artery disease Postmenopausal hypertension Thromboembolic events Reduces aortic stiffening, glycoxidative damage, and permeability [14]	Coronary artery disease Thromboembolic events [14]	Reduces myocardial ischemia in men with coronary artery disease Improves brachial arterial vasoreactivity in men with coronary artery disease [14]

effects of estrogen on vascular contraction [2]. Interestingly, vascular protective effects of testosterone have also been described [2,17]. For example, testosterone induces pulmonary and coronary dilation [2]. The testosterone-induced vasorelaxation appears to be a structurally specific effect of the androgen molecule, which is enhanced in more polar analogs that have a lower permeability to the VSM cell membrane [14].

5. Effect of sex hormones on VSM growth

In many cells, estradiol activates signaling pathways that lead to cell proliferation; however, the steroid inhibits signaling and growth in other cell types such as VSM. Sex differences in VSM growth and proliferation have been reported in Wistar–Kyoto (WKY) and spontaneously hypertensive (SHR) rats. The rate of growth in cells derived from female aorta is significantly slower than that of male aorta, and the difference has been attributed to antiproliferative effects of endogenous estrogen in females [14]. Also, 17β-estradiol inhibits growth factor-induced proliferation and migration in human female aortic smooth muscle cells in culture. Interestingly, β-estradiol inhibits proliferation in coronary smooth muscle cells isolated from both female and male pigs, suggesting beneficial coronary vascular effects in both sexes. These estrogen-induced effects on smooth muscle proliferation and migration may lead to inhibition of arterial intimal hyperplasia, which characterizes obstructive atherosclerotic lesions. The inhibitory effects of 17β-estradiol on VSM cell growth are blocked by ER antagonists such as ICI 182,780 (Table 1), suggesting that these functions are mediated by ER. One pathway involved in the proliferation is mitogen-activated protein (MAP) kinase. Estrogen inhibits this pathway, an effect which is blocked by ICI 182,780, suggesting that inhibition of the MAP kinase pathway via ERs contributes to the inhibitory effects of 17β-estradiol on smooth muscle cell growth (Fig. 1) [3]. The inhibitory effects of estrogen on VSM cell growth may also be related to interactions of estrogen with signal-modulating proteins (Fig. 1). Estrogen may also antagonize the growth-promoting effect of angiotensin II on VSM via the induction and activation of protein phosphatases through genomic as well as non-genomic mechanisms [13].

Progesterone also inhibits VSM cell proliferation and migration and may facilitate the inhibitory effects of estrogen [7]. Progesterone may mediate its inhibitory effects on VSM cell growth by reducing MAP kinase activity [14].

Some studies have suggested that androgens accelerate vascular growth by stimulating the proliferation of VSM cells, with the mitogenic activity of dihydrotestosterone being more potent than that of testosterone. However, androgens may control the proliferation of their target cells by first increasing cell proliferation and later by inhibiting the proliferation of those same cells [14]. For example, dihydrotestosterone may modulate human umbilical VSM cell proliferation in a dose-dependent manner, with low concentrations (3 nM) stimulating [^{3}H]thymidine incorporation, whereas high concentrations (300 nM) inhibiting [^{3}H]thymidine incorporation, suggesting a role of androgens in remodeling the responses to vascular injury. An important specific target for androgens in males is the vasculature of the prostate. Androgens most likely affect the

vasculature indirectly by modulating the expression of angiogenic factors in prostate cells particularly the smooth muscle.

6. Effect of sex hormones on VSM contraction

In addition to the long-term genomic effects of sex hormones, rapid non-genomic vascular effects have been described (Fig. 1) [2,14]. For example, estrogen produces rapid vasodilation of various vascular beds. The acute vasodilator effects of estrogen may be influenced by the vessel type, sex, estrous cycle, and previous exposure to estrogen. The vasodilator effects of estrogen have been ascribed, in part, to endothelium-dependent vascular relaxation (see Chapters 4 and 6). However, estrogen also causes vasodilation in de-endothelialized vessels, suggesting that the estrogen-induced inhibition of vascular tone involves direct action on VSM [2,4]. For example, 17β-estradiol has been shown to produce endothelium-independent relaxation of human coronary arteries [14]. Also, estrogen causes vasodilation in de-endothelialized rabbit carotid and coronary artery and porcine coronary artery precontracted by endothelin-1, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and high KCl depolarizing solution. The vasodilator effects of estrogen do not appear to be mediated by the classic cytosolic-nuclear ER or stimulation of protein synthesis, suggesting that the estrogen-induced inhibition of vascular tone involves direct action on VSM (Fig. 2) [2,4].

In rat aorta, 17β-estradiol causes greater relaxation in males than females. Among female rats, the largest 17β-estradiol-induced vasodilation is seen in the tail and mesenteric arteries from females at the pro-estrous stage. However, the magnitude of relaxation in microvessels of estradiol replaced OVX female rats is smaller than that of nonreplaced OVX rats, suggesting that chronic estradiol replacement may downregulate the acute non-genomic vasorelaxation effects of estrogen in small arteries of OVX rats [14]. These differences in vasorelaxation may be related to possible effects of genetic sex or endogenous sex hormones on the number of ERs in blood vessels.

The effects of progesterone on vascular reactivity are less clear and range between no effect, inhibition of vasorelaxation in some vascular beds, and potent vascular relaxation in other vascular beds [2,14]. Progesterone may cause endothelium-independent relaxation of VSM, although it is smaller than that induced by estrogen (Fig. 2) [2]. Progesterone may also induce relaxation of primate, porcine, rabbit and rat coronary artery [2]. The vasodilator effect of progesterone in isolated VSM suggests that its benefits in hormone replacement therapy may be related to its non-genomic vascular relaxant effects.

Testosterone may enhance vascular contraction either by inhibiting endothelium-dependent relaxation or by directly stimulating the smooth muscle. For example, pretreatment of porcine coronary arteries with physiological nanomolar concentrations of testosterone impairs bradykinin- and A23187-induced endothelium-dependent vascular relaxation. Also, testosterone enhances thromboxane A2-induced coronary vasoconstriction in guinea pigs. However, other studies have shown that testosterone induces relaxation of rabbit coronary artery and aorta, rat aorta and canine and porcine coronary artery (Fig. 2) [2,17]. The physiological significance of this difference is unclear at this time. A significant portion of the testosterone-induced vascular relaxation appears to be

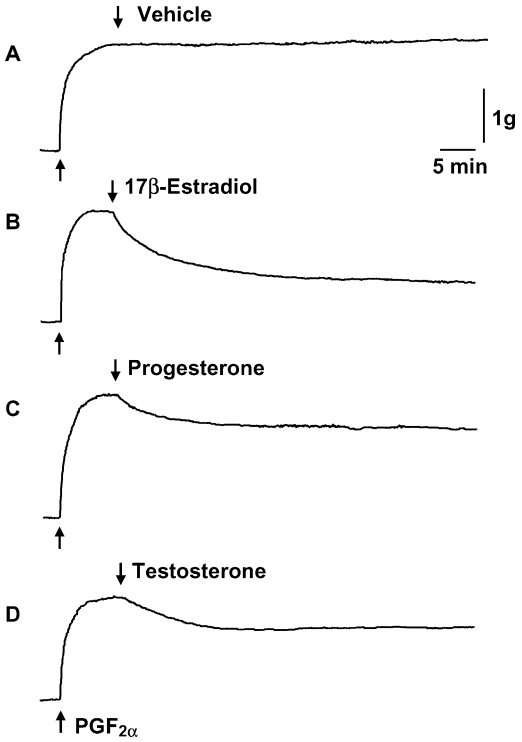

Fig. 2. Effect of sex hormones on prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)-induced contraction in porcine coronary artery. Endothelium-denuded coronary artery strips were stimulated with $PGF_{2\alpha}$ (10^{-5} M), then treated with the vehicle ethanol (A) or with 10^{-5} M 17β-estradiol (B), progesterone (C) or testosterone (D) (reproduced from Ref. [2]).

endothelium-independent because minimal differences could be observed between the relaxation in vessels with or without endothelium. Also, inhibition of synthesis of endothelium-derived factors such as NO, prostaglandin, and guanylate cyclase do not affect the vasorelaxing effect of testosterone [17], providing further evidence that a significant component of testosterone-induced relaxation is endothelium-independent and involves direct action on VSM.

Although both the endogenous presence and exogenous application of sex hormones may be associated with reduction in vascular contraction, the mechanisms of hormone-induced relaxation in isolated vascular strips or cells and the possible vasorelaxant effects of the hormone in vivo may not be identical. The acute effects of estrogen on vascular contraction in vitro are often observed at micromolar concentrations, which are several-fold higher than the physiological nanomolar concentrations observed in vivo. Although a genomic action of physiological concentrations of estrogen might underlie the reduced cell contraction in VSM cells of intact females, it is less likely to account for the acute inhibitory effects of exogenous micromolar concentrations of 17β-estradiol on vascular contraction. The acute vasorelaxant effects of exogenous estrogen may represent additional non-genomic effects of estrogen on the mechanisms of VSM contraction [2].

However, in general most of the sex hormones tested appear to cause vascular relaxation and inhibit VSM contraction, the vascular relaxant effects of estrogen significantly surpass those of progesterone or testosterone. Since the estrogen levels are greater in females compared with males, this could well explain the gender differences in vascular tone and the reduced vascular contraction in females compared with males (Fig. 3). However, since the expression of sex hormone receptors in arterial smooth muscle may vary depending on the sex and the status of the gonads [14], sex differences in vascular contraction may be related to the relative abundance of sex hormone receptors. This is supported by reports that females have higher levels of ERs in their arteries than males [14]. The sex differences in vascular contraction could also be related to effects of sex hormones on the gene expression of the specific receptors of vasoconstrictor agonists such as angiotensin II (Fig. 3). Western blot analyses in VSM have revealed that estrogen induces a downregulation and progesterone an upregulation, of the angiotensin AT1 receptor protein. Also, 17β-estradiol decreases the AT1 receptor mRNA half-life, whereas progesterone induces a stabilization of AT1 receptor mRNA. Other studies have shown that progesterone replacement in OVX monkeys decreases thromboxane A2 receptors in coronary arteries. However, sex differences in vascular contraction could also be due to differences in the signaling mechanisms of VSM contraction downstream from receptor activation as described below.

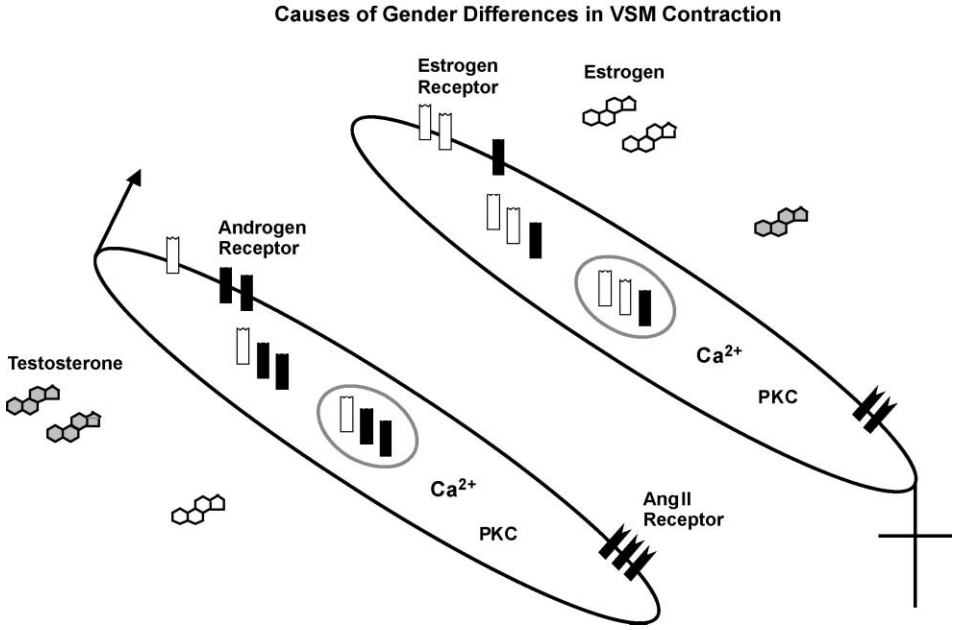

Fig. 3. Possible causes of sex differences in VSM contraction. The greater VSM contraction in males (*left*) compared with females (*right*) could be related to differences in the level of the sex hormones estrogen and testosterone, the amount of sex hormone receptors, or the expression of specific receptors of vasoconstrictor agonists such as angiotensin II (Ang II) or their post-receptor signaling mechanisms such as Ca^{2+} and PKC.

7. Signaling mechanisms of VSM contraction

It is widely accepted that VSM contraction is triggered by increases in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) due to initial Ca^{2+} release from the intracellular stores and maintained Ca^{2+} entry from the extracellular space [10]. Also, activation of protein kinases such as myosin light chain (MLC) kinase, Rho kinase and MAP kinase as well as inhibition of MLC phosphatase may contribute to smooth muscle contraction (Fig. 4). Additionally, the interaction of an α-adrenergic agonist such as Phe with its receptor is coupled to increased breakdown of plasma membrane phospholipids and increased production of diacylglycerol (DAG). DAG binds to and activates protein kinase C (PKC). PKC is mainly cytosolic under resting conditions and undergoes translocation from the cytosolic to the particulate fraction when it is activated by DAG or phorbol esters (Fig. 4). PKC is now known to be a family of several isoforms that have different enzyme properties, substrates and functions and exhibit different subcellular distributions in the same blood vessel from different species and in different vessels from the same species.

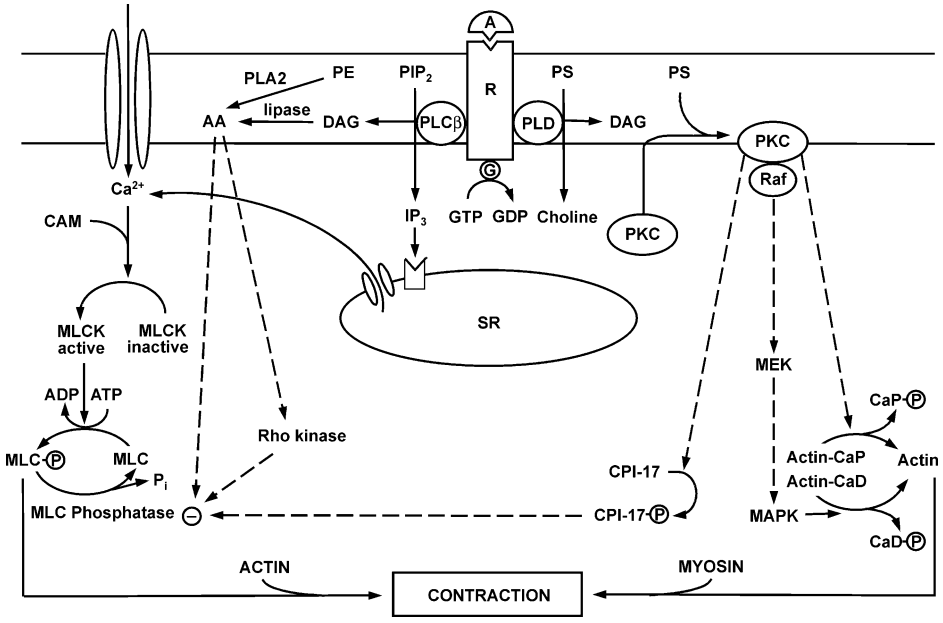

Fig. 4. Signaling mechanisms of VSM contraction. An agonist (A) activates a specific receptor (R), stimulates membrane phospholipases (PLCβ and PLD), and increases the production of inositol-1,4,5-tisphosphate (IP_3) and DAG. IP_3 stimulates Ca^{2+} release from the sarcoplasmic reticulum (SR). Also, Ca^{2+} enters the cell through Ca^{2+} channels. Ca^{2+} binds calmodulin (CAM), activates MLC kinase, causes MLC phosphorylation and initiates VSM contraction. In the presence of phosphatidylserine (PS) and Ca^{2+}, DAG causes activation and translocation of PKC from the cytosol to cell membrane. PKC phosphorylates CPI-17 and calponin (CAP), and activates a protein kinase cascade involving Raf, mitogen-activated protein kinase (MAPK) kinase (MEK) and MAPK, leading to phophorylation of caldesmon (CAD), and an increase in the myofilament force sensitivity to Ca^{2+}. Agonist-induced formation of arachidonic acid (AA) and activation of Rho kinase also increase the force sensitivity to Ca^{2+} by inhibiting MLC phosphatase.

8. Effect of sex hormones on $[Ca^{2+}]_i$

Since $[Ca^{2+}]_i$ is important for the initiation of smooth muscle contraction, several studies have used isolated vascular strips and smooth muscle cells from intact and gonadectomized male and female experimental animals to investigate the effect of sex and sex hormones on $[Ca^{2+}]_i$ and the Ca^{2+} mobilization mechanisms of smooth muscle contraction, i.e. Ca^{2+} release from the intracellular stores and Ca^{2+} entry from the extracellular space [2,10,18].

Studies in isolated VSM cells have shown that the resting cell length is longer and the basal $[Ca^{2+}]_i$ is smaller in intact female rats compared with intact males, suggesting sex differences in the Ca^{2+} handling mechanisms [10]. Sex differences in resting cell length and $[Ca^{2+}]_i$ appear to be related to estrogen because the cell length and $[Ca^{2+}]_i$ are greater in OVX females compared with intact females, but not different between OVX females with 17β-estradiol implants and intact females, or between castrated and intact males (Fig. 5) [10].

In cells incubated in the presence of external Ca^{2+}, Phe causes an initial peak in $[Ca^{2+}]_i$ due to Ca^{2+} release from the intracellular stores, followed by a smaller but maintained increase in $[Ca^{2+}]_i$ due to Ca^{2+} entry from the extracellular space (Fig. 5) [10]. In Ca^{2+}-free solution, Phe causes a transient increase in smooth muscle contraction and $[Ca^{2+}]_i$ that are not different between intact and gonadectomized male and female rats, suggesting that the inositol 1,4,5-trisphosphate-mediated Ca^{2+} release is not involved in the sex differences in cell contraction and $[Ca^{2+}]_i$ [10]. Also, caffeine, which stimulates the Ca^{2+}-induced Ca^{2+} release mechanism, causes a small cell contraction and a transient increase in $[Ca^{2+}]_i$ that are similar in magnitude in smooth muscle from intact and gonadectomized male and female rats, suggesting that the sex differences in cell contraction and $[Ca^{2+}]_i$ are not related to the Ca^{2+}-induced Ca^{2+} release mechanism [10].

On the other hand, the maintained Phe-induced $[Ca^{2+}]_i$ in VSM cells incubated in the presence of external Ca^{2+} is greater in smooth muscle from intact male than from intact female rats, suggesting sex differences in the Ca^{2+} entry mechanism of VSM contraction. The maintained Phe-induced $[Ca^{2+}]_i$ is enhanced in OVX compared with intact females, but not different between OVX females with estrogen implants and intact females, or between castrated and intact males, suggesting that the sex differences are more likely related to estrogen than androgen (Fig. 5) [10].

Other experiments also support the hypothesis that the sex differences are more likely related to endogenous estrogen. For example, membrane depolarization by high KCl mainly stimulates Ca^{2+} entry from the extracellular space. KCl-induced smooth muscle contraction, Ca^{2+} influx, and $[Ca^{2+}]_i$ are greater in smooth muscle cells from intact males than intact females [10]. Also, the KCl-induced cell contraction and $[Ca^{2+}]_i$ are enhanced in OVX females compared with intact females, but there is no difference between OVX females with estrogen implants and intact females. The causes of the sex differences in the Ca^{2+} entry mechanism are not clear, but may be related to the plasmalemmal density and/or the permeability of the Ca^{2+} channels depending on the presence or deficiency of endogenous estrogen. This is supported by reports that the expression of the L-type Ca^{2+} channels in cardiac muscle is substantially increased in ER-deficient mice, and that

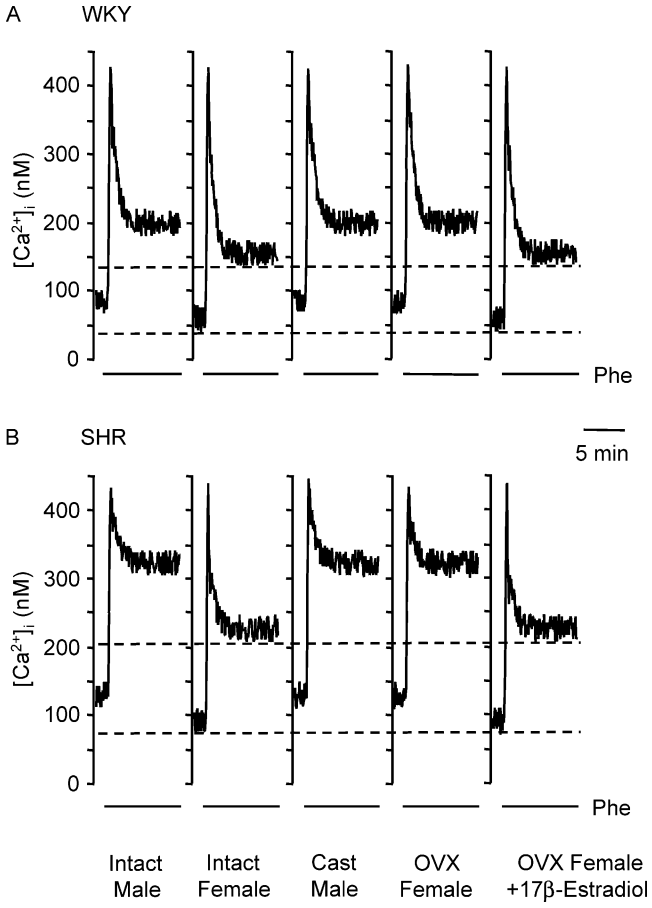

Fig. 5. Effect of Phe (10^{-5} M) on $[Ca^{2+}]_i$ in aortic smooth muscle cells isolated from intact and gonadectomized, male and female WKY and SHR rats, and OVX female rats with 17β-estradiol implants, and incubated in Hank's solution (1 mM Ca^{2+}). Dashed lines are drawn at the smallest basal and maintained Phe-induced increase in $[Ca^{2+}]_i$ in cells of intact females in order to facilitate comparison with the other groups (reproduced from Ref. [10]).

the L-type Ca^{2+} current is significantly greater in coronary smooth muscle of males compared with females [14].

Since VSM contraction and $[Ca^{2+}]_i$ are often enhanced in animal models of hypertension, any sex differences in the Ca^{2+} mobilization mechanisms of VSM contraction are expected to be more apparent in hypertensive SHR than normotensive WKY rats. Aortic strips of SHR show greater vascular contraction and Ca^{2+} entry than those of WKY rats [10]. Also, VSM cells of SHR show shorter resting cell length, greater basal $[Ca^{2+}]_i$ and greater Phe- and KCl-induced contraction and $[Ca^{2+}]_i$ than those of WKY rats (Fig. 5) [10]. Additionally, the reduction in vascular contraction, Ca^{2+} entry, and $[Ca^{2+}]_i$ in intact females or OVX females with estrogen implants compared with intact males or OVX females is greater in SHR than WKY rats, suggesting possible differences

in the number of ERs or the number and permeability of the plasma membrane Ca^{2+} channels [10]. It should be noted, however, that sex differences in the mechanisms of Ca^{2+} mobilization into VSM could be due to a multitude of simultaneous effects of sex hormones in vivo. On the other hand, 17β-estradiol causes relatively rapid relaxation of isolated vascular strips of rabbit, porcine, and human coronary artery [2] (see Fig. 2), suggesting that it may be mediated by an effect on Ca^{2+} mobilization and/or fluxes.

Several studies have shown that estrogen does not inhibit caffeine- or carbachol-induced smooth muscle contraction or $[Ca^{2+}]_i$ in Ca^{2+}-free solution, suggesting that it does not inhibit Ca^{2+} release from the intracellular stores [2]. However, supraphysiological concentrations of estrogen may inhibit thromboxane A2-induced Ca^{2+} release in porcine coronary artery [14]. On the other hand, estrogen inhibits the maintained $PGF_{2\alpha}$- and thromboxane A2-induced contraction, Ca^{2+} influx, and $[Ca^{2+}]_i$, suggesting inhibition of Ca^{2+} entry from the extracellular space. Also, estrogen inhibits the high KCl-induced contraction, Ca^{2+} influx, and $[Ca^{2+}]_i$, suggesting that it may act by inhibiting Ca^{2+} entry through voltage-gated channels (Fig. 6) [2].

Whether estrogen inhibits Ca^{2+} entry by direct or indirect action on plasmalemmal Ca^{2+} channels is unclear. Some studies have shown that estrogen blocks Ca^{2+} channels in cultured A7r5 and aortic smooth muscle cells [18]. Other studies have shown that estrogen activates BK_{Ca} channels in coronary smooth muscle cells which could lead to hyperpolarization and decreased Ca^{2+} entry through voltage-gated channels [15]. However, estrogen-induced vasorelaxation and inhibition of Ca^{2+} influx into VSM in the absence of activation of K^+ efflux has been reported, lending support to possible direct effects of estrogen on Ca^{2+} channels.

Estrogen may also decrease $[Ca^{2+}]_i$ by stimulating Ca^{2+} extrusion via plasmalemmal Ca^{2+} pump. However, this mechanism seems less likely since the rate of decay of caffeine- and carbachol-induced contraction and $[Ca^{2+}]_i$ transients in smooth muscle incubated in Ca^{2+}-free solution, which are often used as a measure of Ca^{2+} extrusion, are not affected by estrogen [2].

In contrast to estrogen, the effect of progesterone on $[Ca^{2+}]_i$ is not clearly established. However, several studies have shown that acute application of progesterone decreases Ca^{2+} influx and $[Ca^{2+}]_i$ in rabbit and porcine coronary artery smooth muscle (Fig. 6) [2]. There have also been inconsistent reports on the effects of testosterone on VSM $[Ca^{2+}]_i$. However, the majority of studies suggest that testosterone has a potent vasorelaxant effect in the rabbit coronary artery and aorta and porcine coronary artery and that testosterone decreases VSM $[Ca^{2+}]_i$ by inhibiting Ca^{2+} entry from the extracellular space (Fig. 6) [2,17]. It has been shown that the relaxing effect of testosterone is attenuated by K^+ channel blockers, suggesting that stimulation of K^+ conductance through specific K^+ channels, e.g. voltage-dependent (delayed-rectifier) K^+ channel may be involved in the inhibitory effects of testosterone on $[Ca^{2+}]_i$ [17].

Although progesterone and testosterone inhibit smooth muscle contraction, the progesterone- and testosterone-induced inhibition of $PGF_{2\alpha}$-induced contraction is greater than the inhibition of the KCl-induced responses [2]. These data suggest that progesterone and testosterone not only inhibit Ca^{2+} entry through voltage-gated channels, but may also inhibit additional contraction mechanisms activated by $PGF_{2\alpha}$ such as PKC. Additional evaluation of hormone ion channel function can be found in Chapter 8.

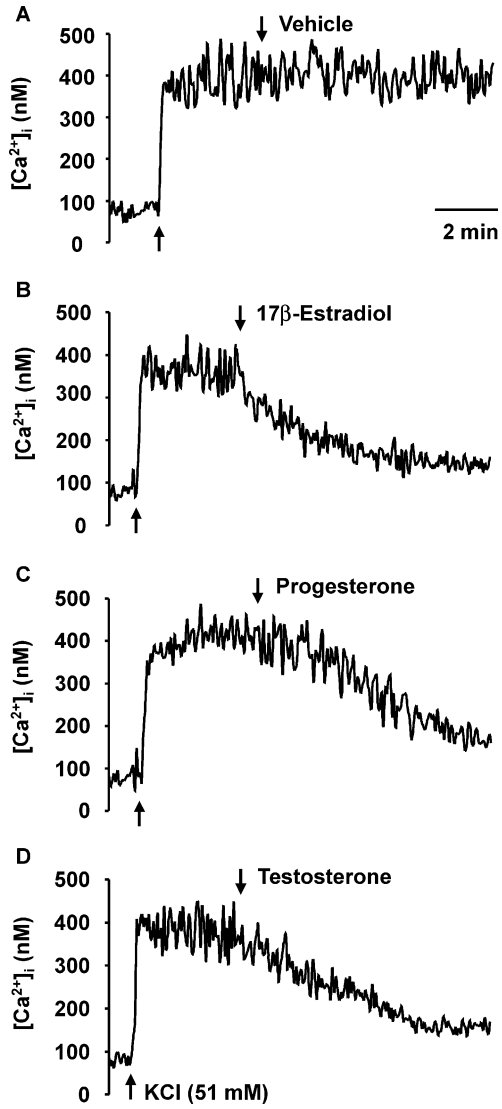

Fig. 6. Effect of sex hormones on KCl-stimulated $[Ca^{2+}]_i$ in male porcine coronary smooth muscle cells. Cells were stimulated with 51 mM KCl solution for 5 min. The cells were then treated with the vehicle ethanol (A) or 10^{-7} M 17β-estradiol (B), progesterone (C) or testosterone (D) (reproduced from Ref. [14]).

9. Effect of sex hormones on PKC

Recent studies have investigated whether the sex differences in vascular contraction reflect differences in the expression and activity of PKC isoforms in VSM. Phorbol esters, which activate PKC, produce greater contraction in isolated vessels from intact male than

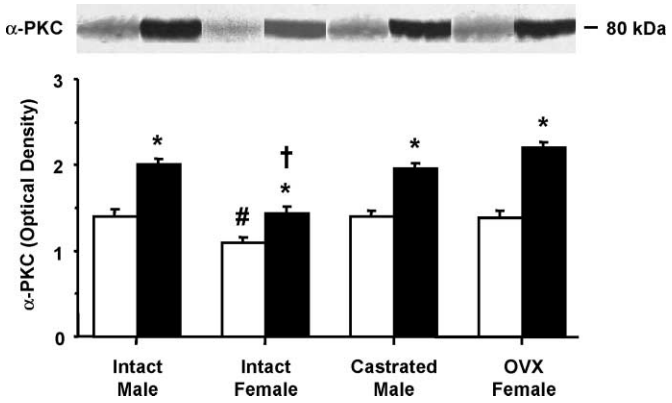

Fig. 7. Expression of α-PKC in aortic strips of intact and gonadectomized male and female WKY (open bars) and SHR rats (solid bars). Western blot analysis was performed using whole tissue homogenate of rat aortic strips, and anti-α-PKC antibody. Position of the molecular mass marker is shown on the right. The amount of α-PKC is expressed as optical density. *SHR significantly different ($p < 0.05$) from WKY. #Significantly different from other WKY. † Significantly different from other SHR (reproduced from Ref. [6]).

from intact female rats [6]. The greater Phe- and phorbol ester-induced contraction and PKC activity in intact male compared with intact female rats have suggested sex differences in the PKC-mediated pathway of VSM contraction [6], which may be related to differences in the amount of PKC expressed in VSM and/or the sensitivity of the PKC pathway to endogenous sex hormones.

Immunoblot analysis in aortic smooth muscle of intact male WKY rat has shown significant amounts of α-, δ- and ζ-PKC (Fig. 7). In the same preparation, both Phe and phorbol ester cause activation and redistribution of α- and δ-PKC from the cytosolic to the particulate fraction. The amount of α-, δ- and ζ-PKC, and the Phe- and phorbol ester-induced redistribution of α- and δ-PKC are reduced in tissue from intact females compared with intact males, suggesting that the sex differences in vascular contraction are related, in part, to underlying changes in the amount and activity of α-, δ- and ζ-PKC (Fig. 7) [6].

The Phe- and phorbol ester-induced contraction and PKC activity are not different between castrated and intact male rats, but greater in OVX than intact females, suggesting that the sex differences in vascular contraction and PKC activity are more likely related to estrogens than androgens. This is supported by reports that treating OVX female and castrated male rats with implants releasing 17β-estradiol is associated with reduction in vascular contraction and PKC activity [6].

Previous studies have shown that vascular PKC activity is augmented in smooth muscle of SHR rats. Also, the reduction in vascular contraction and PKC activity in smooth muscle from intact females compared with intact males is greater in SHR than WKY. The greater reduction in vascular contraction and PKC activity in smooth muscle from intact female SHR compared with WKY could not be explained by differences in their plasma estrogen levels, but could be related to inherent differences in the amount of PKC isoforms expressed in VSM (Fig. 7) [6].

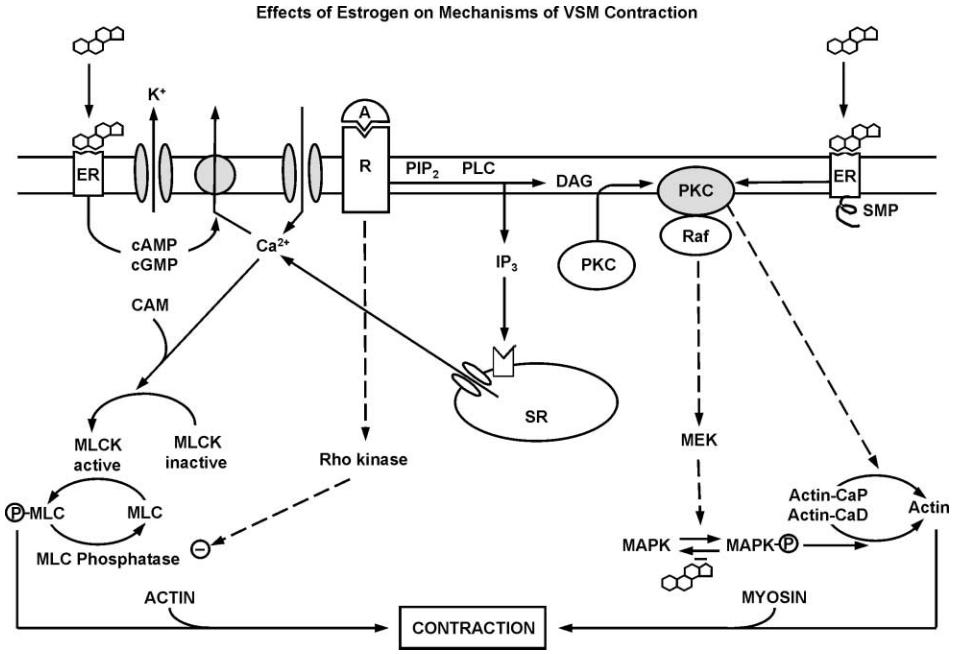

Fig. 8. Effects of estrogen on the mechanisms of VSM contraction. Possible effects of estrogen include activation of K^+ channels leading to membrane hyperpolarization and inhibition of Ca^{2+} entry through voltage-gated Ca^{2+} channels. Estrogen may also increase cAMP or cGMP, which may inhibit Ca^{2+} entry through voltage-gated Ca^{2+} channels or stimulate Ca^{2+} extrusion via the plasma membrane Ca^{2+} pump. The estrogen-induced decrease in $[Ca^{2+}]_i$ leads to the inhibition of the Ca^{2+}-dependent MLC phosphorylation pathway and inhibition of VSM contraction. Estrogen may also inhibit PKC and the MAP kinase pathways, and thereby further inhibiting VSM contraction.

A genomic action of estrogen on the expression of PKC isoforms in VSM might well underlie the reduction in vascular contraction and PKC activity observed in smooth muscle from intact females compared with intact males. However, additional non-genomic effects of estrogen on the PKC molecule or its lipid co-factors or other protein kinases upstream from PKC cannot be excluded (Fig. 8). Although, a direct effect of estrogen on PKC activity has not been established, progesterone has been shown to inhibit phorbol ester-induced contraction and PKC translocation in VSM, suggesting an effect of progesterone on PKC activity. The progesterone-induced inhibition of PKC may be mediated by increasing cAMP levels in smooth muscle and may be more important in females than males.

10. Summary

1. The greater incidence of hypertension and coronary artery disease in men and postmenopausal women compared with premenopausal women has suggested possible vascular protective effects of the female sex hormone estrogen. However, vascular

effects of the female sex hormone progesterone and the male sex hormone testosterone have also been suggested.

2. Estrogen, progesterone and testosterone receptors have been identified in the plasmalemma, cytosol and nuclear compartments of various vascular cells including the smooth muscle suggesting that all three of these hormone receptors may be involved in the regulation of VSM.
3. The interaction of sex hormones with their specific receptors triggers long-term genomic effects that often lead to inhibition of growth and proliferation of VSM cells.
4. Recent studies have also suggested acute non-genomic responses to sex hormones that may inhibit the signaling mechanisms of VSM contraction such as $[Ca^{2+}]_i$, PKC and other protein kinases.
5. The sex hormone-induced inhibition of the cell growth as well as the contraction of VSM may contribute to the gender differences in vascular tone, and may represent potential beneficial vascular effects of hormone replacement therapy during natural and surgically-induced deficiencies of gonadal hormones.

11. Future directions

It is apparent that there is an array of factors contributing to the greater incidence of cardiovascular disease in men and postmenopausal women compared with premenopausal women. One contributing factor is sex differences in the regulation of vascular tone. Numerous studies have suggested both genomic and non-genomic effects of sex hormones on VSM, but there are yet many unanswered questions.

One important question is related to the subtypes, distribution and function of sex hormone receptors in vascular cells. In blood vessels of wild-type mice, estrogen attenuates vasoconstriction by an ER-β mediated increase in inducible NO synthase expression. Initial studies in ER knock-out mice have shown that deficiency of ER-β renders the aortic wall supersensitive to relaxation by 17β-estradiol, but does not change the vascular wall morphology, suggesting that ER-β may not be involved in vascular structure development. Other studies have shown that ER-β deficient mice develop sustained systolic and diastolic hypertension as they age, and their blood vessels show multiple abnormalities in ion channel functions [9], supporting a role for ER-β in the regulation of vascular function and blood pressure. However, complex tissue-specific effects of sex hormones may be mediated by the expression of heterogeneous forms of their cognate receptors. Variant estrogen and progesterone receptor transcripts are expressed in human VSM and may be of importance in altering the physiological effects of estrogens or progestins on VSM.

In addition to the nuclear ERs that mediate the classic transcriptional effects of estrogen, ERs may associate with the cell membrane, and a subpopulation of these membrane-bound ERs may mediate the rapid effects of estrogen. However, little is known regarding the pathways that regulate the distribution of ER between the nuclear and membrane fractions. Studies in human VSM cells transiently transfected with ER-α have shown translocation of ER-α from the membrane to the nucleus. Nuclear localization of ER-α was blocked by both pharmacologic and genetic inhibition of MAP kinase. Also,

constitutive activation of MAP kinase resulted in nuclear translocation of ER-α. These studies suggest that MAP kinase-mediated phosphorylation of ER-α induces nuclear localization of ER [8]. Although sex differences in vascular contraction may be related to effects of sex hormones on vascular $[Ca^{2+}]_i$ or PKC activity, other protein kinases such as MLC kinase, Rho kinase and tyrosine kinase as well as MLC phophatase could regulate smooth muscle contraction (Fig. 8). Whether the expression and activity of smooth muscle protein kinases and phosphatases differ with sex and by the presence or deficiency of gonadal hormones is unclear and should be examined in future investigations.

Another question is related to the effect of sex hormones on cell growth and proliferation. Why estrogen enhances the proliferation of breast or uterine cells, but inhibits the proliferation of VSM cells remains an enigma, and should represent an important area for future studies.

The rapid vasodilator effects of estrogen have suggested other mechanisms in addition to the classic genomic pathway of steroid action, possibly involving effects on the cellular mechanisms of vascular relaxation and/or contraction. Although evidence indicates Ca^{2+} and K^+ channels in VSM cells mediate in part estrogen-induced relaxation of many vascular beds, elucidating the signal transduction mechanisms coupling ER-α and ER-β activation to the generation of second messengers and effector mechanisms remains an area of intense study.

There is considerable evidence that both female and male sex hormones affect the mechanisms of vascular contraction; however, the vascular effects of sex hormones may not be uniform. Preliminary studies suggest sex differences in effects of estrogen on the mechanisms of vascular contraction [14]. However, this concept should be examined more thoroughly.

Since the vascular effects of estrogen, and perhaps progesterone, may involve modulation of the Ca^{2+} channels, hormone therapy (HT) may represent a relatively natural approach to decrease the severity of certain forms of hypertension that are responsive to Ca^{2+} channel blockers. To use or not-to-use HT in postmenopausal women with hypertension or coronary artery disease is still controversial. Some studies have shown no significant differences in arterial pressures with HT, suggesting that, with careful supervision, it may be safe in hypertensive women. Other studies have demonstrated that postmenopausal HT is accompanied with a reduction in arterial pressure when natural hormones are used in a manner that avoids first-pass liver effects and in doses that produce hormone levels similar to those that exist in the premenopausal state. Finally, estradiol metabolism may be an important determinant of its cardiovascular protective effects. Effects of nonfeminizing estradiol metabolites to confer cardiovascular protection in both sexes should be explored.

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Cardiovascular membrane excitability and the influence of sex and sex steroids

Douglas K. Bowles and Meredith Hay

1. Introduction

The physiology of nerves and muscles are dependent on the electrical excitability of their composite cells and the maintenance of an electrical potential across their cell membranes. Sex and sex hormones have been found to potentially affect the function of both nerves and muscles. Interest in the effects of sex hormones on neuronal and vascular tissue arose primarily from epidemiological evidence that pre-menopausal females are protected from some forms of cardiovascular disease, including coronary heart disease, relative to post-menopausal females and males. Based on these findings, initial hypotheses predicted that estrogen would produce beneficial effects on vascular and neuronal cells. Conversely, it was assumed that testosterone would produce detrimental effects on vascular and neuronal cells. Evidence is mounting that these initial assumptions may be overly simplistic. First, in contrast to epidemiological studies, recent large scale clinical trials have not shown that hormone therapy (HT) protects females from cardiovascular disease, underscoring the lack of understanding with regard to sex and sex hormone effects on the cardiovascular system and its regulation by the central nervous system. Second, in vitro, both estrogen and testosterone produce similar, potentially beneficial effects [3,5]. Furthermore, recent studies in humans have supported a beneficial effect of testosterone. For example, acute administration of testosterone can increase the anginal threshold in men with coronary heart disease [18]. Third, the beneficial effects of sex hormones may be sex specific. Often the beneficial effects of estrogen are specific to females, with no effect in males. Similarly, the beneficial effects of testosterone have been observed in males, but not in females. For example, in animal models of atherosclerosis, testosterone appears beneficial in males, yet detrimental in females. The complex, sex-specific effects of sex hormones on vascular and neuronal tissue are just now being recognized and yet to be fully understood.

Understanding the effects of sex and sex hormones on key target cells of the cardiovascular system, including vascular smooth muscle and neurons, will be essential to understanding sex differences in the cardiovascular system as a whole. The mechanisms

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underlying these sex and sex hormone effects include actions at the level of the excitable cell membrane. Often, these effects influence the electrical potential difference across cell membranes and the activity of ion channels involved in the activation or inhibition of nerve and muscle cell function. The major goal of this chapter is to explain how sex and sex hormones may modulate the function of nerves and vascular cells; specifically how sex and sex steroids may act at the level of the ion channels to alter nerve and vascular smooth muscle activities.

2. Ionic mechanisms of membrane potentials

The ability of excitable cells to function as such is dependent upon the generation of an electrical potential difference across the plasma membrane. Generally, in most cells, the cytoplasm of a cell has a negative charge relative to the extracellular environment. This voltage potential difference across the cell membrane is referred to as the *resting membrane potential*. The maintenance of the resting membrane potential is required for the nerve or muscle cell to generate an *action potential*. The action potential is an electrical event across the cell membrane that generates a biological signal for communication both within and between cells. For example, action potentials are used by neurons to communicate with other neurons and by muscle cells to depolarize the entire surface of the cell.

Resting membrane potential. The resting membrane potential is due to the relative distribution of various ions across the cell plasma membrane. In most nerves and muscles this potential is rather large and, when expressed by conventional means with the charge of the cytoplasm relative to that of the extracellular fluid, is usually in the range of -70 mV in neurons and -50 mV in smooth muscle. In vascular smooth muscles, estrogen is known to increase the resting membrane potential, i.e. render it more negative [8]. Estrogen-induced increases in vascular smooth muscle resting membrane potential contribute to estrogen's vasodilatory actions by inhibiting calcium influx through voltage-gated calcium channels.

The two membrane proteins that are primarily responsible for the maintenance of the membrane potential are the Na^+–K^+-ATPase, which actively transports Na^+ out of the cell and K^+ into the cell, and a K^+ leak channel that allows for the diffusion of K^+ out of the cell. The Na^+–K^+ pump serves to maintain the ion concentration gradient by pumping three Na^+ out for every two K^+ it brings in. In the cardiac cell, estrogen stimulates the Na^+–K^+-ATPase activity [6]. This effect on the Na^+–K^+ pump may contribute to estrogen's protective effects on the heart by hyperpolarizing the cell membrane away from the threshold for action potential generation. This will decrease the action potential firing rate, decrease heart rate and lower energy demand by the heart. A lower energy demand during times of ischemia can increase the chance that the myocardial tissue will survive.

Via the K^+ leak channel, potassium is allowed to travel out of the cell down its concentration gradient (outward) but this outward movement of K^+ is opposed by its electrical gradient (inward). A potassium balance or equilibrium is reached when its tendency to follow its concentration gradient outward is offset by its tendency to follow the electrical gradient inward. The membrane potential at which this balance is achieved is

referred to as the K^+ equilibrium potential. The relationship between the concentration gradient and the electrical gradient at equilibrium for an individual ion can be calculated by using the *Nernst equation*. In most cells, the cell membranes are very permeable to K^+ and therefore the resting membrane potential is near the K^+ equilibrium potential, which is very negative. However, there are additional ions that also contribute to the resting membrane potential. These are Na^+, Cl^- and to a lesser extent, Ca^{2+}. The *Goldman equation* uses the Nernst equation and individual ion gradients and permeabilities to predict the resting membrane potential of a cell (Fig. 1).

The action potential. The action potential is the mechanism by which nerve cells communicate and conduct information and muscle cells are induced to contract. Different cells types have characteristically distinct action potentials. The characteristics of the action potential are defined by the compliment of voltage-gated ion channels expressed in each individual cell. Some cells, such as neurons and cardiac cells, display an "all-or-none" action potential, whereas other cell types, such as vascular smooth muscle, display a graded response. An all-or-none, or phasic, response is defined as a rapid depolarization of the membrane potential followed by a repolarization back to the membrane potential maintained at rest. The rapid depolarization of a phasic response is due to the presence of voltage-gated Na^+ channels. When the membrane potential reaches the threshold value for opening the Na^+ channels, these channels open rapidly allowing a large flux of Na^+ into the cell due to the large electrical and chemical gradient for Na^+ across the cell membrane. This large Na^+ influx produces a strong depolarization of the membrane potential. In contrast, in a graded, or tonic, response, membrane potential can be maintained over a wide range of potentials without triggering a rapid depolarization. This graded response of the cell membrane can only occur in cells that do not contain voltage-gated Na^+ channels. In many cells, the characteristics of the action potential are influenced by sex and sex hormones. Sex hormones are able to increase and decrease the excitability of nerves and muscles via modulation of various ion channel conductances.

The action potential in each type of excitable cells can be described by five distinct electrically and chronically defined events: *subthreshold responses*, the *threshold*

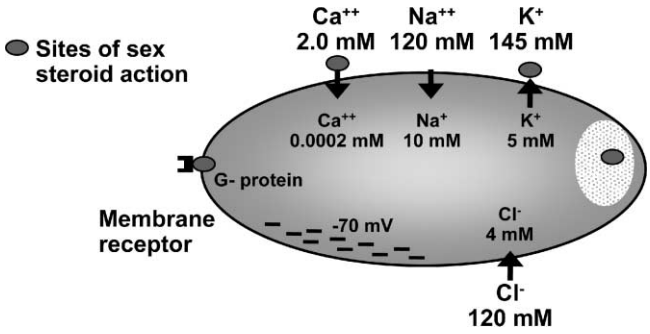

Fig. 1. The relative distribution of ions and related concentrations between the extracellular fluid and the cytoplasm in a mammalian cell. Sex steroids are capable of influencing ionic distribution across the cell membranes at a number of sites including the calcium channels, potassium channels, membrane neurotransmitter/modulator G-protein linked receptors and within the cell nucleus.

potential, the *overshoot*, *action potential duration* and the *after-hyperpolarization* or refractory period (Fig. 3). Each of these can be electrical events governed by specific ion channel events and can be targets of sex steroids. The subthreshold responses involve subtle increases (hyperpolarization) and decreases (depolarization) in membrane potential. These evoked depolarization potentials are summed in a graded fashion until the threshold for evoking an action potential is reached (Fig. 4). Testosterone has been shown to facilitate the subthreshold excitatory events in neurons in the hippocampus and may contribute to sex differences in excitability in these neurons [15].

The action potential is evoked when the membrane potential reaches the threshold for all-or-none depolarization. At this point, the depolarization results in a very large, fast response that actually "overshoots" the zero potential and cell membrane potential momentarily reverses sign (i.e. negative to positive). The action potential is driven by the gradient for Na^+ and the rapid but transient activation of the voltage-gated Na^+ channels. The rapid inactivation of the voltage-gated Na^+ and the slower, but simultaneous activation of voltage-gated K^+ channels are responsible for the return of the membrane potential to resting levels. The duration of the action potential is an important regulator of rate of cell repolarization and thus the cell firing rates of cells. Estrogen increases the cardiac action potential duration in females. This increased cardiac action potential duration in females contributes to sex differences in susceptibility to arrhythmias and some cardiac diseases such as long-QT syndrome [16] (see also Chapter 9). The after-hyperpolarization period of the action potential contributes to the relative refractory period of the action potential in which the ability of the cell to fire an additional action potential is markedly decreased. Generally, this phase is due to increased K^+ conductance that works to return the membrane potential back to rest, but overshoots the resting period briefly rendering the membrane potential more negative than that observed at rest.

3. Ionic mechanisms regulating membrane potential

Section 3.1 focuses on the effects of sex and sex hormones on specific ion channels that regulate membrane potential and the physiological outcomes of these sex differences.

3.1. Voltage-gated ion channels

Na^+ channel. As described earlier, the voltage-gated Na^+ channel plays a pivotal role in the initiation of the action potential in excitable cells. By influencing the characteristics of this channel, sex has the potential to greatly alter the cellular and integrative function of the neural and vascular system. For example, changes in the excitation threshold for Na^+ channel activation could make a neuron more or less apt to fire in response to a stimulus. Unfortunately, no information exists regarding the role of sex or sex hormones in influencing Na^+ channel expression or activity in neuronal or vascular smooth muscle cells. The sex hormones, estrogen and progesterone, do influence both the activity and expression of voltage-gated Na^+ channels in other cell types; so the possibility does exist for an influence of sex on this channel in vascular smooth muscle and neurons.

K^+ channel. In many cell types, including neurons and vascular smooth muscle, K^+ channels represent the dominant ion conductance and therefore define the membrane potential. Membrane potential changes, in turn, affect numerous cell responses including activation of voltage-gated Ca^{2+} channels, calcium influx and gene expression. In vascular smooth muscle, K^+ channels regulate vessel diameter through effects on membrane potential, voltage-gated Ca^{2+} channels and calcium influx. In neurons, the resulting Ca^{2+} influx triggers exocytosis of neurotransmitters and neuron-to-neuron communication. Numerous types of K^+ channels have evolved with varying characteristics, including differences in voltage-sensitivity and voltage- and time-dependent activation and inactivation. The complex nature of the K^+ channel families allows various cells exquisite control over membrane potential changes, for example the shaping of the action potential described earlier. Four types of K^+ channels that control membrane potential of arterial smooth muscle and neurons are: Ca^{2+}-activated K^+ channels (K_{Ca}), voltage-dependent or delayed rectifier channels (K_v), inward rectifier K^+ channels (K_{IR}) and ATP-sensitive K^+ channels (K_{ATP}). Activation of these K^+ channels produce hyperpolarization due to the negative equilibrium potential of K^+. This hyperpolarization produces vasodilation in blood vessels and inhibits neuron firing due to the subsequent inactivation of voltage-dependent Ca^{2+} channels. Sex and sex hormones are known to influence both the expression and activity of numerous vascular K^+ channels, thus sex has a profound influence on vascular and neuronal function [9]. The immediate (acute) and long-term (chronic) effects of sex hormones on K^+ channels are discussed below.

Acute effects. Traditionally, sex hormone effects are thought to be initiated by the sex hormone binding to its respective receptor. This hormone/receptor complex then enters the nucleus to regulate gene expression, thus these traditional responses are termed *genomic*. Since genomic responses require gene transcription and protein synthesis, they require relatively long periods of time (hours to days) to appear. However, some effects of sex hormones occur rapidly, within minutes and are therefore considered to act via *non-genomic* mechanisms. These acute, non-genomic responses may result from the binding of sex hormones to a different type of membrane-associated hormone receptor or, in some cases, the direct action of hormones on target proteins, such as ion channels (see also Chapters 3 and 4). Relaxation of vascular smooth muscle in response to acute application of both estrogen and testosterone are partly due to effects on ion channel activity. Both estrogen and testosterone stimulate Ca^{2+}-activated K^+ channels [5,17,19]. Estrogen and estrogen-like compounds increase Ca^{2+}-activated K^+ channel activity through stimulation of both guanylate cyclase and direct interaction with the channel [17,19]. In addition, estrogen increases calcium transport out of the cell, which would increase vasodilation in blood vessels and reduce neurotransmitter release in neurons [14,15]. Many of these effects have been reported in cells and tissues obtained from both females and males. This is not surprising for effects due to direct interaction of estrogen and K^+ channels as the target channels expressed in both sexes are identical. While many of these rapid, acute effects have been obtained in vitro using pharmacological doses of hormones, many recent reports have demonstrated similar effects at physiological levels. Thus, K^+ channels are the targets for many acute, presumably non-genomic, effects of sex hormones on vascular and neuronal tissue.

Chronic effects. While acute effects of sex hormones are primarily studied by administering sex hormones in vitro to isolated cell or tissue preparations, chronic effects of sex hormones, or the influence non-hormone related sex differences, are best studied in vivo. Recent advances in genetic models, e.g. mice lacking certain receptors, have allowed study of complex interactions of sex and sex hormonal status on ion channel function. Mice lacking a form of the estrogen receptor (estrogen receptor β-null mice) have elevated blood pressure (i.e. hypertension) and smooth muscle cells from these animals show profound reduction in K^+ channel activity compared to mice with normal expression of the receptor [20]. Thus, normal estrogen function is imperative for normal vascular K^+ channel function and blood pressure regulation. Loss of estrogen, or its receptors, can lead to loss of K^+ channel function and hypertension. Both male and female mice were used in this study indicating that estrogen receptors, which are expressed by cells of both sexes, play a role in regulating vascular function in both females and males. In fact, the increase in blood pressure due to loss of the estrogen receptor was greater in males compared to females.

Ca^{2+} channel. Voltage-gated Ca^{2+} channels in vascular smooth muscle play a central role in the regulation of arterial tone and, thus, blood flow and blood pressure. In neurons, calcium influx through these channels is required for exocytosis, neurotransmitter release and cell-to-cell communication. Neural, hormonal and local agents (e.g. serotonin, endothelin, norepinephrine) influence vascular and neuronal cell function through modulation of voltage-gated Ca^{2+} channels.

Acute effects. As described earlier, the effects of sex hormones on K^+ channels and membrane potential alter cell function through their subsequent effects on voltage-gated Ca^{2+} channels. Thus sex hormones can influence voltage-gated Ca^{2+} channel activity in a secondary, indirect manner. However, sex and sex hormones also affect Ca^{2+} channel activity directly, independent of effects on K^+ channels [1]. Estrogen inhibits calcium influx during depolarization due to a direct inhibition of L-type voltage-gated Ca^{2+} channels [3,13]. This inhibition of Ca^{2+} channel activity affects both arterial diameter and neuronal conduction by inhibiting contraction and neurotransmitter release, respectively. As mentioned previously, Ca^{2+} channel activation results in increased vasoconstriction and, thus increases blood pressure. In neuronal cells, calcium influx through Ca^{2+} channels is required for the release of neurotransmitters that increase vasoconstriction to increase blood pressure. Thus, the estrogen effect on vascular calcium channels has the potential to reduce peripheral vascular resistance and lower blood pressure. In neuronal tissues, this effect may play a role in sex differences in cardiovascular reflex responses, for example, the baroreflex response. Integrated neural–vascular processes, like regulation of blood pressure, may be affected by sex hormones at multiple levels. For example, inhibition of Ca^{2+} channel activity by estrogen at both the neuron and vascular smooth muscle cell level would be expected to have an additive effect on reducing blood pressure. Unfortunately, the literature is somewhat unclear as to whether this estrogen effect is common to both sexes. Studies either do not indicate the sex of the animal from which the cells were obtained, or more often, use cell lines in culture with no reference to the chromosomal sex of the cells.

Chronic effects. While numerous reports exist regarding the acute effects of sex hormones on vascular tone, fewer and more equivocal studies exist regarding long-term

effects. Chronic effects of sex hormones are most often studied using animal models involving the removal of sex organs (gonadectomy) and subsequent hormone replacement. This paradigm has shown that sex differences in Ca^{2+} channel activity contribute to differences in vascular function between males and females [4]. When voltage-gated Ca^{2+} channels are stimulated by depolarizing solutions containing high extracellular K^{+}, subsequent intracellular calcium and contractile responses are greater in vessels from male rats compared to females. This sex difference is due to the presence of estrogen in females. Ovariectomy in females eliminates this sex difference and estrogen replacement in ovariectomized females restores the sex difference. On the contrary, removal of testosterone in males by castration had no effect on the sex difference [3,4,12]. Unfortunately, the corollary experiment of giving estrogen to males was not done; thus it remains unknown whether providing estrogen to males will inhibit calcium and contractile responses similar to females. These studies demonstrate the strong influence of estrogen on vascular reactivity being mediated through actions on ion channels, specifically a reduction in calcium channel activity and/or expression by estrogen. Inhibition of calcium channel synthesis by estrogen likely occurs through a classical genomic mechanism as functional estrogen receptors are required for this effect [10].

Chronic interactions of sex and sex hormones on calcium channels may be more complex than expected. Some studies have observed that when estrogen is given to ovariectomized female rats, calcium channel numbers in aortic smooth muscle are increased [2]. Interestingly, pharmacological stimulation of these channels with the drug,

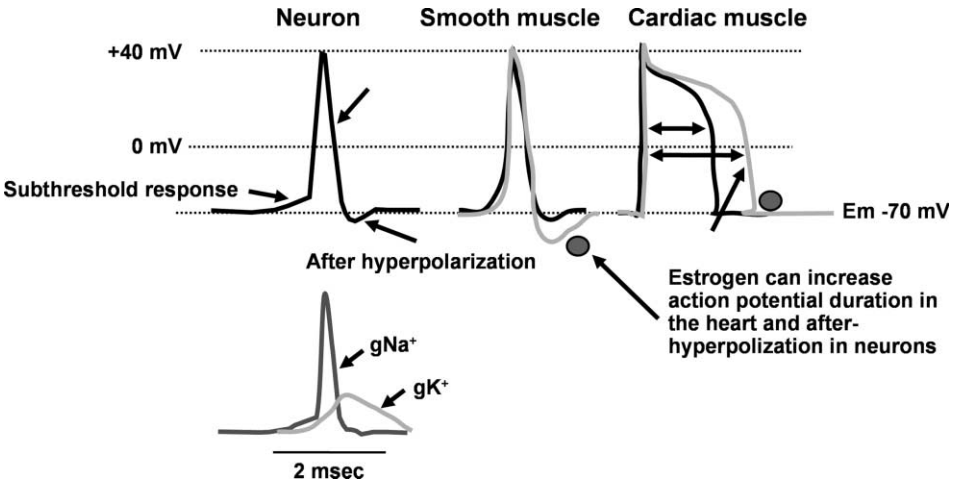

Fig. 2. Illustration of typical action potentials from three different types of excitable cells: the neuron, the smooth muscle cell and the cardiac muscle cell. The principal currents underlying the neuronal action potential include the rapid and transient conductance of Na^{+} (gNa^{+}) and the delayed conductance of K^{+} (gK^{+}). Sex steroids such as estrogen can modulate the intrinsic properties of the action potential and thus alter the excitability of the cell. For example, estrogen is known to decrease smooth muscle excitability by increasing the after-hyperpolarization. In heart cells, estrogen can increase the duration of the action potential and thus influence the activity of these cardiac myocytes.

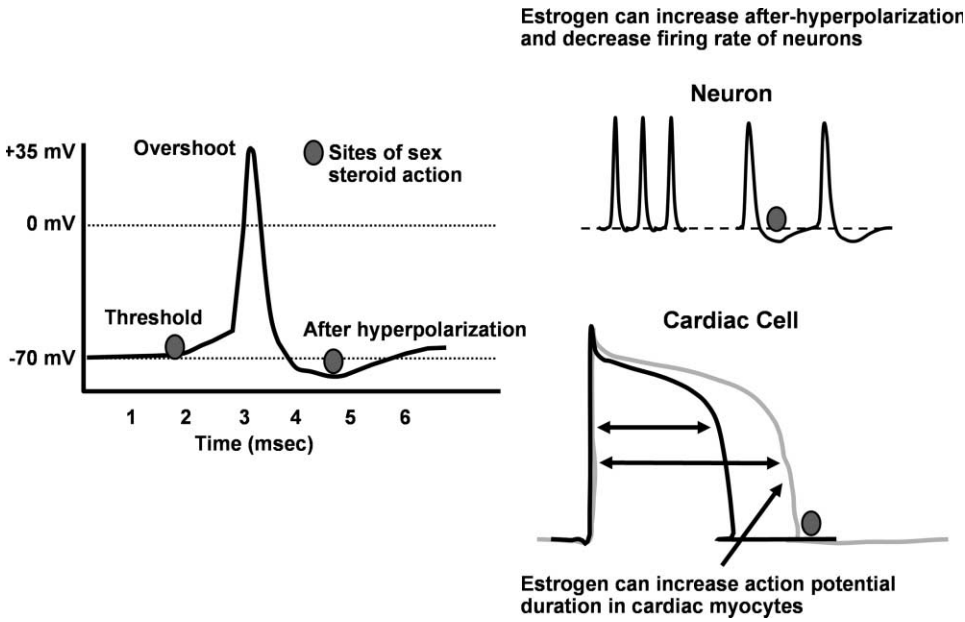

Fig. 3. Characteristics of an action potential and effects of sex steroid actions. In neurons, sex steroids can increase after-hyperpolarization, while in cardiac cells the action potential duration can be increased. Both of these effects are due to changes in K^+ channel activity.

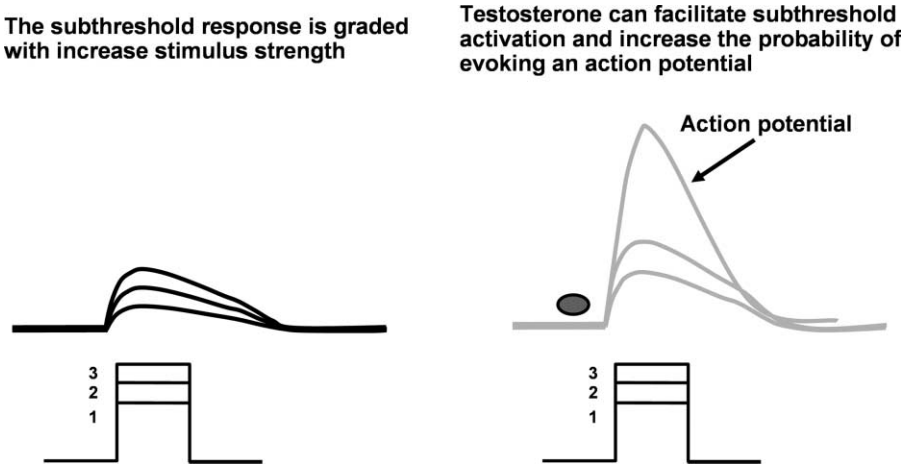

Fig. 4. The subthreshold response for depolarization is graded with increased stimulus strength (left panel). Testosterone can facilitate subthreshold activation and increase the probability of evoking an action potential (right panel).

Bay K 8644, produced contractions that were not different between estrogen treated and untreated vessels. To reconcile this paradox, it was proposed that the Ca^{2+} channels expressed by estrogen were either non-functional or that a compensatory mechanism was also upregulated to offset increased calcium influx. Thus, estrogen may have complex, multi-targeted effects on cell calcium regulation. Similarly, testosterone increases calcium channel activity in certain experimental situations. Subcutaneous administration of testosterone increased depolarization responses of coronary arteries in both males and females [7,11].

4. Summary

(1) Sex and sex steroids are potent modulators of membrane excitability.
(2) Within the components of the action potential, sex steroids are known to affect subthreshold responses, action potential duration and the after-hyperpolarization (Figs. 2–4).
(3) The ion channels underlying these events of the action potential are direct sites of action for both the acute and chronic effects of sex steroids.
(4) Both estrogen and testosterone can stimulate Ca^{2+}-activated K^+ channels and normal estrogen function is important to normal K^+ channel expression.
(5) Estrogen and testosterone have been shown to modulate voltage-gated Ca^{2+} channel function and expression.

5. Future directions

Plasma membrane ion channels and transporters are central to regulation of membrane potential and excitability in both neuronal and vascular cells. The firing of neuronal action potentials and smooth muscle contractility are central to cardiovascular control. A growing body of scientific studies clearly demonstrates that sex and sex hormones affect ion channels in both cell types. Thus, understanding sex and sex hormone effects on cardiovascular neuronal and smooth muscle cells is vital to understanding sex differences in blood pressure regulation, blood flow and cardiovascular disease risk. Although much has been learned in the past decade or two, much remains unknown in this area. The potential for sex hormone regulation of Na^{2+} channels is an area of great potential in which little has been done. In addition, much of the knowledge in the area derives from acute, short-term exposure of cells and tissues to sex hormones, often at levels above that seen in the body. More scientific investigation is needed to examine the long-term role of sex hormones on vascular and neuronal voltage-gated Ca^{2+} channels and the complex integration of these effects on vascular and neuronal function. Finally, more rigorous examination of the potential for sex-specific effects of sex hormones is needed. Many studies to date have not examined whether the effects occur similarly in both males and females. This is of great importance if the beneficial effects of sex hormones, or pharmacological substitutes, are going to be exploited to treat diseases.

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Sex-differences in electrophysiology of the heart and cardiac arrhythmias

Thai V. Pham and Michael R. Rosen

1. Introduction

It has long been noted that there are male/female differences in cardiovascular physiology and pathophysiology and in recent years there is increased awareness/appreciation of the influence of a patient's sex on presentation of various cardiac arrhythmias (Table 1). One of the most dramatic and important differences between men and women regarding sex and arrhythmia is the greater risk of arrhythmia induced by drugs. It is now recognized that a wide spectrum of drugs that prolong ventricular repolarization and QT interval [6], including certain antiarrhythmics, antihistamines and antipsychotics, induce a malignant cardiac rhythm disturbance, torsades de pointes (TdP), more frequently in women than men (Table 2). Although the mechanisms underlying sex-related differences in the presentation of different types of arrhythmias are largely unknown, they are thought to be associated with fundamental differences in the electrophysiology of male and female hearts. Recent clinical and experimental studies have begun to identify some key factors related to sex-differences in the risk of acquired (or drug-induced) long QT and TdP. These studies suggest that gonadal steroids are important factors or modulators of sex-related differences in arrhythmias. This chapter will consider (1) the sex- and hormonal-related differences in the electrophysiology of the heart; (2) differences in the responses to drugs that induce QT prolongation; and (3) the potential sex-related differences in the mechanisms underlying the induction of TdP.

2. Overview

The heart is a functional syncytium of excitable muscle cells organized to provide a pump mechanism for the circulation of blood. The contractions of these muscle cells are controlled electrically. In the normal heart, the overall regulation of contraction is via an orderly pattern of conduction of electrical impulses (i.e. action potentials) throughout the myocardium [7]. The spread of excitation in the heart can be assessed via an electrocardiogram (ECG) as illustrated in Fig. 1.

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Table 1
Male–female presentation of cardiac arrhythmias

Arrhythmia type	Male predominance	Female predominance
Bradyarrhythmia	Atrioventricular block	Sinus node disease
Supraventricular tachyarrhythmia	Atrial fibrillation	Inappropriate sinus tachycardia
	AVRT	AVNRT
	WPW syndrome	
Ventricular tachyarrhythmia	Ventricular tachycardia	Congenital LQTS
	Sudden cardiac death	Acquired LQTS
	SUDS	
	Brugada syndrome	

AVNRT, atrioventricular node re-entrant tachycardia; AVRT, atrioventricular reentrant tachycardia; LQTS, long QT syndrome; SUDS, sudden unexplained death syndrome; WPW, Wolf–Parkinson–White.
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The ECG can be considered as a summation of electrical activities of all cardiac myocytes during the excitation (systole) and relaxation (diastole) of the heart. The impulse originates in the cells of the sinoatrial node and is conducted to the atrial muscles and the atrioventricular (AV) node. The AV node introduces a delay as it propagates the impulse to the bundle of His and the Purkinje fibers, from which the impulse is rapidly conducted to the ventricle. The P wave on the ECG signifies the excitation of the atria. The QRS complex represents ventricular excitation/depolarization and the T wave corresponds with

Table 2
Examples of drugs that prolong cardiac repolarization and induce TdP

Class	Drug	TdP reported
Antiarrhythmic drugs	Amiodarone	+
	Dofetilide	+
	Procainamide	+
	Quinidine	+
	D,L-sotalol, D-sotalol	+
Vasodilators/anti-ischemic agents	Bepridil	+
	Prenylamine	+
Psychiatric drugs	Clomipramine	+
	Haloperidol	+
	Lithium	+
	Thioridazine	+
Antimicrobial and antimalarial drugs	Erythromycin	+
	Halofantrine	+
Antihistaminics	Astemizole	+
	Diphenhydramine	+
	Terfenadine	+

Adapted from Cardiovasc. Res. 2000, 47, 219–233.

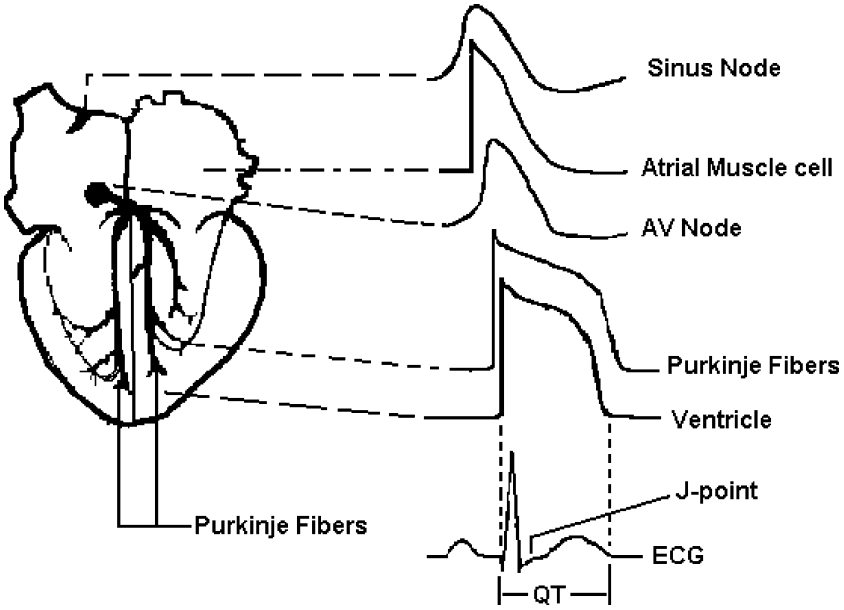

Fig. 1. Regional electrical waveforms of the heart. The electrical waveforms on the right and above the ECG are local action potentials recorded from the different regions of the heart. The QT interval on the ECG reflects the action potential duration of millions of individual ventricular cells in situ. Measurements of the QT interval correspond with the repolarization time of the ventricle (Modified with permission after Netter, F.H., 1971. The CIBA Collections of Medical Illustrations: Heart. Vol. 5. p. 49 [11]).

ventricular repolarization (i.e. recovery from excitation). The ST segment is the part of the ECG tracing immediately following the QRS complex and the point at which it starts from the QRS complex is known as the J point. The QT interval reflects the duration of activation and repolarization of the ventricle while the JT interval reflects repolarization only (Fig. 1).

Ionic basis of the cardiac action potential. The unequal distribution of Na^+, K^+, Ca^{2+} and Cl^- across the cell membranes creates an ionic concentration gradient that give rises to the electromotive forces of these various ionic species. These ions carry/conduct electrical currents across the cell membranes as they pass through different classes of ion channels. The regulation of ionic currents across the cardiac cell membranes maintains the cell's resting membrane potential and excitability.

Specific ionic currents contribute to each phase of the cardiac action potential (Fig. 2). The current waveforms shown above the action potential are those of inward/depolarizing currents (Na^+ current [I_{Na}] and L-type Ca^{2+} current [$I_{Ca,L}$] and the sodium calcium exchanger [$I_{Na/Ca}$]) and the current waveforms below the action potential reflect outward ionic (predominately K^+ repolarizing) currents responsible for repolarization of the action potential. The different cardiac action potential shapes and waveforms (Fig. 1) arise as a result of different currents and regulation of the ion channels in the various types of cardiac cells.

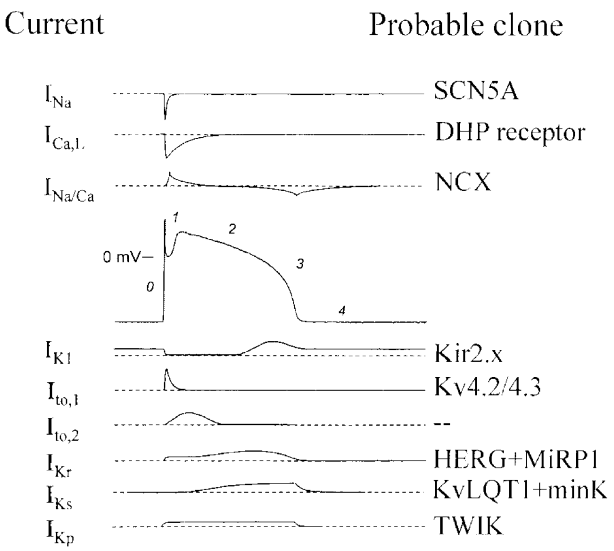

Fig. 2. Ionic basis of the cardiac action potential. A representative action potential is shown in the middle with the numbers indicating the different phases of the depolarization and repolarization of the action potential. Nomenclatures on the left denote the ionic currents and on the right indicate the molecular clones of the channels. The electrical waveforms above and below the action potentials show the relative ionic currents contributing depolarization and repolarization, respectively (Printed with permission from Cardiovasc. Res. 2000, 47, 219–233).

Action potential duration (APD) is a measure of repolarization of the cell. Ventricular APD to 90% (APD_{90}) repolarization is used as a surrogate to approximate the QT interval (Fig. 1). With respect to repolarization it is important to bear in mind that increasing the magnitude of depolarizing current such as $I_{Ca,L}$ will lengthen the action potential (or prolong repolarization) while increasing repolarizing currents such as K^+ currents will shorten repolarization.

Basic principles of sex-related arrhythmias. There are many causes of arrhythmia, including cardiac disease (i.e. myocardial infarction), abnormal electrolytes, or toxic effects of drugs; however, the underlying mechanism of arrhythmia most often lies with abnormalities in electrical activities of the cardiac cells and the ability to conduct impulses through the heart. Female sex is an important risk factor for cardiac arrhythmias with the major risk associated with congenital and acquired long QT syndromes. The mechanisms for the signature arrhythmia associated with long QT syndrome, TdP, are referred in Fig. 3.

Torsades de pointes is a polymorphic ventricular tachycardia characterized by a progressively changing amplitude and contour of the QRS complexes that appear to twist around the isoelectric line. As a general rule, drug-induced TdP is associated with QT prolongation (i.e. prolonged ventricular repolarization). Drug-induced prolongation of repolarization, in most cases, results from reduction in outward repolarizing K^+ current, especially the rapidly activating delayed rectifier K^+ current (I_{Kr}). However, toxins or drugs that increase inward currents such as the sodium current can also prolong ventricular repolarization.

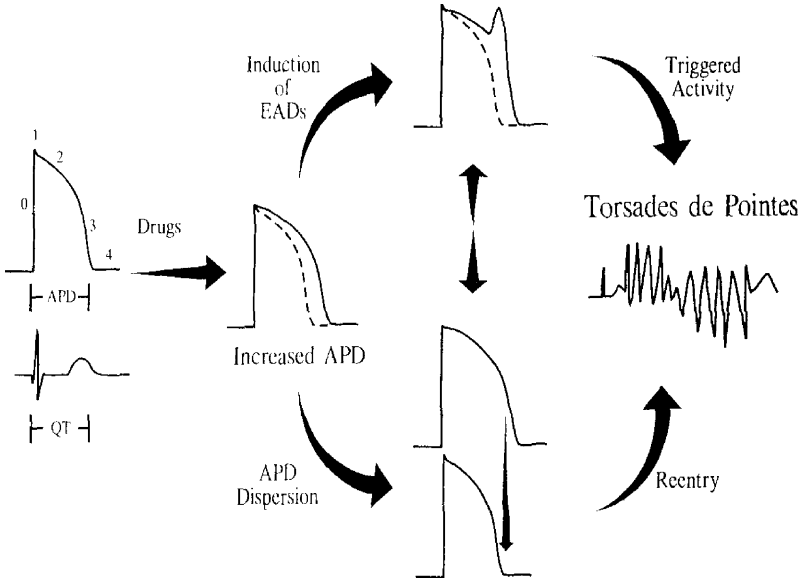

Fig. 3. Mechanisms underlying the induction of torsades de pointes by drugs that prolong repolarization (Printed with permission from J. Women's Health 1998, 7(5), 547–557).

When ventricular repolarization is excessively prolonged, induction of early after-depolarizations (EAD) can occur in the ventricle. These EAD are oscillations thought to be caused by increased inward current through the L-type calcium channels and/or the T-type calcium channels or sodium channels. EAD can initiate premature action potentials and resultant triggered activity if they depolarize and induce action potentials that propagate through the ventricles. Alternatively, excessive QT prolongation can exaggerate regional dispersion of electrical activity (i.e. epi-endocardial dispersion) creating conditions that elicit reentry. In other words, induction of TdP can be influenced by abnormal impulse initiation (i.e. EAD) and/or abnormal conduction (action potential dispersion and re-entry).

3. Sex, hormones and cardiac electrophysiology

3.1. Clinical studies

Sex-related differences in the electrocardiogram are noted in Table 3. Women have faster resting heart rates and longer rate-corrected QT intervals (QTc) than men [10]. Sex-related differences in the QT-RR relationship (rate-adaptation) may contribute to the increased length of the QTc in women, because the sex-based differences in the QT intervals on the ECG are more pronounced at slow heart rates than at rapid rates. In addition to QT duration differences, the maximum instantaneous slope of the ascending and descending limbs of the T wave is less steep in women than in men. These data suggest

Table 3
Gender-related differences in ventricular repolarization in humans

Measurement of repolarization	Condition or treatment	Summary
QTc duration	Normal	QTc duration is longer in women than men
	Rate dependence	Steeper QT/RR ratio in women than men
	Age to	
	1 ~ 12 yrs	QTc duration is the same
	~ 13 to 50 yrs	QTc duration is longer in women than men
	> 50 yrs	QTc duration is the same
ST duration	Normal	ST duration is longer in women than men
T wave	Ascending and descending slope	Less steep in women than men
JT duration	Gonadal steroids	
	Virilization	JT duration is shorter in virilized women than normal women
	Orchiectomy	JT duration is shorter in normal men than orchiectomized men
Risk of developing TdP	Drugs that prolong repolarization (i.e. quinidine, sotalol, etc.)	
	Normal	Lower risk for developing TdP in men than women
	Menstrual cycle	Lower risk for developing TdP in women during luteal phase than menstruation and ovulation phases

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that biological sex influence the early and late phases of repolarization and, potentially, dispersion of repolarization.

Sex hormones and repolarization (*QT interval*). Sex-associated differences in QTc intervals appear to have a developmental/hormonal component. No sex-related difference in heart rate or QT interval is observed in children before the age of 10 (Table 3). However, from puberty through their adult years, women have faster heart rates with correspondingly longer QTc than men (Fig. 4). The duration of the QTc in men decreases at puberty and then gradually increases until the sixth decade when the QTc approaches that of women [16]. The shortening of the QT interval during and after puberty in males implies that androgen (specifically testosterone) rather than estrogen may contribute to sex differences in QTc. This hypothesis is further supported by a recent study showing

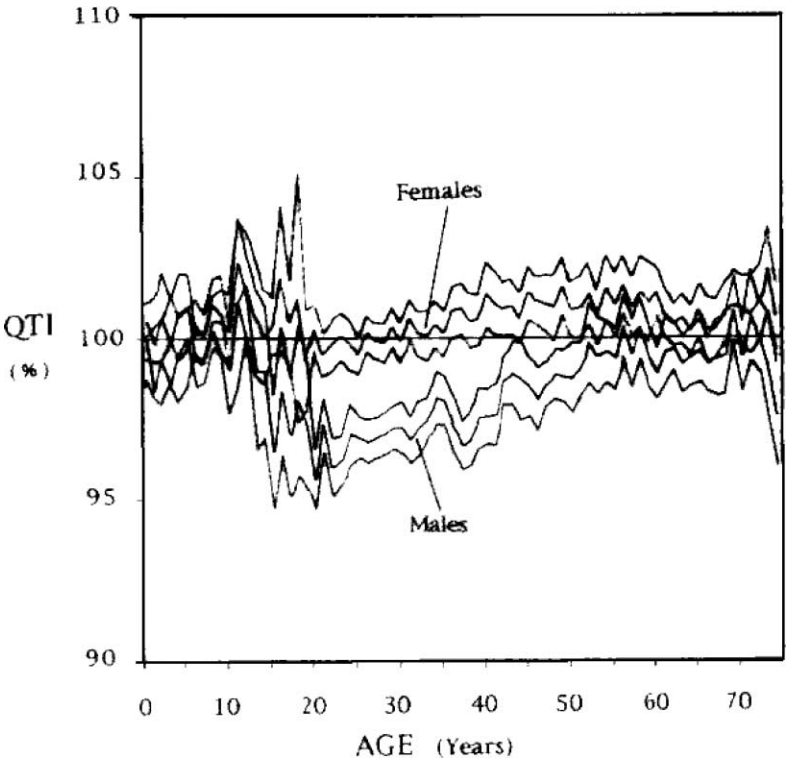

Fig. 4. Age–sex associated differences in QT interval. Mean values of QT index demonstrate a shortening in men's QT index during puberty which then increases over time. (Printed with permission from Can. J. Cardiol. 1992, 8, 690–695).

a tendency for longer JT intervals in orchiectomized as compared to non-orchiectomized men [1]. This same study reported that women with virilization have shorter JT intervals than castrated men and normal women (Table 3).

Sex, hormones and TdP. The most pronounced and potentially lethal sex-specific difference with regards to arrhythmia is the female predisposition toward drug-induced life-threatening arrhythmia, TdP [10]. There is a marked overall female predominance in reported TdP cases with approximately 70% of all cases relating to antiarrhythmic agents occurring in females. Similarly, 67% of all reported cases of TdP induced by non-antiarrhythmic drugs were women.

It is evident that women more often than men develop TdP after taking a variety of drugs, including some antiarrhythmics, antipsychotics, antibiotics, antimalarials, antihistamines and various other drugs (see partial listing in Table 2). All these drugs from markedly varying classes have in common the effect to prolong cardiac repolarization and the QT interval on the ECG by blocking the delayed rectifier potassium current, I_{Kr}.

Female sex hormones may be important factors contributing to drug-induced arrhythmia in women, because women taking oral contraceptives are at greater risk of ventricular ectopy [18] and women are most at risk of drug-induced QT prolongation during the menstruation and ovulatory phases, but not the luteal phase of the menstrual cycle [17].

3.2. Experimental studies

Recent experimental studies, largely in rabbits, have begun to address the cellular and ionic basis of biological sex and gonadal steroids on differences in cardiac electrophysiology and drug-induced arrhythmias [4,12]. Similar to humans, there are sex-related differences in rabbit QT interval, rate-dependence of QT duration and susceptibility to drug-induced arrhythmia. Female rabbits have a steeper cycle length dependence of QT such that at slow heart rates the QT intervals of female rabbits are longer than those in males (Table 4).

3.3. Effects of repolarization prolonging drugs

In rabbits, female sex is associated with a higher incidence of TdP and potassium channel blockade and QT prolongation appear to be contributory factors (Table 5). Drugs that prolong repolarization, such as quinidine and D-sotalol, cause greater QT prolongation in females [9]. In addition I_{Kr}-blocking drugs (dofetilide) induce greater prolongation of repolarization (i.e. APD_{90}), a higher incidence of EAD and greater transmural dispersion of repolarization in female than in male rabbits.

3.4. What is the role of sex hormones in cardiac repolarization and arrhythmia?

Gonadectomy and repolarization. In both male and female rabbits, gonadectomy has little or no effect on baseline APD_{90} at CL = 1000 ms (Fig. 5A, Table 5). However, gonadectomy had a dramatic effect on the response of rabbit papillary muscles to the I_{Kr}-blocking drug, dofetilide (Fig. 5B). In male rabbits, orchiectomy resulted in an increase in the dofetilide-induced prolongation of APD and incidence of EAD; whereas, in female rabbits oophorectomy reduced the risk for dofetilide-induced APD prolongation and EAD. Because the study [15] determined that estradiol levels did not differ in female and oophorectomized female rabbits, the data argue against a unique estrogenic basis for the greater risk of females to the proarrhythmic effects of an I_{Kr} blocker. Thus, it is probable that non-estrogenic ovarian factors contribute to the promotion of a proarrhythmic response.

Sex hormone replacement. In oophorectomized female rabbits, chronic estradiol and DHT treatment resulted in significantly longer QT intervals than in placebo-treated rabbits. Further, hormone treatment can modify the rate dependence of ventricular repolarization (Table 5), such that at cycle lengths of 300 ms differences in APD_{90} were negligible while at cycle lengths greater than 500 ms the estradiol-treated group exhibited significantly longer APD_{90} than the DHT-treated group [5].

Table 4
Gender-related differences in ventricular repolarization in the rabbit

Preparation	Measurement of repolarization	Condition or treatment	Summary
Male/female rabbits isolated Langendorff hearts	QT duration	CL = 400 ms	QT is equal in males and females
		CL = 2300 ms	QT is longer in females than males
	ΔQT duration	D-Sotalol	ΔQT ↑ is greater in females than males
		Quinidine	ΔQT ↑ is greater in females than males
Male/female rabbits isolated right ventricle	APD_{30}	CL = 330, 500, 1000 ms	APD_{30} is greater in females than males
	APD_{90}	CL = 330, 500, 1000 ms	APD_{90} is equivalent between females and males
	ΔAPD_{90} CL = 1000 ms	Dofetilide (10^{-8} M)	ΔAPD_{90} ↑ is greater in females than males
	Incidence of EAD (CL = 1000 ms)	Dofetilide (10^{-6} M)	Greater incidence of EAD in females than males
	Transmural dispersion of APD_{90}	Control	Epicardial APD_{90} is equivalent to endocardial APD_{90} in both females and males
		Dofetilide (10^{-6} M)	APD_{90} transmural dispersion is greater in females than males

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Table 5
Effects of sex hormones on ventricular repolarization in the rabbit

Preparation	Measurement of repolarization	Condition or treatment	Summary
Gonadectomized male/female rabbits: isolated right ventricle	APD_{90}	CL = 330, 500, 1000 ms	OVX-females = ORCH-males
	ΔAPD_{90} ↑	Dofetilide (10^{-8} M); CL = 1000 ms	ORCH-males > OVX-females
	Incidence of EAD	Dofetilide (10^{-6} M); CL = 1000 ms	ORCH-males > OVX-females
OVX-female rabbits treated with either EST, DHT or placebo (PLA): isolated right ventricle	APD_{90}	CL = 330 ms	EST = DHT = PLA
	APD_{90}	CL = 500–2000	EST > DHT
	Incidence of EAD	CL = 5000	EST > DHT = PLA
		E4031 (10^{-6} M); CL = 1000 ms	EST > DHT = PLA
		E4031 (10^{-6} M); CL = 2000 ms	EST > DHT = PLA
ORCH-male rabbits treated with either EST, DHT or placebo: isolated right ventricle	APD_{90}	Control; CL 1000 ms	EST = DHT = PLA
	ΔAPD_{90} ↑	Dofetilide (10^{-8} M); CL = 1000 ms	PLA = EST > DHT
	Incidence of EAD	Dofetilide (10^{-8} M); CL = 1000 ms	PLA = EST > DHT
Female treated with DHT	APD_{90}	Control; CL = 1000 ms	Female > female + DHT
	ΔAPD_{90} ↑	Dofetilide (10^{-8} M); CL = 1000 ms	Female > female + DHT
	Incidence of EAD	Dofetilide (10^{-6} M); CL = 1000 ms	Female > female + DHT

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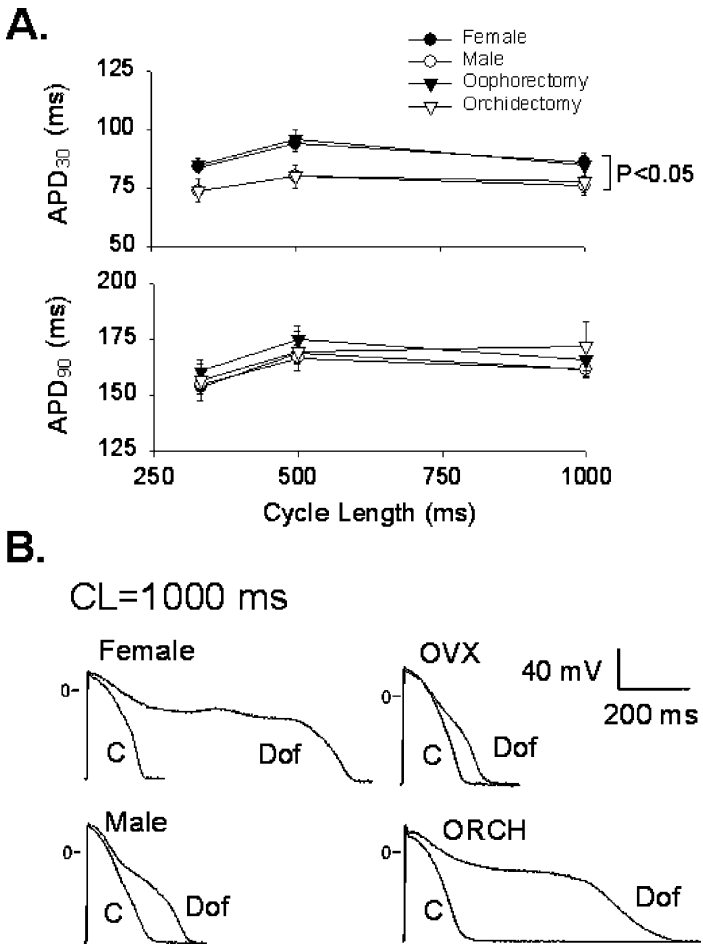

Fig. 5. Effects of gonadectomy on APD and response to dofetilide. (A) Predrug control measurement of APD. (B) The sexual differential response to dofetilide (10^{-6} M) is reversed by gonadectomy. OVX and ORCH indicate oophorectomy and orchiectomy, respectively (Modified from Circulation 2001, 103, 2207–2212).

Gonadal steroids also modulate the response to drugs that block the delayed rectifier K^+ current (I_{Kr}). I_{Kr}-specific blocking drugs cause more extensive prolongation of repolarization and higher incidence of EAD in estradiol-treated oophorectomized female rabbits than placebo- and DHT-treated groups [3] (Table 5).

As noted earlier, testosterone may protect against drug-induced excessive prolongation of repolarization and EAD [8,13]. Recent experimental studies have provided direct evidence for this by evaluating the effects of chronic administration of dihydrotestosterone (DHT) on repolarization and drug response in orchiectomized male rabbits and normal female rabbits. Castration of male rabbits resulted in an increase in both dofetilide-induced action potential prolongation and incidence of EAD (Table 5). These effects of dofetilide

were diminished upon DHT replacement to levels equivalent to that present in normal male rabbits (Table 5). Similarly, chronic DHT treatment in female rabbits resulted in shortening of ventricular repolarization under control conditions. In the presence of dofetilide, DHT treatment decreased the action potential prolonging effects of dofetilide and decreased the incidence of EAD induced by dofetilide compared to normal females (Table 5).

To summarize, gonadal steroids are important determinants of sex-specific differences in repolarization. The basic electrophysiologic characteristics such as QT interval (increased), APD (prolonged at slow heart rate) and response to I_{Kr} blockers (exaggerated) expressed in the estrogen-treated rabbits are analogous to those of female rabbits. Moreover, the electrophysiologic characteristics in DHT-treated rabbits are analogous to those seen in control males. Finally, testosterone can modify baseline repolarization such that the response to repolarization prolonging drugs is diminished.

3.5. *Sex-specific ionic basis of repolarization*

Thus far, experimental studies in rabbits have only examined in detail the L-type calcium current ($I_{Ca,L}$) and some of the repolarizing K^+ currents (Table 6 and 7).

L-type calcium current. Transmural dispersion of $I_{Ca,L}$ occurs in female but not in male rabbit ventricle [14]. This gradient is due to greater whole cell $I_{Ca,L}$ conductance in the epicardium than in the endocardium of female ventricle. The $I_{Ca,L}$ transmural gradient in females may contribute to the greater transmural (epi-endocardial) dispersion of repolarization and increased occurrence of EAD in female rabbit ventricles.

Repolarizing potassium currents. Currently, only three major repolarizing K^+ currents have been examined: the transient outward current (I_{to}), the inwardly rectifying K^+ current (I_{K1}) and I_{Kr}. There is no sex-based difference in I_{to} density [9]. However, I_{Kr} density was approximately 20% lower in females than males. Further, there is less outward I_{K1} density in female than male rabbits [2]. The smaller I_{Kr} and I_{K1} densities in female rabbits may contribute to the gender disparity in QT interval. In addition, the lower I_{Kr} density may

Table 6
Gender-related differences in ionic currents in the rabbit ventricle

Preparation	Ionic current	Property	Summary
Male/female rabbits disaggregated LV myocytes	$I_{Ca,L}$	$I_{Ca,L}$ density	Transmural gradient in females; absent in males
	I_{Kr}	Current density	Males ~20% greater than females
	I_{K1}	Current density	Greater in males than females at 50 mV; at all other voltage points males and females have equal I_{K1} density

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Table 7
Effects of gonadal steroids on ventricular ionic currents in the rabbit

Preparation	Ionic current	Property	Summary
OVX-female rabbits treated with either EST, DHT, or PLA: disaggregated LV myocytes	$I_{Ca,L}$	$I_{Ca,L}$ density: transmural gradient	EST = DHT > PLA
ORCH-male rabbits treated with either EST, DHT or PLA: disaggregated LV myocytes	$I_{Ca,L}$	$I_{Ca,L}$ density: transmural gradient	PLA = EST = DHT
OVX-female rabbits treated with EST, DHT or PLA: RNA from ventricle	HERG (I_{Kr})	mRNA levels	PLA = EST = DHT
	KCNE1 – regulatory subunit of I_{Ks}	mRNA levels	PLA > EST = DHT
	Kv1.5; I_{Kur}	mRNA levels	PLA = EST = DHT
ORCH-male treated with DHT	I_{Kr}	I_{Kr} density	ORCH + DHT > ORCH
	I_{K1}	Outward I_{K1} density	ORCH + DHT > ORCH

Adapted with permission from Cardiovasc. Res. 2000, 53, 740–775.

explain the steeper cycle length dependence of the QT interval in females given that at rapid heart rates there is more I_{Kr} than at slow rates.

Effects of gonadectomy and hormone replacement on $I_{Ca,L}$. Gonadectomy, estradiol or DHT treatment have no effect on $I_{Ca,L}$ density or other $I_{Ca,L}$ properties in male rabbits [14]. However, oophorectomy eliminates the transmural $I_{Ca,L}$ gradient in female rabbits. This result suggests that the ovaries contribute hormonal factors important for the modulation of $I_{Ca,L}$ in female rabbits. Moreover, there are modulatory effects of estradiol and DHT on regional $I_{Ca,L}$ properties. In vivo estradiol and DHT treatment of oophorectomized female rabbits partially restores the $I_{Ca,L}$ transmural gradient.

Because levels of estradiol and DHT in normal females are similar to those of oophorectomized female rabbits, it is likely that estradiol and DHT are not unique modulators of $I_{Ca,L}$ under physiologic conditions. Furthermore, effects of estradiol and DHT on $I_{Ca,L}$ kinetics are different from those observed in normal females. These data suggest that other factors (e.g. progesterone, GnRH, LH or FSH) may contribute to the modulation of $I_{Ca,L}$.

Effects of gonadectomy and hormone replacement on K^+ currents. In oophorectomized female rabbits, chronic estradiol and DHT treatment downregulated the message

levels of two potassium channels [3]: Kv1.5, a clone of the ultra-rapidly activating delayed rectifier (I_{Kur}) and KCNE1, a modulatory subunit of the slowly activating delayed rectifier, I_{Ks}. Thus far, only two K^+ currents (I_{Kr} and I_{K1}) have been evaluated with respect to DHT replacement in orchiectomized male rabbits [2,8]. Chronic DHT treatment increases the maximum I_{Kr} density and outward I_{K1}. These effects would decrease APD and contribute to the shorter QT interval of the male. Since the hormones change in mRNA levels of the two potassium channels, it is likely that the hormones act through classical genomic mechanisms of hormone treatment. However, the full impact of hormonal treatment and biological sex on changes in ionic channels remains to be determined and goals of future studies.

3.6. Ionic mechanism of sex-related differences in repolarization and proarrhythmia

Sex-related differences in the $I_{Ca,L}$ transmural gradient combined with smaller I_{Kr} and I_{K1} densities in female compared to male rabbit ventricle could result in prolonged repolarization and greater transmural dispersion of repolarization in female hearts. These conditions in the presence of I_{Kr}-blockade may cause higher incidences of EAD in females. Prolonged repolarization, transmural dispersion and EAD are important factors for the induction of TdP and together these factors create conditions that put females at greater risk for proarrhythmic effects of drugs (Fig. 3).

Role of testosterone. Clearly, there is sufficient evidence for the protective role of DHT in males and females against the risk for drug-induced excess APD prolongation and EAD. That DHT has no effect on $I_{Ca,L}$ in males implies that the protective action of DHT is not through changes in $I_{Ca,L}$ but might occur via modulation of other ionic currents (i.e. I_{Kr}). Other currents contributing to repolarization such as the persistent sodium current, I_{to}, or the slowly activating potassium delayed rectifier (I_{Ks}) remain to be examined.

4. Future directions

4.1. Are there male and female hearts?

Clearly there are now ample data hinting at unique male and female hearts. Although the baseline cardiac electrophysiological differences are subtle and complex, the expressed phenotypes (i.e. risk of ventricular arrhythmias) are dramatic. Clinical data indicate that women are at greater risk for drug-induced arrhythmias (TdP) and that female sex is an independent risk factor for syncope and sudden death. These observations suggest that the higher propensity towards arrhythmias in females is associated with fundamental differences in ventricular cellular repolarization in the normal heart such that rate-corrected QT intervals are longer in females than males. In addition, both clinical and experimental data implicate gonadal steroids (estradiol and testosterone) as determinants of sex-related differences in repolarization.

The findings from in vitro studies outlined only provide a glimpse of the intricate mechanisms underlying the impact of biological sex and gonadal steroids on ventricular

repolarization and arrhythmias. The results indicate that estradiol and DHT are not unique determinants of sex-related differences in cardiac ventricular repolarization because there is evidence indicating involvement of non-estrogenic ovarian factors (i.e. progesterone) or pituitary–hypothalamic factors in the proarrhythmic responses to I_{Kr} blockers.

Although estradiol and DHT may not be the only determinants of sex-related disparities in repolarization, they are important modulators of the proarrhythmic response to drugs that block I_{Kr}. In orchiectomized male and normal female rabbits, testosterone protects against I_{Kr} blockade-induced excessive prolongation of repolarization and drug-induced high incidence of EAD. The protective mechanism provided by testosterone remains unclear, but may be via modulation of I_{Kr} and I_{K1}. The mechanism(s) underlying hormonal regulation of repolarization in the females appears to be more complex and remains to be elucidated.

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Sex differences in cardiac muscle and remodeling

Brian L. Stauffer and Leslie A. Leinwand

1. Introduction

Retrospective analysis of large clinical epidemiology databases has identified numerous sex-dependent differences in cardiac muscle and its adaptations to various perturbations. Physiologic challenges (i.e. exercise) and pathologic stimuli (i.e. pressure or volume overload, myocardial infarction) have been shown to result in sex-specific responses in the heart. This variability between the sexes has been identified at the hemodynamic and whole organ level and more recently at the cellular and subcellular levels, but the mechanisms underlying them remain unknown. An understanding of the basis for these differences may lead to the development of treatment or prevention of numerous cardiovascular and myocardial diseases. Therefore, there is intense clinical and scientific interest in characterizing these changes. For example, left ventricular (LV) hypertrophy (which differs between men and women) is a strong independent predictor of morbidity and mortality in clinical populations [38]. Moreover, there are some data that suggest that there is a larger relative risk of death associated with LV hypertrophy in women than in men [39]. Thus, regression of hypertrophy may prevent numerous clinical events. This chapter will explore differences and similarities between males and females in cardiac function and responses to exercise and disease.

2. Sex differences in normal cardiac structure and function

Structural differences between male and female hearts appear late in childhood. Although the normal ranges for LV mass in children tend to be higher for boys than for girls [42], up to the age of 12 there are no statistically significant differences in LV mass, mean wall thickness, or diastolic dimension. The lack of difference is also true for neonatal rodents [55]. These observations imply that initially there is a similar number of cardiac myocytes in males and females.

During puberty there is a greater increase in absolute LV mass in boys compared with girls. This increase is due to a concomitant increase in mean LV wall thickness and

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diastolic chamber dimension, and persists through old age [15]. Normal adult cardiac mass in men is 15–30% greater than in women. This difference is most likely due to a greater degree of hypertrophy of male cardiomyocytes since widespread cell division is arrested in the first year of life in both sexes. Regardless of sex, linear growth (height) and weight appear to be major factors in predicting LV mass.

3. Factors that influence sex differences in cardiac mass

Since normal cardiac mass in adults is dissimilar between the sexes, an important question to be asked is how do we compare any changes that might occur? One method to accomplish this is to find a normalization which is comparable in men and women. In healthy clinical populations, height to the 2.5–2.7 correlates with LV mass. However, even when correcting for height with this model, weight still has an excess influence on LV mass in young adults. In fact, in this young population, weight is more influential than blood pressure on LV mass [63]. To further complicate this issue, it has been demonstrated that age is significantly related to LV mass in women but not in men [12]. The increase in LV mass that directly correlates with age in women may be due to increases in cell size over time without a concomitant decrease in cell number. In contrast, the maintenance of LV mass over time in men may be due to the cardiac myocyte attrition noted in aging, with augmented hypertrophy of the remaining cells (see discussion later, Fig. 1). In elderly populations (age 65–100), weight and body surface area, not height, more closely correlate with LV mass [22]. In some adult populations normalization of LV mass to lean body mass has been found to eliminate the sex difference [16,17]. Unfortunately, lean body mass measurements are rarely performed in cardiovascular studies.

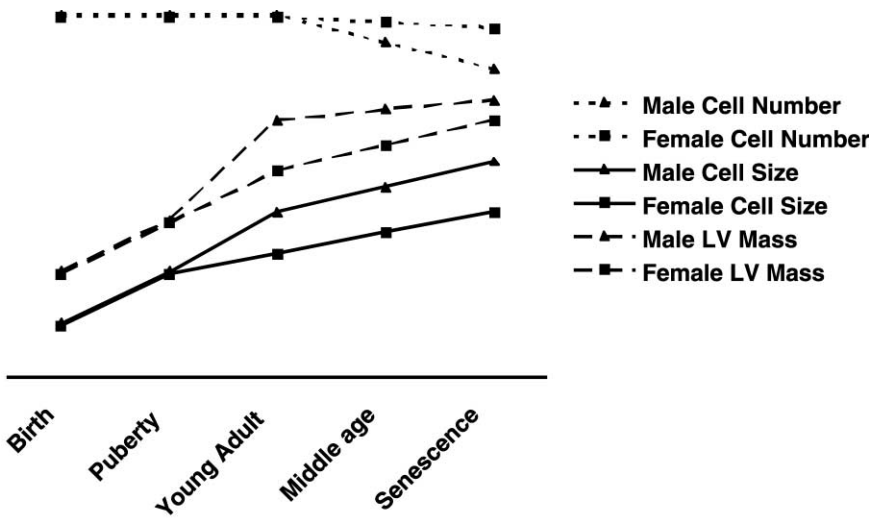

Fig. 1. Relative changes with age in cell number, size, and left ventricular (LV) mass between males and females.

Using ballistocardiography, men were initially found to have significantly higher peak aortic flow velocities than women, suggesting a greater intrinsic acceleration of part or all of the cardiac muscle fibers in men. An augmentation of early ventricular filling in diastole was also found in men implying improved diastolic function compared to women [27]. In contrast, echocardiography has demonstrated better diastolic function in young women when compared to age-matched men [25]. With aging, there is a progressive slow down of relaxation in both sexes; however, men appear to have a concomitant decrease in systolic function that is not paralleled in the female population [25]. Associated with this decrease in systolic function in men is an increase in LV chamber dimension. The absence of these changes probably contributes to the decrease in end-systolic wall stress observed in women as they age. Changes in wall stress may be an initiating factor in the cellular response to stressors.

With normal aging there appears to be a loss of myocytes in the male but not female heart [2,6,48]. This cell loss in males may contribute to the lack of augmentation of LV mass with age and/or systolic function (noted earlier, Fig. 1). In addition, there appears to be an increase in myocyte volume of the remaining myocytes with age in men which is not observed in women. A decrease in tissue factor (TF), a transmembrane receptor that supports cell adhesion, with age is also observed in men, but not women [41]. Since TF is predominantly expressed at the site of cell-to-cell contact, this decrease in TF may be due to changes in cell number in men. The cytoplasmic portion of TF is associated with actin in the *fasciae adherens* of the contractile apparatus which may allow TF to serve as a mechano-transducer for extracellular stimuli, thereby contributing to sex differences in phenotype.

4. Cellular mechanisms underlying sex differences in the heart

Analysis of gene expression in adult male and female Fischer rats has shown higher levels of mRNAs encoding for several key proteins in both muscle and extracellular matrix compartments of the hearts of female compared to male rats. The α- and β-myosin heavy chain (MHC) mRNA abundance is significantly higher in ventricular tissue from female animals when normalized to total RNA [55]. In human nonfailing myocardium, the ratio of α-MHC to total MHC protein in males is approximately twice that observed in females (10% vs. 5%). This isoform of MHC becomes virtually undetectable in heart failure (HF) in both men and women [46]. Messenger RNA for both sarcomeric and cytoskeletal actin, connexin 43, TGF-β1, and pro α2 (I) collagen are all higher in ventricular tissue from adult female animals relative to the males. Interestingly, there are no sex-associated differences in the expression of these genes in prepubertal animals [55]. These data suggest that there are sex-associated differences in adult cardiac myocytes that are not present in neonatal or prepubertal states. These changes may make the female myocardium more resistant to tissue injury.

A number of kinases and phosphatases have been implicated in cardiac hypertrophy with a sex difference demonstrated in one of them. The serine-threonine protein kinase called Akt (or protein kinase B) when expressed as a transgene in constitutively active form can induce hypertrophy that is greater in females than males when normalized to body weight (BW) [44]. The active, phosphorylated form of Akt (phospho-Akt) is

increased in young women when compared to age-matched men (5-fold) and postmenopausal women (16-fold). In cell culture, nuclear localization of Akt is induced by estrogen receptor agonism. This induction suggests that sex hormones in females play a role in the induction of the hypertrophy [8].

Several ion channels have been evaluated in cardiac myocytes isolated from both sexes. An extensive evaluation of voltage sensitive K^+ channels has revealed a down regulation in Kv1.5, responsible for the ultrarapid delayed rectifier K^+ current, at both the mRNA and protein level in the female ventricle when compared to males [62]. The K^+ current, I_{Kur}, from this channel is also significantly lower in the female mouse ventricle [62]. This difference may be responsible for the increase in action potential duration noted in female myocytes. No sex difference in many other K^+ channels, including Kv4.2, Kv4.3, and Kir2.1, as well as inward currents I_{Na} and $I_{Ca\text{-}L}$ has been noted [62]. In contrast, an increase in functional ATP-sensitive K^+ channels has been observed in female myocytes. This increase in functional units is predominantly due to a significant increase in the SUR2A subunit protein and is associated with a decrease in Ca^{2+} loading after ischemic stress [53]. It is hypothesized that this change is responsible, in part, for making female hearts more resistant to metabolic insults.

5. Acute changes in gross morphology from exercise

Conflicting data on the acute adaptation of male vs. female hearts to bouts of exercise have accumulated. There is some evidence to suggest that women perform isometric exercise with a relatively lower afterload than men secondary to the decrease in skeletal muscle mass involved in the exercise [56]. This difference in afterload may change the stress seen by the myocardium, leading to divergent adaptive pathways. Acute adaptation to anaerobic exercise is different between men and women. Women exhibit a blunted HR response to anaerobic exercise and men develop a decrease in stroke volume (SV) resulting in no net difference in cardiac output (CO) between the two groups [7]. One study observed that during aerobic exercise, women have increased end-diastolic volume without a change in ejection fraction, while men increase ejection fraction without changing end-diastolic volumes; thus both groups maintain adequate SV response for CO [29]. It is clear that the response to activity depends on the type of activity (aerobic, anaerobic, chronic, acute). It is unclear whether these documented differences contribute to, or are a result of, the differential remodeling seen between the sexes.

6. Chronic adaptation to regular exercise

Similar to humans, exercise trained male and female rats develop different patterns of hypertrophy depending on the exercise paradigm. Exercised male animals have similar heart mass when compared to sedentary controls regardless of exercise paradigm. Female rats exercise trained by running, have a similar heart mass to female sedentary rats. In both sexes, run training causes a relative hypertrophy when heart weight (HW) is normalized for BW. The hearts of male rats adapt to physical training by running with improved intrinsic performance, whereas the hearts of female rats do not [57]. Swim trained females

Table 1a
Adaptation of rodent heart and hemodynamics to chronic swimming and running

Rats	HW	BW	HW/BW	PLVP	SW	EF	CF	EDV
Run trained male	⟷	↓ ~22%	↑ ~22%	⟷	↑ ~20%	↑ ~20%	⟷	⟷
Run trained female	⟷	↓ ~9%	↑ ~15%	⟷	⟷	⟷	⟷	⟷
Swim trained male	⟷	↓ ~14%	↑ ~21%	⟷	↑ ~30%	↑ ~24%	⟷	⟷
Swim trained female	↑ ~33%	⟷	↑ ~31%	↑ ~13%	↑ ~14%	↑ ~20%	↑ ~45%	⟷

HW, heart weight; BW, body weight; PLVP, peak LV pressure; SW, stroke work; EF, ejection fraction; CF, coronary flow; EDV, end-diastolic LV volume.

exhibit a substantial increase in absolute heart mass, associated with an increase in tension development in both an isolated papillary preparation and a whole heart model (Table 1a).

In general with chronic exercise training, the heart enlarges to compensate for the increased demands [19,43,50,51,59]. This includes greater right ventricular (RV) and LV end-diastolic chamber dimensions (LVIDd) with compensatory wall hypertrophy and a resultant greater LV mass (Fig. 2b). In addition, there are greater LV passive filling

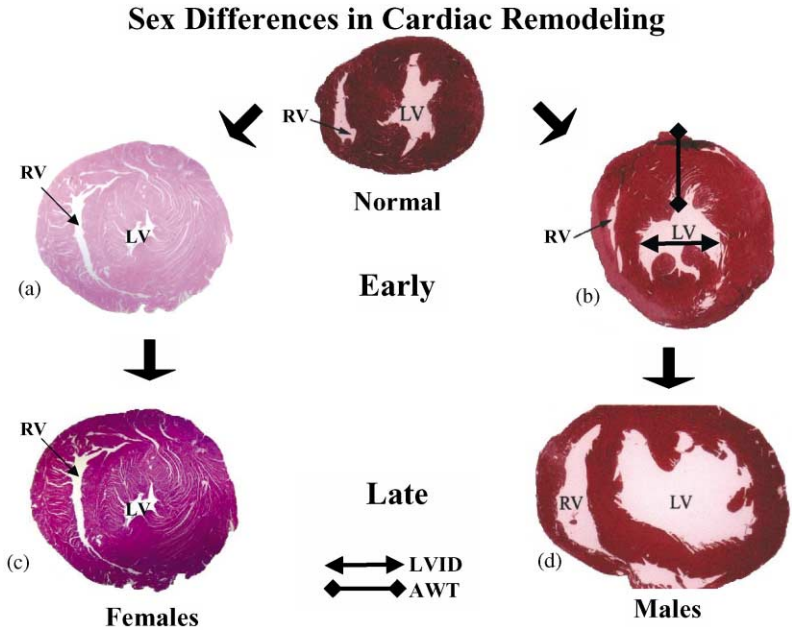

Fig. 2. Overview of sex dependent differences in phenotype. In general the female heart exhibits concentric remodeling and is relatively resistant to dilation. In contrast, the male heart initially shows concentric remodeling; however, there is a tendency toward eccentric remodeling and ventricular dilation. AWT, anterior wall thickness; LVID, left ventricular internal dimension; RV, right ventricle; LV, left ventricle.

Table 1b
Adaptation in echocardiographic indices in the human heart to exercise training

Humans	LVIDd	Stroke volume	LV mass	IVSd
Male athletes	↑ ~10%	↑ ~26%	↑ ~24%	↑ ~9%
Female athletes	↑ ~8%	↑ ~15%	↑ ~36%	↑ ~16%

LV, left ventricle; LVIDd, LV end-diastolic dimension; IVSd, diastolic septal thickness.

velocities with a lower atrial filling component, most likely secondary to improved relaxation. This hemodynamic measure changes similarly between the sexes. In contrast, absolute changes in LV dimension are significantly greater in trained male populations when compared to trained females (Table 1b). This difference is sustained when the changes are normalized to height, but there is some evidence that it is not present when normalized to body surface area (i.e. the relative change in female LV chamber size is greater than in the male) [50]. However, the relative changes that are induced when trained populations are compared to sex-matched untrained healthy controls are not significantly different. This contrast suggests that some of the morphologic sex differences may be due to inadequate normalization.

In habitually trained adults, hemodynamic evaluation reveals a persistently lower SV in women athletes compared with age-matched men. This difference in SV appears to be the primary contributor to the documented decrease in maximal CO when women are compared to men [47]. During recovery from acute bouts of exercise the decrease in SV is accelerated in females which may also contribute to an increase in post-stress hypotension during inactive recovery [10].

On a metabolic level, women appear to provide a large proportion of their energy requirements through aerobic pathways [7]. Exercise increases free radicals in rodent myocardium with a complementary increase in catalase, glutathione reductase, and superoxide dismutase. However, no difference between sexes has been documented [61]. In contrast, acute exercise induces a 2-fold increase in heat shock protein (HSP) 70 in male and ovariectomized females, but not in hormonally intact female rats (either unoperated or pharmacologically replaced with estrogen) when compared to unexercised controls. HSP 70 expression is associated with improved cardioprotection. In this model, the animals expressing increased HSP 70 had significant improvement in their cardiac function after an *ex vivo* ischemic insult [49]. These data contradict prior data suggesting an increase in susceptibility of the male myocardium to ischemic injury [5,11]. However, this may be a unique, time-dependent situation and in general other processes may overcome this benefit.

7. Overview of changes with hypertrophy and heart failure

In large population studies several sex differences in HF have been identified. In the general population, males have a lower ejection fraction, greater LV mass, higher renin, and lower ANP and BNP compared to female subjects. In the presence of moderate to

severe LV dysfunction, irrespective of the cause, a marked increase in LV mass, ANP, BNP, and cGMP is observed in men [18]. In contrast, only a small percentage of women with systolic impairment exhibit a mild increase in LV mass. Moreover, increases in LV chamber dimensions, wall thickness, and cardiac natriuretic factors are markedly attenuated in these females with LV dysfunction. These characteristics reinforce previous data suggesting women, when compared with men, are characterized by smaller LV end-diastolic volumes, smaller LVs, less eccentric LV remodeling, and preserved contractility in the face of pathologic stress (Fig. 2). Several of the largest epidemiology studies [1,30, 58] have reported a better prognosis in the setting of HF in women when compared with men. While this may seem to be due to the previously mentioned differences in remodeling, the benefit has been maintained even when adjusted for similar degrees of LV dysfunction. However, it is clear that each pathologic stimulus elicits a unique cellular response.

In human HF several cellular and molecular sex differences have been observed. Similar to Ca^{2+} handling changes with hypertrophy, there are sex-dependent differences in intracellular Ca^{2+} mechanics in human HF. Myocardial sarcoplasmic reticulum Ca^{2+} ATPase (SERCA) protein content is diminished in samples from failing ventricles of both males and females when compared to nonfailing sex-matched controls. In contrast, phosphorylated phospholamban was reduced only in the failing male samples. This leads to increased inhibition of SERCA and is associated with lower SR Ca^{2+} uptake in the male myocardium, which contrasts with the lower Ca^{2+} transient observed in normal female myocytes [13]. In addition to the increase in cell loss in men with age, there is a 2-fold increase in cell loss, both from necrosis and apoptosis, in men with HF when compared to women with HF [26]. These differences may be responsible for some of the mechanical changes noted in HF such as diminished systolic function.

8. Aortic stenosis

A number of clinical studies have evaluated macroscopic myocardial changes associated with pressure overload from valvular aortic stenosis (AS) [4,9,20,35,37,45, 54,65]. Sex-dependent differences in myocardial adaptation are seen in asymptomatic patients with mild to moderate valve disease. Women in this stage of the disease have significantly smaller LVIDd and LV end-systolic dimension (LVIDs), LV mass, stroke volume, and CO, but have increased relative systolic wall thickness, which is indexed to chamber dimensions, when compared to men with similar degrees of AS. These women also have an increase in mitral flow velocities suggesting better diastolic myocardial properties than their male counterparts. At this stage both men and women are able to increase their CO depending on heart rate response. However, during exercise testing women exhibit impairment in their CO augmentation, which may be a manifestation of the smaller LV volumes noted earlier. These differences at the structural level may translate into more clinical complications for women. For example, female sex is an independent risk factor for poor outcomes after balloon aortic valvuloplasty, and patients with higher relative wall thickness and smaller LV dimensions have a significantly increased risk of death following aortic valve replacement for AS [37].

In more advanced disease, women have a greater degree of LV hypertrophy and improved systolic function when compared to men with similar severity of AS. Moreover, women appear to lack any change in LV chamber size while men have a significantly higher prevalence of LV dilatation [9,35]. When these patients were reevaluated based on similar systolic function, there were no sex differences in LV hypertrophy, chamber geometry, or a variety of other hemodynamic measures. In advanced disease, an increase in myocardial stiffness has been identified in men which is complementary to the increased mitral flow previously noted in women. This evidence of diastolic dysfunction in men was associated with a significant increase in abnormal myocardial collagen architecture, consisting of endocardial fibrosis and/or increased collagen fiber cross-hatching. While not all evaluations have had exactly the same morphometric conclusions [45], the majority of the clinical studies suggest that women adapt to pressure overload from AS with LV hypertrophy, lack of chamber dilatation, and preserved systolic function more frequently than men [3,4,9,20,45]. These changes occur early in the disease and are due to sex-dependent alterations in LV geometry and not changes in LV mass. In clinical populations, sex has its major influence on the likelihood of the LV response to AS; a dilated LV with poor systolic function at one end of the spectrum and a small, thick-walled ventricle with preserved systolic function at the other end (Fig. 2(d) vs. (c)).

9. Hypertension-induced changes in gross morphology

A number of sex-dependent differences in myocardial remodeling have been observed in hypertension (HTN). Women with concentric or eccentric LV hypertrophy secondary to HTN have improved contractility (higher % fractional shortening) when compared to men with similar elevation in blood pressure [24]. However, similar to AS, women with isolated systolic HTN have increased LV wall thickness and mass without LV chamber enlargement while men with a similar degree of HTN exhibit LV dilatation and increased LV mass without increased wall thickness. Once again, this translates into more concentric remodeling in women and more eccentric remodeling with increased wall stress in men [28,36] (Fig. 2). Interestingly, there is some evidence that this sex dimorphism is not seen when the HTN is adequately medically managed [67].

Spontaneously HTN rats have also exhibited sex differences in myocardial adaptation. In this animal model, both sexes develop LV chamber enlargement with age; however, at the onset of symptoms of HF, fractional shortening had declined in the males but not in the females. Hormonal manipulation by surgical gonadectomy revealed that a lack of sex hormones causes a decrease in HW in both HTN and normotensive female animals which can be reversed with exogenous estrogen supplementation. However, surgical gonadectomy in males causes only a small decrease in HW in HTN animals and no change in normotensive animals [66]. Individual cardiac myocytes have a larger cross-sectional area in young HTN males than the young HTN females. However, similar to AS, at the time of symptomatic HF in this model, there are no significant differences in cell size between the sexes. This again suggests an increase in the hypertrophic reserve of the female myocytes (40% increase in size with age in the HTN females vs. a 12% increase in the males) [60].

It may be this apparent arrest in myocyte growth as wall stress continues to climb which leads to HF with disease progression.

10. Sex-dependent differences in adaptation to myocardial infarction

After a myocardial infarction (MI), LV remodeling consists of progressive chamber dilation, infarct expansion, and compensatory hypertrophy of the noninfarcted myocardium. In contrast to the smaller LV chamber size previously noted in female rats, 6 weeks after an MI involving a similar proportion of the LV there were no sex differences in chamber size. In addition, at this same timepoint, the females had minimal hypertrophy of the uninfarcted myocardium while the males developed significant hypertrophy in the noninfarcted ventricular tissue. This difference in myocardial thickness is paralleled at the cellular level with myocytes from male myocardium having a larger diameter. One week following a MI, the posterior wall of the female heart exhibits augmented thickening which is absent in the males [40]. In fact, this reproduces a phenomenon seen in clinical studies post-MI and disappears by 6 weeks after the infarction. The increase in wall thickness at 6 weeks post-infarction in the males is accompanied by impairment in diastolic function (Table 2). The restrictive cardiac physiology also observed in the male animals may result mainly from increases in LV chamber stiffness, and may explain why there appears to be a larger benefit of inhibition of angiotensin converting enzyme (ACE) in these male animals when compared to the females. These results suggest a unique pattern of remodeling dependent on the sex of the animal [40].

As mentioned previously, multiple stimuli may have synergistic or antagonistic effects on cardiac remodeling. This has been tested in the male and female salt-sensitive rats that were made hypertensive from a high-salt diet approximately 2 weeks after a large anterior MI. In this model, the male rodents develop eccentric hypertrophy with LV dilation, and thinning of the LV scar. However, in the females, hypertension post-MI results in concentric hypertrophy without chamber dilation or scar thinning. Perhaps more importantly, this concentric hypertrophy results in augmented contractile function which is absent in the hypertensive males [31]. Unfortunately, no cellular or molecular changes were investigated in this system.

In a rat model of MI, the sex difference in *in vivo* hemodynamics is maintained following at least a moderate sized (>31% of the LV myocardium) MI. Male animals

Table 2
Adaptation of human myocardium after myocardial infarction

Sex	LVH of uninfarcted myocardium	Early diastolic dysfunction	Myocyte hypertrophy	Augmented PW shortening at 1 week	Augmented PW shortening at 6 weeks
Male	+	+	+	−	−
Female	−	−	−	+	−

LVH, left ventricular hypertrophy; PW, posterior wall.

have persistently lower peak cardiac index and peak SV index to a saline infusion than female animals. However, there is no sex difference in response to an acute increase in afterload (aortic occlusion) [52].

11. Ischemia/reperfusion

Evidence suggests a sex difference in the susceptibility of the myocardium to ischemic injury. In rodent ischemia/reperfusion models the length and severity of ischemia must be greater in female animals to produce pathology to a similar extent as males [5]. In an animal overexpressing the sodium–calcium exchanger (NaCaX), increased functional cardiac impairment is seen in male and ovariectomized female transgenic animals. This is presumed to be secondary to the Ca^{2+} overload associated with ischemia in this model. The functional impairment correlates with diminished myocardial ATP and phosphocreatine during reperfusion/recovery. These data suggest a modulating effect of a substance produced by the ovary, presumably estrogen, on energy production during recovery [11].

12. Idiopathic dilated cardiomyopathy

The prevalence of idiopathic dilated cardiomyopathy is 2–4 times higher in men than women. However, women with the disease have larger LV chamber dimensions, increased wall thickness, and worse symptoms than men. These changes are associated with a decrease in exercise tolerance. In addition, there is evidence that women with this disease have worse outcomes than men [14]. There is no documentation of sex differences at a molecular level in this disease. In contrast to several other types of cardiac disease, there is no age-associated sex difference in diagnosis, suggesting a lack of sex hormone contribution to the development of the disease [14].

12.1. Animal models of hypertrophic disease with sex-dependent cardiac dimorphism

Cardiac evaluations in humans allow description of myocardial changes due to hypertrophic stimuli; however, there is little mechanistic evaluation possible at a cellular or molecular level. Animal models, specifically transgenic mouse models, have emerged as a more convenient way to evaluate specific molecular or cellular pathways implicated in cardiac remodeling. A mouse overexpressing TNF-α in the heart develops a sex-dependent difference in cardiac phenotype by 6 weeks of age. Males have decreased fractional shortening and LV chamber dilation associated with a decrease in β-adrenergic responsiveness. Females do not start to develop these features associated with HF until 12 weeks of age. Even at this age females maintain a greater degree of functional augmentation to β-adrenergic stimulation. All of these abnormalities are normalized with 6 weeks of TNF-α blockade [32]. At a cellular level, both wild type and transgenic males express more TNF-α receptor mRNA and protein in the heart. However, no difference between sexes are noted in multiple downstream effectors such as TGF-β1, IL-1β, and MCP-1 [33].

Sex differences in cardiac phenotype have also been documented in several mutant MHC transgenic models [23,64]. In one model, male mice develop earlier and more consistent evidence of atrial and ventricular disease [23]. In a second model, both sexes develop LV hypertrophy in young adulthood. However, by 10 months of age, the males progress to a dilated cardiomyopathy while the females maintain hypertrophy through 12 months of age [64]. An interesting observation in this model is the effect of diet on these animals. A diet free of phytoestrogens prevents the dilated cardiomyopathy in the male animals through 12 months of age (unpublished observations). This additional modifier may assist in identifying some cellular mechanisms contributing to these differences.

13. Alcoholic cardiac disease

In addition to sex differences in response to mechanical stressors, cellular toxins have been observed to have a sex-dimorphic effect on the myocardium. Chronic high-level ethanol consumption leads to a progressive cardiomyopathy characterized at a functional level by depressed CO, hypertrophy, fibrosis, and congestive HF. Structural cellular changes associated with this disease include a decrease in the number of cardiomyocytes, increased collagen deposition, and disruption and swelling of mitochondria and sarcoplasmic reticulum. In population studies, the prevalence of alcoholic women with dilated cardiomyopathy is similar to that of alcoholic men; however, women require a lower lifetime dose of ethanol to develop the disease [21]. These clinical data would suggest that the ventricular myocardium in women is more sensitive to ethanol-induced damage than male tissue. However, evaluation of atrial tissue in a rodent model of alcoholic cardiomyopathy is inconsistent with this statement. Atrial tissue from male animals subjected to chronic alcohol exposure exhibit diminished cardiac contractile force when compared to nonexposed controls. There is no difference in muscle mechanics between exposed and unexposed atrial tissue in the female animals [34]. Given this relatively unique situation, examination of ventricular tissue in this model may be of considerable interest. The clinical observations are contrary to much of the previously reported data which suggest that male hearts are more sensitive to tissue damage and the female myocardium is better prepared for repair.

14. Summary

1. Evaluations of cardiac adaptation at the macroscopic level are confounded by sex differences in baseline parameters.
2. Characterization of clinical and hemodynamic sex dimorphism reveals a propensity for the female heart to undergo concentric remodeling while the male heart has eccentric remodeling (Fig. 2).
3. Female myocardium has an increase in hypertrophic reserve when compared to age and disease matched males (Fig. 1).
4. Cellular remodeling with age involves cell loss and hypertrophy of remaining cardiomyocytes in males, but not in females.

5. Changes in wall stress, mechanotransduction, and endogenous hormones may contribute to the sex differences in remodeling.
6. Gene expression profiles have not been systematically explored to discover pathways leading to the sex differences noted in cardiac phenotype. However, the emergence of animal models of these changes should promote the molecular characterization of the sex dimorphic phenotype.

15. Future directions

Numerous sex-dependent differences have been noted in the macroscopic cardiac phenotypes of men and women due to stressors. While more attention has recently been directed towards the molecular changes leading to the differences in cardiac phenotype, significantly more investigation into the cellular signaling resulting in the molecular activation is also needed. It remains unclear whether there are differences in receptor stimulation, mechanical force transduction, or additional mechanisms which result in the sex-dependent phenotypes previously noted. A systematic approach will be necessary to describe the mechanisms responsible for these phenotypic differences. Hopefully additional animal models of this paradigm will be identified to allow further characterization of these differences.

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Reflex control of the circulation

Christopher T. Minson

1. Introduction

The central question of this chapter is "Are there sex differences in reflex control of the circulation, and if so, how do these differences manifest in daily life?" Certainly many sex differences exist in terms of the circulation, but does this necessarily mean that neural control of the circulation is different? In other words, is neural control of the circulation *functionally* different in men and women, or are the differences in neural regulation only to compensate for differences in vascular function? As will be discussed, and is a central theme to this chapter, the same *net* regulation of blood pressure may occur in males and females, but it is now clear that this is achieved by different mechanisms. Specifically, a greater dependence on heart rate may cause women to be more susceptible to fainting spells, but a greater reliance on peripheral vascular changes in men is associated with higher rates of cardiovascular disease.

The challenge in reviewing the issue of sex differences in reflex control of the circulation is that there have been relatively few studies specifically addressing the issue. Thus, there are few data available that consider how fluctuations in sex hormones impact circulatory function temporally and mechanistically. More work has been directed towards investigating the effects of estrogen and progesterone on neural control of circulation than sex differences per se. Of the studies that investigated sex differences, most have not adequately characterized the menstrual cycle to control how female sex hormones might impact circulatory function. Along these lines, many studies on sex and the circulation only study women during the early follicular phase of the menstrual cycle, during which the levels of estrogen and progesterone are low. As women are typically in the early follicular phase for only 7–10 days a month, it is not appropriate to draw conclusions about differences in sex when any conclusions only relate to women less than one-third of each month. Furthermore, there is sufficient evidence that exogenous forms of estrogen and progesterone, as taken in oral contraceptives and hormone replacement therapy, may have different bioactivity or even biological actions from the endogenous forms. Lastly, a vast majority of work has been performed in animals, and findings may not be generalized to humans, as differences exist in the human menstrual cycle and the estrous cycle of animals. In attempting to find answers to these questions, one can see why research in

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the area of sex differences in the circulation is so challenging. The goal of this chapter is to discuss sex difference with respect to reflex control of the circulation in humans, to put these differences in perspective of how they may impact daily life, and to identify specific areas and problems where research is lacking.

2. Overview of the circulatory system

The human cardiovascular system is highly organized to meet the needs of various tissues during significant challenges. Although many different factors contribute to the maintenance of blood pressure and blood flow, overall regulation of the cardiovascular system is controlled by the autonomic nervous system. This requires integration of afferent signals from the periphery, and continual efferent neural outflow to regulate blood flow to the various regions of the body and, therefore, maintain blood pressure. A simplistic overview of this concept is displayed in Fig. 1. The primary "integration center" of the autonomic nervous system is the nucleus tractus solitarius (NTS). Located in the medulla, the NTS receives direct neural information from the periphery and higher centers of the brain. The various neural inputs to the NTS include afferent nerves arising from the aortic and carotid baroreceptors, the cardiopulmonary receptors, chemoreceptors, metaboreceptors, and thermoreceptors. Each of these afferent signals and their impact on blood pressure regulation is discussed in detail below. The NTS must integrate, or interpret, the incoming signals, make a "decision" about changes that must be made in the circulation, and alter efferent signals accordingly.

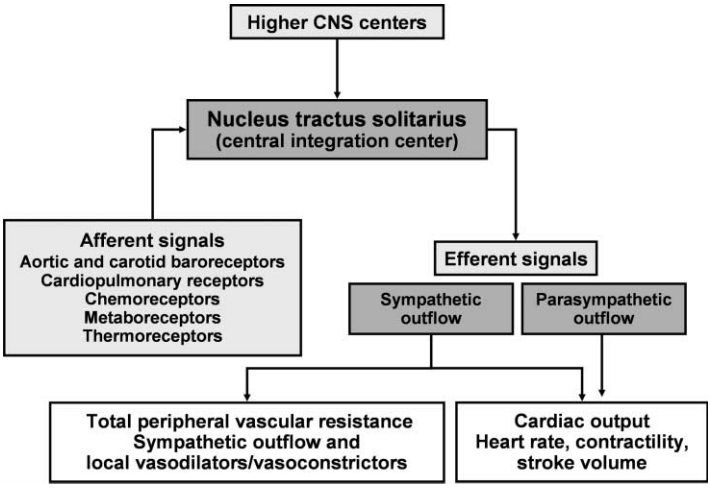

Fig. 1. *Simplistic overview depicting neural control of the circulation*. The NTS is the integration center of the autonomic nervous system. The NTS receives neural information from afferent nerves and from higher brain centers. The NTS interprets and integrates these signals and makes appropriate adjustments in sympathetic and parasympathetic outflow to change total peripheral resistance or cardiac output accordingly.

The two efferent branches of the autonomic nervous system involved in control of the circulation are the *sympathetic nervous system* and the *parasympathetic nervous system*. Sympathetic outflow is directed to the peripheral vasculature and the heart, whereas parasympathetic outflow is only directed to the heart. Total peripheral vascular resistance thereby represents the balance of sympathetic outflow and local vasodilator and vasoconstrictor substances in all of the vascular beds. Cardiac output is determined by ventricular filling pressure and the balance of the sympathetic and parasympathetic influences on heart rate and contractility. A more detailed depiction of cardiovascular control by the autonomic nervous system is presented in Fig. 2. This figure is referred to throughout the chapter, and serves as the template for discussion on sex differences in neural control of the circulation.

The classic depiction of the sympathetic nervous system in undergraduate physiology courses is to describe it as the "flight or fight" response. In this scenario, a person is

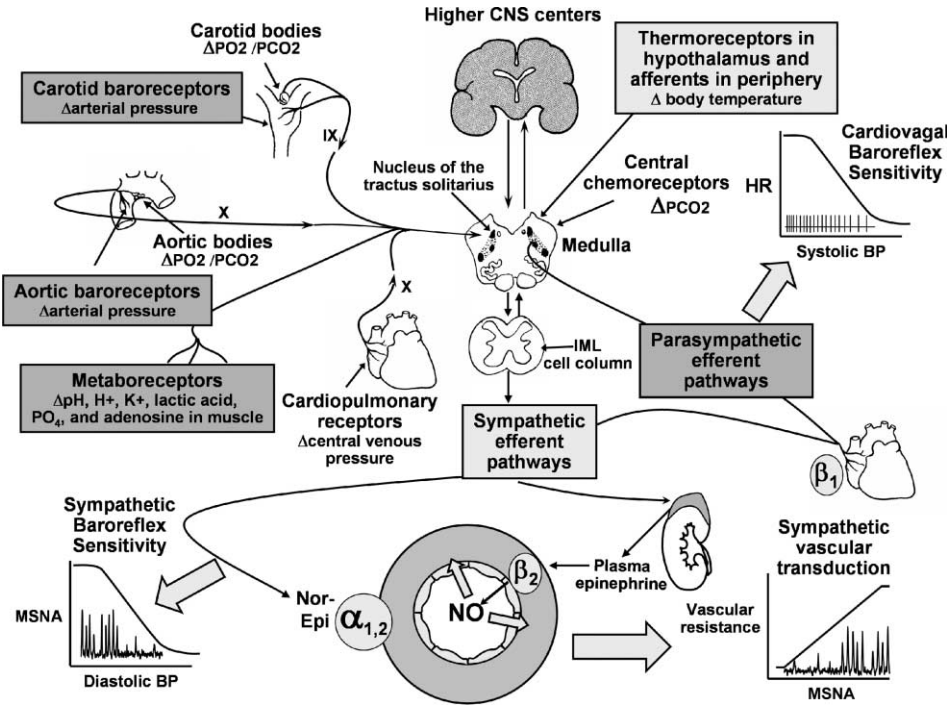

Fig. 2. *Neural control of the circulation*. Afferent signals that arise from the arterial and cardiopulmonary baroreceptors, metaboreceptors, thermoreceptors, and chemoreceptors are integrated in the NTS of the medulla. Parasympathetic outflow is directed to the heart (which lowers heart rate) and sympathetic outflow is directed to the heart and the peripheral vasculature to raise cardiac output and increase peripheral vascular resistance. Increased sympathetic outflow to the adrenal gland will stimulate epinephrine to be released into the blood. Cardiovagal and sympathetic baroreflex sensitivities and sympathetic vascular transduction relationships are displayed. PO_2 and PCO_2 are the partial pressure of oxygen and carbon dioxide, respectively. MSNA is muscle sympathetic nerve activity as directly recorded by microneurography.

walking in the jungle, a tiger jumps out, and the person has two choices – to fight or to flee. The sympathetic nervous system prepares the cardiovascular system by increasing heart rate, increasing blood pressure, and decreasing blood flow to inactive muscles. This example suggests that the sympathetic nervous system is like a switch that gets flipped on, initiating mass sympathetic outflow to the heart and all the vascular beds simultaneously. However, this is a misconception. Sympathetic outflow is very specific and targeted, depending on the incoming signals to the NTS. Furthermore, the sympathetic nervous system is always active, even when resting supine. It is also important to realize that *both* branches of the autonomic nervous system work together to maintain circulatory function.

Without the sympathetic nervous system, the simple act of standing would cause blood to rapidly pool in the feet (instantly decreasing venous return, causing cardiac output and blood pressure to drop), quickly leading to collapse. Sympathetic outflow is needed to raise blood pressure and avoid collapse, but unfortunately, this system is relatively slow, requiring approximately 10–15 s to be fully activated. The parasympathetic nervous system, on the other hand, responds very fast (within one heart beat), and serves to *lower* heart rate when activated. It is by *decreasing* activity of the parasympathetic nervous system (or decreasing "vagal tone" as these nerve fibers are part of the vagus nerve) that causes the rapid increase in heart rate upon standing to minimize changes in blood pressure. One problem, however, is that heart rate simply cannot increase for very long without making adjustments in the periphery to maintain blood flow back to the heart. For example, consider what happens when you quickly stand after lying down for a prolonged period of time. The brief "dizzy" feeling occurs because blood pressure has decreased, despite heart rate being elevated, as the sympathetic nervous system is not fully activated. The dizzy feeling disappears once sympathetic outflow is sufficient to increase peripheral vascular resistance and aid blood return to the heart. Therefore, the simple act of standing is an example of how the sympathetic and parasympathetic systems work in concert to maintain adequate blood pressure. If these two systems are not tightly controlled, blood pressure will continue to fall while standing and the person will faint (termed "orthostatic intolerance"). While orthostatic intolerance may not seem to be a common occurrence in most otherwise healthy individuals, orthostatic intolerance is the most common blood pressure-related disorder after hypertension, and women have much higher rates of orthostatic intolerance than men [1].

2.1. Overview of cardiac output, peripheral vascular resistance, and blood pressure

In terms of neural control of the circulation, blood pressure is the primary variable being regulated. When maintenance of blood pressure is challenged, the cardiovascular system can alter the various components of blood pressure according to the formula

$$\text{Blood pressure} = \text{cardiac output} \times \text{peripheral vascular resistance}$$

Cardiac output is the product of heart rate and stroke volume, and peripheral vascular resistance is equal to the sum of all the resistances in the vasculature. As *pressure* is equal to *flow* × *resistance*, it follows that blood pressure is equal to cardiac output times

peripheral vascular resistance. Thus, to increase blood pressure, either cardiac output or peripheral vascular resistance can be increased.

As the heart can only pump what it receives, it follows that venous return is an essential determinant of cardiac output. If venous return is compromised, the heart can maintain cardiac output by increasing heart rate to compensate for reduced ventricular filling, but only for a limited time. The ability to maintain heart rate in the face of diminished venous return is limited to the blood contained in the pulmonary system, termed "central blood volume". In the example of changing from supine to standing, the heart has about 10 beats before central blood volume is depleted. Thus, sympathetic outflow must increase to raise peripheral vascular resistance to maintain blood pressure. Importantly, the increase in peripheral vascular resistance will also have the *passive effect* of minimizing pooling of blood in the legs which serves to limit the decrease in venous return.

2.2. Measuring sympathetic activity in humans

It would be very difficult, if not impossible, to study the various afferent limbs of the cardiovascular system and how they interact at the level of the NTS in humans. Thus, scientists are relegated to measuring an index of sympathetic outflow. For example, arterial blood pressure can be manipulated via activation of various pressor responses or pharmacological interventions, and subsequent changes in norepinephrine spillover from sympathetic nerves can be measured in the blood. However, plasma norepinephrine concentrations are not a precise indicator of sympathetic outflow, and more direct measures are required to make comparisons between groups of individuals or between men and women. The need for a more direct measure in humans led to the development of a technique called "microneurography", first discovered accidentally by Swedish neurophysiologists in the 1960s. This technique allows the direct measurement of sympathetic nerve activity to the muscle and cutaneous vascular beds. Although the technique is minimally invasive, there are a number of limitations. Microneurography is technically challenging, cannot be used to quantify sympathetic activity to other vascular beds or organs such as the kidneys and heart, and can only be used in stationary subjects. Furthermore, there is large interindividual variation in resting levels of sympathetic outflow, decreasing the power to identify differences between groups of subjects. Despite these limitations, the development of the technique has greatly increased our understanding of sympathetic activity in humans.

2.3. Baroreflex control of the circulation

As shown in Fig. 2 and as discussed above, the NTS is the primary cardiovascular control center and receives sensory input from a variety of peripheral sensory receptors. The primary reflex pathways for homeostatic control of blood pressure are the baroreceptor reflexes, or simply "baroreflexes". Arterial baroreceptors are located in the adventitia of the carotid sinuses and the aortic arch, and are often referred to as the "high-pressure baroreceptors" as they provide information about *arterial* pressure to the NTS. Baroreceptors are mechanosensitive stretch receptors, so an increase in pressure will

increase firing rate of the baroreceptors. Action potentials from the baroreceptors travel to the NTS via sensory neurons. The NTS integrates the sensory input and initiates an appropriate response. There are also receptors widely distributed in the walls of the superior and inferior vena cava, atria, ventricles, coronary arteries, and pulmonary arteries and veins. Receptors in these areas are often referred to as the cardiopulmonary baroreceptors or "low-pressure baroreceptors", although both names are somewhat of a misnomer as they are not purely mechanosensitive. These receptors also provide afferent information to the NTS about venous return, central blood volume, and ventricular filling.

The top of Fig. 3 displays an example of the carotid baroreflex. An increase in arterial pressure stretches the carotid sinus, increasing baroreceptor firing rate to the NTS. The pressure at which these baroreceptors fire is termed the "threshold", and the pressure at which firing is maximized is termed "saturation". In this example, increased firing of

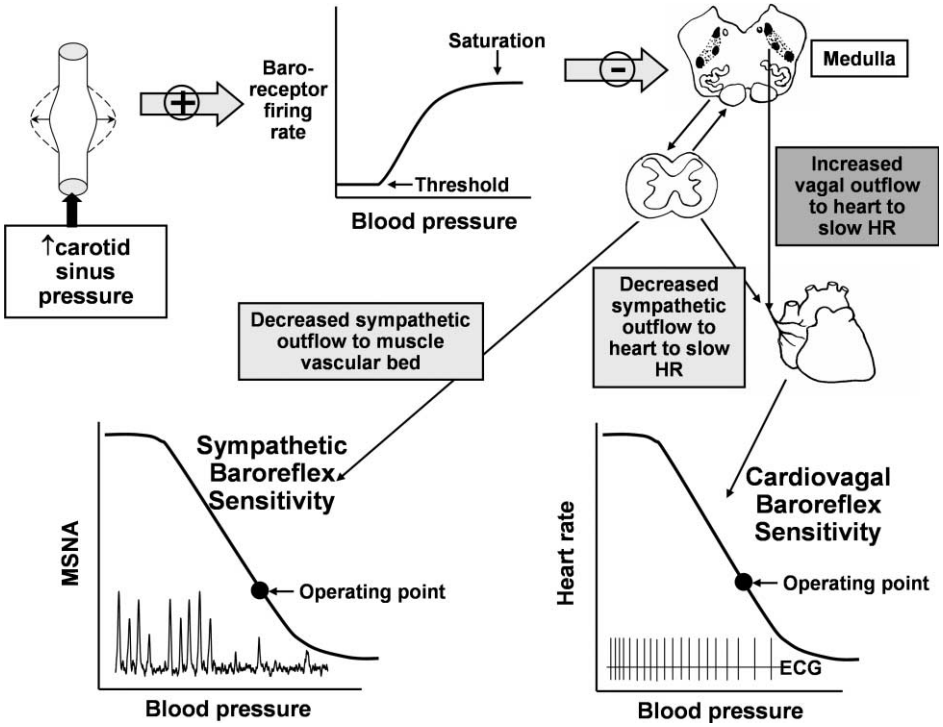

Fig. 3. *Measurement of baroreflex sensitivity in humans*. Baroreflex sensitivity is measured in humans by simultaneous measurement of blood pressure, heart rate, and MSNA. A decrease in blood pressure inhibits the firing rate of the arterial and cardiopulmonary baroreceptors, decreasing vagal outflow to the heart (raising heart rate) and removing the inhibition of sympathetic activity (increasing MSNA and peripheral vascular resistance). A rise in blood pressure increases baroreceptor firing, increasing vagal activity to the heart and decreasing MSNA. The sensitivity of the baroreflex is calculated as the slope of the relationship between blood pressure and HR (termed "Cardiovagal Baroreflex Sensitivity") and between blood pressure and MSNA (termed "Sympathetic Baroreflex Sensitivity").

the baroreceptors will have an *inhibitory* influence on sympathetic outflow and will *increase* parasympathetic activity to the heart. Combined, these responses will decrease peripheral vascular resistance and heart rate, returning blood pressure back to normal. In response to a decrease in arterial pressure, baroreceptors decrease their firing rates, thereby removing the inhibition on sympathetic outflow, and decrease parasympathetic outflow.

Baroreflex sensitivity is measured in humans by simultaneous measurement of blood pressure, heart rate, and muscle sympathetic nerve activity (MSNA) during pharmacological infusions of pressure lowering and pressure raising drugs. This series of infusions results in an initial decrease in arterial pressure followed by an increase in arterial pressure. The initial decrease in blood pressure inhibits the firing rate of the arterial and cardiopulmonary baroreceptors, decreasing vagal outflow to the heart (raising heart rate) and removing the inhibition of sympathetic activity. The subsequent rise in blood pressure to the second drug increases baroreceptor firing, increasing vagal activity to the heart, and decreasing MSNA. The sensitivity of the baroreflex is calculated from the *slope* of the relationship between blood pressure and HR (termed "cardiovagal baroreflex sensitivity") and between blood pressure and MSNA (termed "sympathetic baroreflex sensitivity"). These relationships are displayed in the bottom of Fig. 3. Although the baroreflex is a reverse sigmoidal response, the baroreflex slope in humans is only calculated from the linear portion of the relationship as the resulting changes in pressure to the infused drugs are not great enough to allow measurement of the entire baroreflex. Typically, resting blood pressure is at the lower end of the baroreflex sensitivity curves, termed the "operating point".

The main limitation to this technique is that the baroreflex is viewed in total, and it is not a sensitive enough technique to determine where in the baroreflex that a change may occur. For example, it is possible that progesterone may impact firing rates of baroreceptors, or that estrogen may impact the relationship between afferent signals and the sympathetic response. It cannot be determined in humans where a modification of the baroreflex occurs, so information must be gleaned from animal studies. These studies have provided excellent insight into neural cardiovascular regulation, but there are obvious species differences that, at times, make direct comparisons difficult.

2.4. Sex and baroreflex regulation of blood pressure

There is a substantial base of information about baroreflex regulation of heart rate and sympathetic outflow derived from research in animals, and this body of data comprises much of what is currently known. For example, sex differences in the distribution of neurotransmitters and neuromodulators in the central nervous system and lumbar spinal cord have been found in rats. Neurons of primary cardiovascular centers, such as the NTS, are known to be differentially targeted by estradiol, progesterone and dihydrotestosterone. Specifically, noradrenergic neurons in these areas are targeted by estrogen and progesterone, whereas non-catecholaminergic neurons are targeted by dihydrotestosterone. These differentially targeted medullary neurons are directly or indirectly involved in the baroreflex, and may be partially responsible for sex differences in reflex sympathetic outflow. Furthermore, estrogen and progesterone appear to differentially impact

the baroreflexes. In this context, it is important to note that the net effect of combined estrogen and progesterone on baroreflex sensitivity may depend on the relative concentration of these hormones in the circulation, as well as on differences in central versus peripheral effects.

2.5. *Cardiovagal baroreflex*

Exogenous estrogen supplementation in animals has been shown to enhance the sensitivity of the cardiovagal baroreflex. Infusions of progesterone metabolites, on the other hand, have been shown to shift the baroreflex curve to lower operating pressures independent of changes in mean arterial pressure, resulting in decreased sensitivity of the cardiovagal and sympathetic baroreflex [6]. These changes with exogenous progesterone are similar to those observed in pregnant rats, a condition in which endogenous progesterone levels are increased. The proposed mechanism of progesterone inhibition of the sympathetic baroreflex involves the elevation of gamma amino butyric acid (GABA, an inhibitory neurotransmitter in the medulla) by progesterone.

In humans, cardiovagal baroreflex sensitivity was found to be less in women (during the early follicular phase) when compared to men [2]. However, no difference in cardiovagal baroreflex sensitivity was found between the early follicular phase and the mid-luteal phase in women in a separate study [7]. This may be due to the competing influences of both elevated estrogen and progesterone on cardiovagal baroreflex sensitivity during the mid-luteal phase. Cardiovagal baroreflex sensitivity has not been studied during the late follicular or ovulatory phase, a situation of elevated estrogen and low progesterone. In contrast, the high-hormone phase of oral contraceptive use has been shown to lower cardiovagal baroreflex gain compared to the low-hormone or "placebo-phase" [8]. A graphical comparison of data from various studies is presented on the lower right of Fig. 4. Most likely, the cause for the differences in cardiovagal baroreflex gain with oral contraceptive use versus the menstrual cycle may be due to the higher biological activity of exogenous progesterone than estrogen. These seemingly disparate findings highlight important, but poorly understood differences between endogenous and exogenous forms of estrogen and progesterone on cardiovascular function.

2.6. *Sympathetic baroreflex*

It has been clearly demonstrated in a number of studies that resting MSNA is significantly lower in women than in men. However, the cause of the lower resting sympathetic outflow is still not fully understood. To date, no study has directly compared sex differences in sympathetic baroreflex sensitivity. However, when data from a number of studies were pooled, it appears that women have a lower sympathetic baroreflex gain than men (bottom left of Fig. 4). This may explain, in part, the greater increase in sympathetic outflow in men than in women to various sympathoexcitatory maneuvers. For example, it was recently reported that the rise in sympathetic neural activity and increase in circulating norepinephrine during head-up tilting was lower in women than in men, and that this likely contributed to decrements in blood pressure control during the orthostatic

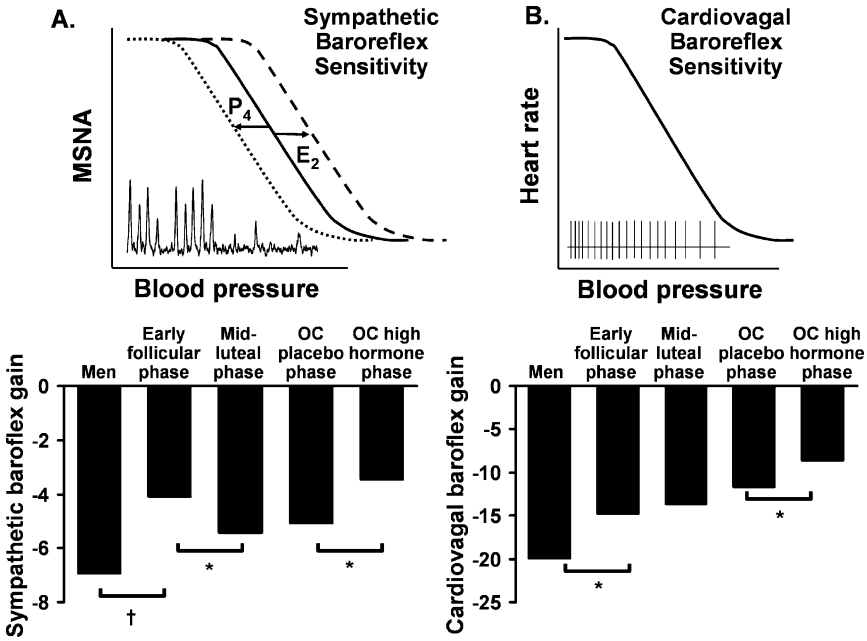

Fig. 4. *Sympathetic and cardiovagal baroreflex sensitivity in humans*. (A) Sympathetic baroreflex sensitivity (or gain) is calculated as the slope of the relationship between blood pressure and MSNA. In animals, progesterone (P_4) has been shown to shift the baroreflex function curve to a lower pressure, whereas estrogen (E_2) shifts the curve to higher pressures. (B) Cardiovagal baroreflex sensitivity (or gain) is calculated as the slope of the relationship between blood pressure and heart rate. Baroreflex sensitivities are different between the sexes, and appear to change across the menstrual cycle or during the course of oral contraceptive use. Data compiled from Refs. [2,7,8].

challenge [9]. However, mechanisms for these differences in baroreflex sensitivity are not known. The degree of distensibility, or "stretch", of the arteries can impact the sensitivity of baroreceptors. Distensibility of the carotid artery is less in women than in men. Since firing of baroreceptor afferents is proportional to stretch, this observation suggests that a greater increase in pressure is required to stretch the baroreceptors and increase their firing rates. If this finding holds true, this may partially explain the decreased sympathetic baroreflex sensitivity in women.

Hormonal changes during the course of the menstrual cycle and oral contraceptive use also impact the sympathetic baroreflex (see bottom right of Fig. 4). In normally cycling women not taking oral contraceptives, sympathetic baroreflex sensitivity is augmented in the mid-luteal phase of the menstrual cycle [7]. Animal experiments have demonstrated that estrogen alone increases, and progesterone independently decreases, sympathetic baroreflex sensitivity. Furthermore, progesterone has been shown to shift the baroreflex function curve to a lower operating pressure. Estrogen may shift the baroreflex function curve to a higher operating pressure, possibly through changes in plasma volume via

effects on plasma renin and aldosterone levels [10]. These independent effects of estrogen and progesterone remain to be studied in humans. The shifts in the baroreflex function curve in humans discussed above are shown in the top of Fig. 4. In contrast to the findings in normally cycling women, in a separate experiment in oral contraceptive users, sympathetic baroreflex sensitivity was *decreased* in the high hormone phase when estrogen and progesterone was elevated as compared with the placebo phase in oral contraceptive users. As discussed above, the reasons for the seemingly disparate findings between normally cycling women and oral contraceptive users is unclear. However, these findings are consistent with the concept that biological activity of exogenous progesterone is greater than the endogenous form, which may relate to the dose and/or formulation of progestins.

2.7. Sympathetic nerve activity and vascular reactivity

The topic of sex and the numerous factors that can impact vascular tone is covered in detail in Chapters 6–8, so only a few general concepts are discussed here. However, it is important to understand that differences in vascular tone or vascular responsiveness will have a large impact on neural control of the circulation. That is, decreased vascular tone, such as observed in women due, in part, to the direct and indirect vasodilator effects of estrogen, must be compensated for by neural modulation by the autonomic nervous system. In other words, to maintain adequate blood pressure, sympathetic neural outflow must be increased to a greater extent in women than men (thereby balancing the vasodilators and vasoconstrictors), or heart rate must be increased to a greater extent. As stated earlier, women will typically respond to sympathoexcitation by greater heart rate responses than men. The main point here is that sex differences in vascular regulation must be viewed in the context of the entire cardiovascular system in order to understand how neural regulation of the circulation may differ between the sexes.

Vascular tone represents the balance between local vasodilator mechanisms which attempt to secure adequate blood flow for metabolic demand and neural vasoconstrictor reflexes attempting to maintain blood pressure. Thus, vascular tone is dependent on the balance between vasoconstrictor and vasodilator influences. In general, there is a linear relationship between sympathetic activity and vascular resistance (see bottom right in Fig. 2). In the absence of sympathetic nerve activity, the vessels maintain a tonic level of vascular tone according to the balance of locally produced vasoconstrictors and vasodilators.

There is sufficient evidence now to support the concept that the female sex steroids favor the balance of vasodilators to vasoconstrictors. This suggests the indirect vasodilator effects of estrogen, most likely through nitric oxide, contribute to the smaller vasoconstrictive responses in women compared to men. Along these lines, serum levels of nitrate and nitrite, metabolites of nitric oxide, increase during the follicular phase of the menstrual cycle in conjunction with rising estrogen levels, and decrease during the postovulatory, high progesterone phase. This is consistent with the concept that estrogen enhances vasodilator and/or inhibits vasoconstrictor activity, whereas progesterone antagonizes estrogen's actions.

Catecholamine release from sympathetic nerves has also been found to be affected by sex. It has been demonstrated in a few studies that presynaptic inhibition of norepinephrine release from sympathetic nerves is greater in female than male rats. In contrast, it has been reported in humans that the change in vascular resistance to infusions of the adrenergic receptor agonist phenylephrine is similar in men and women, suggesting that there are no sex differences in alpha receptor density or sensitivity. However, sex comparisons have only been made during the early follicular phase of the menstrual cycle, and estrogen has been shown to lower alpha receptor density. Furthermore, women may have greater β_2 receptor sensitivity in peripheral vascular beds, which, when stimulated by epinephrine, causes vasodilation [4]. Along these lines, women may have increased β_1 receptor sensitivity in the heart contributing to a greater heart rate response to epinephrine. Combined, these factors may contribute to increased vasovagal (fainting) responses to epinephrine under certain conditions in women.

Taken together, these findings provide evidence that vascular responsiveness to a given rise in sympathetic activity is less in women than in men. Furthermore, there is sufficient evidence to suggest that vascular responsiveness varies during the course of the menstrual cycle. Although these factors may contribute to the decreased tolerance to orthostasis in women, they may also be cardioprotective as increased vascular responsiveness in males has been associated with higher incidence of hypertension and cardiovascular disease.

2.8. Importance of blood volume on neural control of the circulation

Blood volume, even relative to body size, is less in women than it is in men. This difference is due to reduced red blood cells mass in women, and not attributable to differences in plasma volume. Although estrogen administration can lead to significant body fluid retention, it has been found that there is a "plasma volume contraction" during the mid-luteal phase of the menstrual cycle. It is thought that estrogen increases thirst and arginine vasopressin concentrations in the blood (a volume regulating hormone that increases water reabsorption in the kidney) for a given increase in blood tonicity. Progesterone, on the other hand, may inhibit aldosterone-dependent sodium reabsorption in the kidney producing a transient natriuresis. Importantly, the smaller blood volume in women, coupled with a relative plasma volume contraction, may contribute to the decreased orthostatic tolerance in women. Furthermore, resting sympathetic activity during the mid-luteal phase of the menstrual cycle may occur to compensate for the reduced blood volume.

2.9. Other afferent signals modify neural control of the circulation

In addition to baroreceptor afferent signals to the central nervous system, there are a number of other stresses that can impact neural control of the circulation, as shown in Fig. 2. Changes in blood chemistry, such as the partial pressure of oxygen and carbon dioxide, will stimulate peripheral chemoreceptors located in the aortic and carotid bodies and central chemoreceptors located in the medulla. Only recently have baroreflex and chemoreflex interactions become recognized as important modifiers of neural control of

the circulation. However, it is now clear that the chemoreflexes can impact blood pressure and blood flow distribution in humans. Although a few sex differences have been identified, they are still not well understood. Progesterone has long been known to increase the respiratory response to increases in carbon dioxide in the blood, and may impact blood pressure as well. Along these lines, women have demonstrated a greater increase in heart rate than men, and a modest elevation in diastolic blood pressure to hypoxia. A greater pressor response to hypercapnia has been observed during the mid-luteal versus early follicular phase of the menstrual cycle. These mechanisms most likely exist to protect the fetus in pregnant women, as progesterone levels are elevated during pregnancy. Importantly, rates of preeclampsia, a dangerous condition during pregnancy characterized by very high blood pressure, are much greater at high elevations, further suggesting that the female reproductive hormones can impact blood pressure regulation.

2.10. Metaboreflex or "muscle chemoreflex"

Skeletal muscles are innervated by thinly myelinated group III and unmyelinated group IV afferent nerves. These afferents synapse in the dorsal root ganglia and stimulate spinal neurons that travel centrally and activate the brainstem cardiovascular control centers. These afferents are sensitive to various chemical and mechanical stimuli. At rest and during exercise, the group III afferents are primarily mechanosensitive and the group IV afferents are chemosensitive, responding to a variety of metabolic stimuli including potassium (K^+), hydrogen ion (H^+), phosphate (PO_4), and adenosine. It is believed that these afferents sense a mismatch between blood flow and metabolism in the active muscles, such as during isometric and ischemic exercise. Once activated, the metaboreflex causes an increase in arterial pressure in an attempt to improve perfusion of the ischemic muscle and reduce or eliminate the blood flow-metabolism mismatch.

Similar to the findings during other sympathoexcitatory maneuvers, the rise in sympathetic neural activity to the stimulation of the metaboreflex is less in women than it is in men [5]. Importantly, lower production of H^+ and $H_2PO_4^-$ have been observed in women during non-ischemic exercise, suggesting that the stimulus for the metaboreflex is less. It must be pointed out, however, that these are probably not the only metabolites produced under this condition. Taken together, these findings suggest that women may have less production of metaboreceptor stimulants, resulting in an attenuated rise in sympathetic outflow and diastolic blood pressure compared to men. Importantly, these findings were independent of muscle mass, workload, and level of training. However, this only holds true under "free-flow" or non-ischemic conditions, as during ischemic exercise the sex differences were not observed. Ischemic exercise results in a much greater stimulus than non-ischemic exercise, so it is likely that under these conditions the metaboreflex stimulus is maximal for both sexes.

2.11. Heart rate variability and power spectral analysis

Heart rate and blood pressure do not fluctuate randomly under a steady-state condition, but fluctuate with a fairly constant frequency. Power spectrum analysis is a computer-based

technique used to identify the relative preponderance of different frequencies. The high-frequency component occurs in synchrony with respiration, and the low-frequency component is thought to represent sympathetic activity. However, the low-frequency domain in blood pressure variation contains a non-sympathetic component that remains unidentified. Thus, conclusions drawn from heart rate and blood pressure variability must be viewed with caution. Despite these limitations, a number of studies have used these techniques because they are relatively easy to perform and require little in terms of equipment. As there have been a number of studies using these techniques to study sex differences in neural control of the circulation, they merit at least an acknowledgment. In some cases the results from studies using spectrum analysis agree with findings from more direct techniques, and in other cases they are in stark contrast. In general, most studies agree that women tend to have less sympathetic influence on blood pressure and a greater parasympathetic influence on heart rate during challenges to blood pressure regulation.

3. Sex and thermoregulatory reflexes

The influences of estrogen and progesterone on neural control of blood flow were first observed in the context of their influences on temperature regulation. Thus, it is not surprising that more is understood about the effects of these hormones on neural regulation of blood flow of the skin, and in temperature regulation in general, than in neural control of any other vascular bed. However, there are still a number of questions that remain to be answered.

Normal core body temperature in humans is approximately 37 °C (98.6 °F). A major role of the autonomic nervous system is to defend this core body temperature independently of external conditions. Thermoregulation is a classic example of negative feedback. As core body temperature changes, thermoregulatory responses are activated in an attempt to minimize heat loss or lose heat to the environment. This will return core body temperature to normal and the signal for the thermoregulatory reflexes will be removed. In general, thermoregulatory responses are neurally mediated, and can greatly challenge blood pressure regulation during environmental extremes.

An overview of the thermoregulatory reflexes is presented in Fig. 5. Information about the temperature of the body core is provided by thermosensitive neurons in the anterior hypothalamus, spinal cord, and brain stem. Information about temperature in the periphery is provided by themoreceptors in the skin and other areas deep in the body, including muscle. The thermoreceptors can be divided into two very general categories: warm-sensitive neurons and cold-sensitive neurons. The activity of the different types of thermoreceptors is passed to the pre-optic area of the hypothalamus (POAH). This integrated signal is compared to a "set-point" temperature, believed to arise from the posterior hypothalamus. Depending on impending or actual differences between the integrated signal in the POAH and set-point temperature, either heat-loss mechanisms or heat-conservation/generation responses are activated. It is important to note that the "set-point" temperature is not rigidly fixed, but is extremely variable. This implies that the posterior hypothalamus receives input from other areas of the brain, and receives feedback

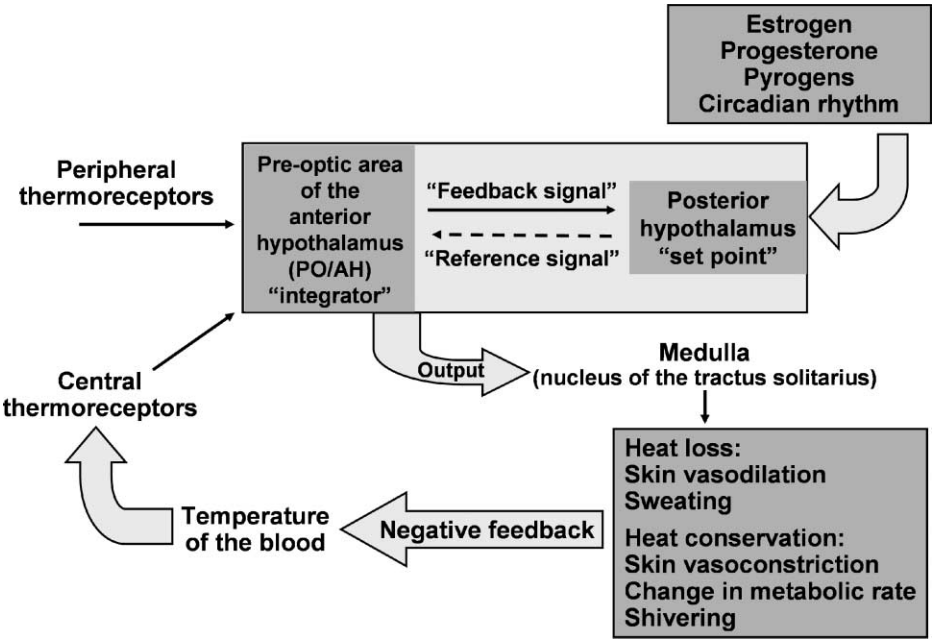

Fig. 5. *Overview of thermoregulatory reflexes*. Afferent information about the temperature of the body core is provided by thermosensitive neurons in the anterior hypothalamus, spinal cord, and brain stem. Afferent information about temperature in the periphery is provided by themoreceptors in the skin and other areas, including muscle. Activity of the different types of thermoreceptors is passed to the POAH, which acts as a "central integrator" for thermoregulatory reflexes. This integrated signal is compared to a "set-point" temperature from the posterior hypothalamus. Either heat-loss mechanisms or heat-conservation/generation responses are activated. Set-point temperature can be influenced by a number of factors, including estrogen and progesterone.

from the POAH. In truth, the concept of an actual "set-point" temperature is passé, although it simplifies the model of thermoregulation for discussion purposes.

Set-point temperature is influenced by a number of factors. Core body temperature fluctuates during the menstrual cycle, and monitoring core body temperature is still commonly used to determine the time of ovulation for the purpose of predicting fertility. It is now clear that these changes in core body temperature across the menstrual cycle are due to the effects of estrogen and progesterone on central regulation of core body temperature. This is not to say that estrogen and progesterone do not influence peripheral mechanisms, as there is sufficient evidence that they may, but that the primary influence is on the set-point temperature. In general, estrogen lowers core body temperature, and progesterone increases core body temperature. The "dip" in core temperature around the time of ovulation is due to elevated levels of estrogen unopposed by progesterone (see Fig. 6). The subsequent increase in core temperature during the luteal phase, in which levels of both estrogen and progesterone are elevated, is due to the greater biological influence of progesterone.

The main autonomic thermoregulatory effector mechanisms are regulation of skin blood flow, sweating, and shivering. Once a difference between set point temperature and

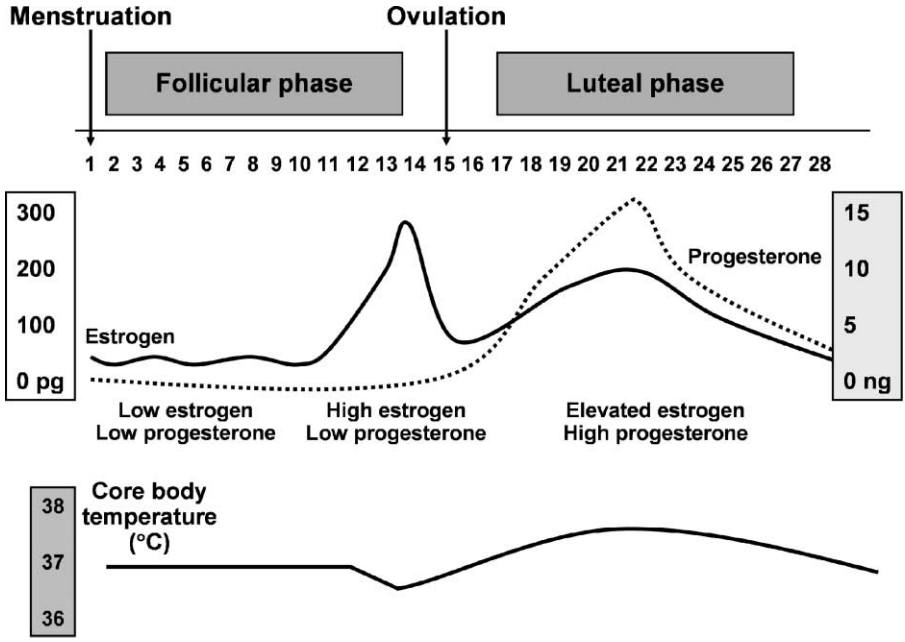

Fig. 6. *Changes in core body temperature during the menstrual cycle*. Estrogen lowers core body temperature, and progesterone increases core body temperature. The "dip" in core temperature around the time of ovulation is due to elevated levels of estrogen unopposed by progesterone. The subsequent increase in core temperature during the luteal phase, in which levels of both estrogen and progesterone are elevated, is due to the dominant influence of progesterone on thermoregulatory control.

the integrated temperature has been sensed by the hypothalamus, efferent sympathetic thermoregulatory responses are initiated. These thermoregulatory signals pass through the NTS of the medulla, providing further evidence that this area of the medulla is an "integration center" and major regulatory site for cardiovascular responses. Thus, it is not surprising to find that thermoregulatory responses are greatly influenced by other inputs to the medulla. Inputs from the baroreceptors, chemoreceptors, and metaboreceptors can all impact the overall thermoregulatory response. In general, any challenge to blood pressure regulation, such as upright posture or dehydration, will result in a rightward shift (to higher core temperatures) for the activation of thermoregulatory responses. These shifts to higher core temperatures before activation of the thermoregulatory responses seem to make sense in terms of protecting blood pressure at the expense of temperature regulation.

In order to compare sex differences in thermoregulation, it is important to state that there are a number of factors that independently affect thermoregulation that may obscure or exacerbate sex differences. For example, it has been reported that maximal aerobic power is a major determinant of differences between individuals in sweat secretion and vasodilation of the skin in response to heat stress. In addition, there are a number of other factors that can impact thermoregulatory responses that may differ between sex, such as body size, blood volume, and body composition (i.e. amount of lean body mass and

subcutaneous fat) that must be considered when making a comparison between sexes. Clearly, the menstrual cycle can impact thermoregulatory reflexes, and results from studies that have not controlled for this issue have lead to erroneous conclusions.

In general, there are a few thermoregulatory differences between men and women when studies appropriately control factors that independently affect thermoregulation, including the menstrual cycle. Individually, estrogen is known to decrease and progesterone is known to increase core body temperature. This has been observed with both the endogenous and exogenous forms of these hormones. In the mid-luteal phase of the menstrual cycle and the "on-phase" of combined oral contraceptive use, when levels of both hormones are increased, the body temperature is elevated by about 0.5 °C. This suggests that progesterone has the dominant thermogenic effect.

3.1. Responses to heat stress

Control of skin blood flow is quite complex and not fully understood. During thermoneutral conditions, the skin maintains a tonic level of vasoconstriction. When core temperature begins to rise, tonic vasoconstriction is released, a "threshold" for vasodilation is reached, and an active vasodilator reflex is activated. The resulting increase in skin blood flow continues to rise linearly with increasing core body temperature. Various factors, such as body posture, level of hydration, and exercise can alter the core temperature–skin blood flow relationship by impacting either the threshold for vasodilation or the sensitivity (slope) of the relationship. As alluded to above, both estrogen and progesterone are known to profoundly alter the threshold for vasodilation as displayed in Fig. 7. It is now clear that estrogen alone will shift the threshold for neurogenic vasodilation to a lower core temperature and that progesterone alone will shift vasodilation to a higher core temperature [3].

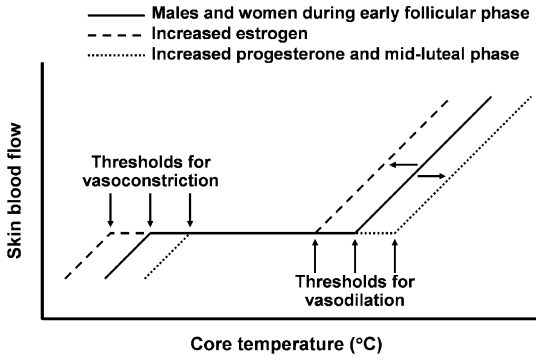

Fig. 7. *Influence of estrogen and progesterone on thresholds for thermoregulatory responses*. Estrogen and progesterone are known to profoundly alter the threshold for heat loss and heat conservation/heat generation mechanisms. Estrogen alone shifts thresholds to lower core temperatures, whereas progesterone alone shifts thresholds to higher core temperatures.

Active vasodilator nerves are believed to be sympathetic cholinergic nerves releasing acetylcholine as the primary neurotransmitter. The acetylcholine binds to muscarinic receptors on sweat glands to activate sweating. However, acetylcholine has been found to be only minimally involved in active vasodilation. Thus, the current theory is that an unknown transmitter is co-released with acetylcholine such that acetylcholine mediates sweating and the unknown neurotransmitter mediates active vasodilation. Nitric oxide, which can be significantly impacted by estrogen, is known to be involved in active vasodilation, but the exact role is unclear. However, the fact that nitric oxide is important in active vasodilation suggests this may be a mechanism by which the female reproductive hormones impact thermoregulatory responses during the course of the menstrual cycle and oral contraceptive use. More research is needed to investigate this possibility.

Although there does not appear to be differences in sweat rate between men and women once confounding factors have been taken into consideration, it has been reported that women tend to have more sweat glands per area of skin than men, but that individual sweat glands in men may produce more sweat. This may cause more sweat to drip off the skin of men, and may give credence to the statement "Men sweat, women glisten".

It has been reported that the core temperature thresholds for sweating is lower during the pre-ovulatory phase, when only estrogen levels are elevated, than during the early follicular phase. This suggests the core temperature threshold for sweating and active vasodilation appear to be similarly affected by the female reproductive hormones. This is not surprising as both of these responses appear to be mediated by the same population of nerves. When postmenopausal women are compared with men, their basal core body temperature and thermoregulatory thresholds are similar to those in age-matched men. Importantly, hormone replacement therapy will return the threshold shifts to those observed in young women. During menopause, however, changes in reproductive hormone levels substantially alter thermoregulatory control of skin blood flow, and most likely contribute to the occurrence of hot flashes. The exact mechanism of hot flashes is not fully understood, but certainly involves the influence of female sex hormones on thermoregulatory control centers and neural vasomotor outflow.

3.2. Responses to cold stress

When core temperature decreases, there is an increase in skin adrenergic sympathetic activity. These cutaneous nerves release norepinephrine which binds to α_1 and α_2 receptors, resulting in vasoconstriction of the skin. This peripheral vasoconstriction serves to keep warm blood near the body's core so less heat is lost to the environment. Along with the increase in core temperature when levels of estrogen and progesterone are elevated, the threshold for *vasoconstriction* also occurs at a higher temperature, as shown in Fig. 7. Although the individual influences of estrogen and progesterone on cutaneous vasoconstriction have not been investigated to date, it is expected that the threshold shifts would be similar to those observed during heat stress if these hormones are truly impacting central thermoregulation as is currently believed.

In general, vasoconstrictor tone to most of the skin is similar in men and women. However, women are much more likely to suffer from Raynaud's disease, a vasospastic

disorder characterized by excessive vasoconstriction of the cutaneous circulation of the extremities. The palms of the hands and soles of the feet do not contain active vasodilator nerves; thus, neural control of these regions is entirely due to the vasoconstrictor nerves. Attacks of Raynaud's disease are precipitated by exposure to cold, and in some cases, mental stress. Under these conditions, sympathetic vasoconstrictor outflow increases to the skin. People suffering from Raynaud's will have an exaggerated vasoconstrictor response, followed by a painful, paradoxical dramatic increase in skin blood flow. The pathology of Raynaud's is still poorly understood, but current evidence suggests that there is a profound sensitivity to a subtype of α-receptors (α_{2C}) in the skin of Raynaud's patients. This suggests that a sex difference in the expression of this receptor may exist. Importantly, Raynaud's is the first symptom of scleroderma, a debilitating and often fatal disease that is more prevalent in women than in men. It is possible that a genetic polymorphism in α-receptors exists, but remains to be clearly identified and shown to be linked to Raynaud's disease and scleroderma.

4. Future directions

Orthostatic intolerance is the most common disorder of blood pressure regulation after essential hypertension, and patients with orthostatic intolerance are most commonly women of childbearing age. Such patients comprise the largest group referred to centers specialized in autonomic disorders. The underlying causes for the greater rate of orthostatic intolerance in women remain unclear and warrant further investigation. In addition, researchers need to carefully characterize the menstrual cycle when studying women, and refrain from simply comparing men to women during the early follicular phase.

It is now clear that sex hormones can greatly impact reflex control of the circulation, and that there may be differences between the endogenous and exogenous forms of these hormones. There is sufficient evidence to state that differences exist in the bioactivity and actions of endogenous and exogenous forms of estrogen and progesterone. As it is estimated that over 11 million women in the United States and 65 million women worldwide are taking oral contraceptives, this issue is not of small consequence. A clearer understanding of how the endogenous and exogenous forms of the sex hormones differentially impact the circulation could lead to improved forms and dosages prescribed to maximize the healthy benefits and minimize the risks. Importantly, there is also a lack of information regarding how hormone replacement therapy impacts neurovascular control in peri-menopausal and post-menopausal women. Along these lines, estrogen replacement therapy is the most effective treatment for hot flashes, but the mechanisms are not fully understood and their use is controversial in breast cancer survivors. Selective estrogen receptor modulators (SERMs) are a class of compounds, including tamoxifene and raloxifene, which are approved for clinical use to treat and prevent breast cancer and osteoporosis. Although these compounds are well tolerated in most patients, one of the most common adverse effects experienced in patients undergoing SERM treatment is hot flashes. Complicating this issue is that SERMs appear to act as estrogen agonists in some tissues but estrogen antagonists in others. Clearly, more research is needed to elucidate

the roles of estrogen, progesterone, and testosterone on autonomic regulation of the circulation.

5. Summary

Fig. 8 summarizes a number of sex differences in neural control of the circulation as discussed in this chapter. It is clear that differences exist in sympathetic nervous system activity at rest and in response to sympathoexcitatory maneuvers. In general, women tend to have lower resting blood pressure and lower peripheral vascular resistance, but higher resting heart rates than men. Furthermore, women tend to respond to various stresses with a greater reliance on heart rate, whereas men tend to respond more by increasing peripheral vascular resistance. The exact causes for these differences are not clear, but may involve differences in the afferent stimulants, distensibility of tissues (i.e. compliance), central integration of afferent signals, efferent sympathetic outflow, release of neurotransmitters, receptor density and sensitivities, or differences in the balance of vasoconstrictors to vasodilators.

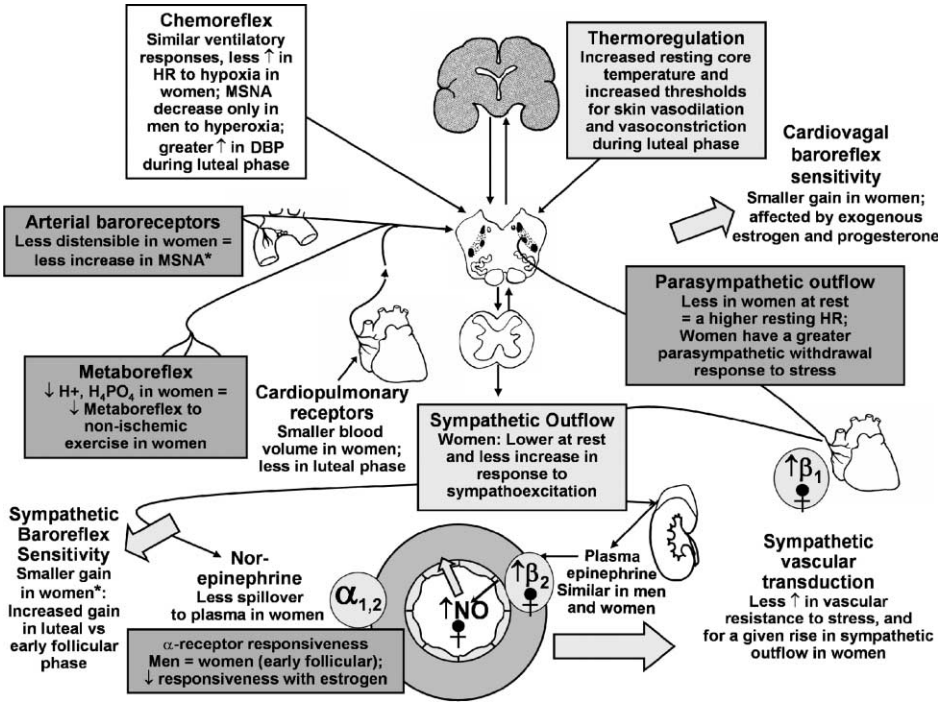

Fig. 8. *Summary of sex differences in neural control of the circulation*. There is evidence of sex differences occurring at numerous places along the cardiovascular reflexes, including differences in afferent signals, central integration, efferent neural output, and vascular responsiveness. *reflects data from combined studies, not directly compared.

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Sex differences in hypertension and renal injury

Jane F. Reckelhoff, Lourdes A. Fortepiani, Licy L. Yanes and Valeria E. Cucchiarelli

1. Introduction

In this chapter some of the possible mechanisms contributing to sex differences in hypertension and renal function and injury will be discussed. Results of both human and animal studies will be evaluated because perturbations in physiological systems can be performed in animals but not humans due to the invasive nature of the studies. In addition, systems must be studied in the context of hypertension and thus with some exceptions that will be discussed later, cell culture is not a viable alternative for study. However, data from mesangial cells in culture will be provided because they are used as a model of renal injury. Most of the data provided have been derived from two types of experimental animals, spontaneously hypertensive rats (SHRs) and Dahl salt-sensitive rats. This chapter does not aim to be an inclusive list of all sex differences in renal function, but rather addresses the possible mechanisms by which hypertensive renal injury may occur. These will include the contributions of androgens, estrogens, the renin–angiotensin system (RAS) and oxidative stress to hypertension and renal dysfunction. The mechanisms responsible for postmenopausal hypertension will not be covered in detail in this chapter, but postmenopausal hypertension is important to mention in any discussion of sex differences in blood pressure control.

Sex differences in humans. Men are at greater risk for cardiovascular and renal disease than women of similar ages. Recent studies using the technique of 24 h ambulatory blood pressure monitoring have shown that blood pressure is higher in normotensive men than in women at similar ages [18]. In addition, in the hypertensive population, the incidence of end-stage renal failure caused by glomerulonephritis and hypertensive glomerular sclerosis is also higher in men than women [25]. In the Modification of Diet in Renal Disease Study, non-diabetic men who underwent serial measurements of glomerular filtration rate (GFR) over 2.2 years were found to experience a more rapid decline in renal function than did the women [3]. The incidence of immune-mediated glomerular diseases is also higher in men than women [25]. Furthermore, aging in men is associated with

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greater decrements in renal function than in women of similar age [13,14]. Finally, many studies have confirmed that the prevalence of hypertension is greater in men with renal disease than in women, but male sex and hypertension each contribute independently to the progression to end-stage renal failure [25].

Following menopause (which occurs, on an average, at 51.4 years of age), blood pressure increases in women. The data from National Health and Nutrition Examination Survey (NHANES) III supported by National Institutes of Health confirmed that by 60–69 years of age non-Hispanic black and Hispanic white women developed a higher prevalence for hypertension than did age- and ethnicity-matched men [18]. The mechanisms responsible for the hypertension in these populations are complicated by co-morbid conditions of obesity and type II diabetes, both of which lead to increases in blood pressure. However, in the non-Hispanic white population, which did not exhibit such a high incidence of obesity and type II diabetes with aging, the prevalence of hypertension also increased in women. So by 60–69 years of age, non-Hispanic white women had similar prevalence of hypertension as men, and by 70–79 years of age, this population of women had a higher prevalence than did men. Interestingly, the increase in BP takes an average of 5–20 years after menopause to develop, and the mechanisms responsible for the increase in blood pressure in postmenopausal women are not clear.

Sex differences in animals. Sex-associated differences in blood pressure have been documented in various experimental animals. For example, male SHRs have higher blood pressure than do females of similar ages [18] (see also Chapter 1). Similar sex differences in development of hypertension are also found in Dahl salt-sensitive rats on a high salt diet, deoxycorticosterone (DOC)- and salt-treated hypertensive rats and New Zealand genetically hypertensive rats [18]. Therefore, as found in humans, in hypertensive rats, males have higher blood pressure than age-matched females. To date, there have been no studies in which a sex difference in blood pressure in normotensive animals has been documented. This is not surprising since in humans, sex differences in blood pressure were only clearly identified using ambulatory blood pressure monitoring techniques. There have been no studies to our knowledge in which blood pressure has been measured 24 h by chronic blood pressure monitoring methods in normotensive male and female rats. From the small differences in blood pressure found in normotensive human subjects, it is clear that blood pressure measurement in conscious rats during acute studies is not sufficient to be able to detect small differences that might be expected in normotensive males and females.

The SHR has been used as a model not only for sex differences in hypertension, but also sex differences in renal function and injury. There are very few studies of sex differences in renal changes in SHR compared to the large number of studies of sex differences in hypertension in this model, however. Male SHR have reduced GFR and renal plasma flow compared to female SHR as early as 14–15 weeks of age [18]. Also males excrete more protein in urine than females. However, despite these changes, there is no renal injury present in male SHR up to 9 months of age. With further aging the renal dysfunction and injury progresses in males despite the fact that the sex difference in blood pressure in SHR is lost by 16–18 months of age. Loss of the sex difference in hypertension is due to an increase in blood pressure in females, not a reduction in blood pressure in males [8]. Therefore, despite similar blood pressures in aged males and females, males have a 50%

reduction in GFR compared to females, and excrete 7-fold more protein than females showing greater renal injury in males. Morphological examination of the kidneys of aged SHRs demonstrate that males have more glomerular sclerosis than do females. The Dahl salt-sensitive rat on a high salt diet also exhibits sex differences in renal injury with males exhibiting more injury than females.

Possible mechanisms responsible for sex differences in hypertension and renal function. The mechanisms responsible for the sex differences in hypertension and renal function are multifactorial. Although much remains to be learned about these mechanisms, below is a discussion of factors that are proposed to be important.

Abnormal pressure–natriuresis in hypertension. Substantial evidence supports the theory that some form of renal dysfunction contributes to the development and maintenance of hypertension [18]. Providing the strongest support for this theory are observations that transplantation of prehypertensive kidneys from SHR to WKY produces hypertension. Similar results have been obtained in renal transplantation studies between Dahl salt-sensitive and salt-resistant rats [18]. Of particular relevance to human hypertension is the study by Curtis et al., which demonstrated that blood pressure returns to normal in hypertensive patients who receive kidneys from normotensive donors [18]. These results indicate that a defect in sodium and water handling within the kidney plays a crucial role in the pathogenesis of hypertension. This defect is present in all types of hypertension studied to date. Under normal conditions, an increase in salt intake leads to increased arterial pressure that elicits a marked increase in sodium excretion, eventually leading to reversal of the increase in blood pressure [18]. According to the renal body fluid

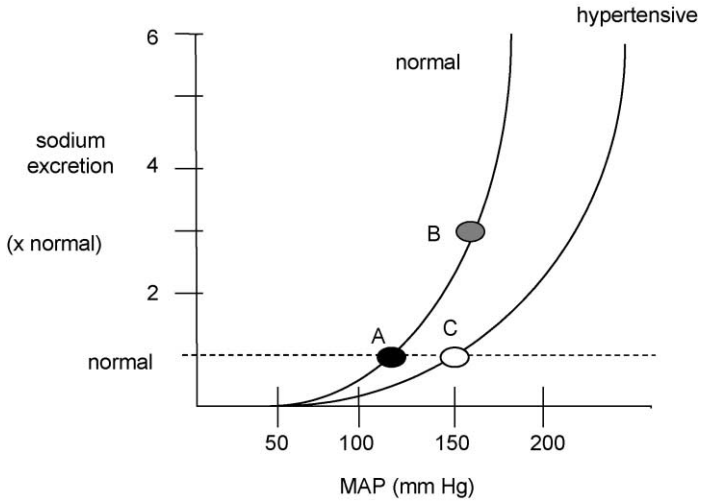

Fig. 1. Schematic diagram describing the pressure–natriuresis relationship. In normotensive individuals (A) who excrete a normal amount of sodium, an increase in sodium intake results in an increase in blood pressure (B), but this increase in blood pressure soon is resolved due to a concomitant increase in sodium excretion. In hypertensive individuals, their blood pressure must be at higher levels (C) just to excrete a normal amount of sodium. This is known as the pressure–natriuresis relationship and hypertensive individuals experience a shift to the right in the relationship when compared to normotensive individuals.

feedback concept, a long-term increase in arterial pressure or hypertension occurs as a result of a reduction in renal excretory function or a rightward shift in the pressure–natriuresis relationship (see Fig. 1). The mechanisms responsible for higher blood pressure in males (human or animal) also result in a shift in the pressure–natriuresis relationship.

1. Sex hormones

Role of androgens in hypertension. There is significant evidence that androgens, such as testosterone, play an important role in sex-associated differences in regulation of blood pressure. For example, studies using ambulatory blood pressure monitoring techniques in children have shown that with increasing age, blood pressure increases in both boys and girls. However, after the onset of puberty, boys have higher blood pressure than do age-matched girls [18]. Blood pressure also does not dip as low at night in postpubescent boys as in girls. These data indicate that with adolescence and puberty when androgen levels are increasing, blood pressure is higher in boys than girls.

Additional evidence that testosterone may contribute to higher blood pressure in males is derived from studies using castrated male experimental animals. Castration of males at a young age (3–5 weeks) attenuates the development of hypertension in SHR, Dahl salt-sensitive rats, and in rats subjected to reduction in renal blood flow to one kidney by clipping the renal artery (Goldblatt maneuver) [18]. Additionally, testosterone supplementation in ovariectomized female SHR causes a dose-dependent increase in blood pressure [18].

Harrap and colleagues reported that when young male SHR kidneys were transplanted into female SHRs, blood pressure in the females did not increase. The blood pressure in female SHR with the male kidneys was similar to blood pressure in female SHRs with female kidneys [18]. Similarly, when female SHR kidneys were transplanted into male SHRs, blood pressure was not attenuated in the male, i.e. blood pressure in male SHRs with female kidneys was similar to blood pressure in male SHRs with male kidneys. Harrap's data indicate that the 25–30 mm Hg higher blood pressure in the male SHR compared to the female is due to some "external factor" (presumably androgens) that further shifts pressure–natriuresis to increase blood pressure. At this time it is not clear if androgens have a direct effect on sodium reabsorption in the proximal tubule or affects sodium reabsorption by its effect on other humoral systems, such as the RAS. However, the androgen receptor is present on proximal tubule cells, and future studies may confirm that androgens do have a direct effect on sodium reabsorption (Fig. 2).

Role of androgens in renal function and injury. There is significant evidence that androgens contribute to renal injury. Castration of male SHRs at 5 weeks of age attenuates the rise in blood pressure and reduces proteinuria. In contrast, ovariectomy of female SHRs has no effect on proteinuria, but testosterone treatment of ovariectomized females does increase proteinuria. In addition, castration as late as 8 months of age in male SHR reverses the reduction in GFR and completely prevents any renal injury that is present in the male SHR by 18 months of age. Other experimental models of hypertension such as Dahl salt-sensitive rats on a high salt diet or rats subjected to renal

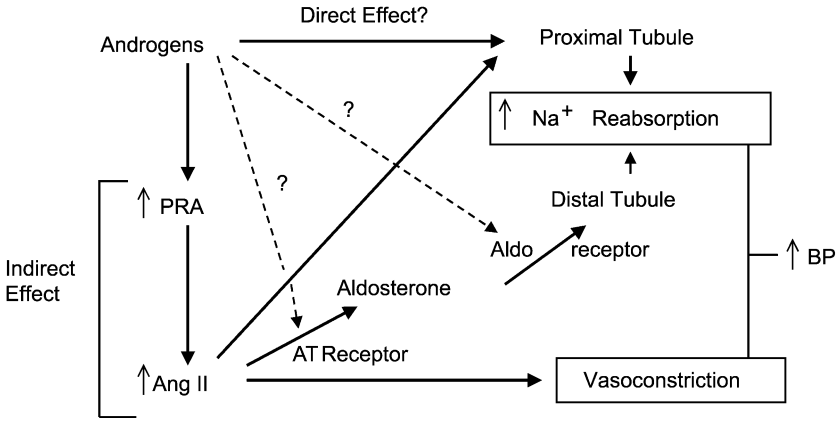

Fig. 2. Possible direct and indirect mechanisms by which androgens could increase blood pressure. Androgens could have a direct effect on sodium reabsorption since androgen receptors have been found in proximal tubule cells. Alternatively, androgens could indirectly increase sodium reabsorption and vasoconstriction by increasing PRA and thereby increasing angiotensin II (Ang II). Ang II directly increases sodium reabsorption in the proximal tubule and causes vasoconstriction. Ang II also causes aldosterone synthesis, and aldosterone increases sodium reabsorption in the distal nephron. PRA, plasma renin activity; Na, sodium; BP, blood pressure.

ablation (removal of nephrons) also exhibit sex differences in the progression of renal injury with males faring worse.

Role of female sex hormones on hypertension. As previously mentioned, women have lower blood pressure throughout life until after menopause, when blood pressure increases. The presence of estrogens has been assumed to be the protective factor in preventing hypertension in women. However, it is not likely to be as simple as the presence or absence of estrogens. For example, hormone therapy (HT), using estradiol and progesterone, has not protected postmenopausal women from either primary or secondary cardiovascular disease [18]. However, whether HT reduces blood pressure in postmenopausal women is controversial. In a study in which blood pressure was measured serially in postmenopausal women for 5 years, HT was not successful in reducing blood pressure and in some women even increased blood pressure compared to untreated control women [18]. Estrogen is not given as a treatment for hypertension in postmenopausal women. Thus, why blood pressure increases in postmenopausal women and why premenopausal women are protected compared to men remains to be determined, but is not likely to be as simple as just the presence or absence of sex hormones. In addition, the presence of obesity, type 2 diabetes and increase in sympathetic tone could also increase blood pressure in postmenopausal women.

Ovariectomy in rats has variable effects on blood pressure. For example, ovariectomy has no effect on blood pressure in young female SHR. However, following cessation of cycling, blood pressure in female SHR increases until the sex difference in hypertension, described above, is lost [8]. In addition, ovariectomy of female SHR when they are 8 months of age prevents the increases in blood pressure found in postmenopausal rats.

The female SHR stops cycling at 10–12 months of age and serum estradiol decreases to the same level as found in age-matched males. However, since ovariectomy of female SHR at 8 months does not result in an increase in blood pressure by 18 months of age, it is likely that estradiol is not the factor protecting female SHR from the age-related increase in blood pressure. However, there are strain differences in the response of to ovariectomy. For example, ovariectomy of female Dahl salt-sensitive rats results in increases in blood pressure even when they are kept on a low salt diet [18]. Mechanisms responsible for the increase in blood pressure with ovariectomy in salt sensitive rats are not known. Therefore, the role, if any, which estrogens play in protecting pre-menopausal women and rats from development of hypertension remains to be elucidated.

1.2. The renin–angiotensin system

Role of the RAS in hypertension. A key system for modulating blood pressure and body fluid volume (i.e. pressure–natriuresis) is the RAS [18]. Long-term pressure–natriuresis is modulated by the RAS. Angiotensin II increases proximal tubular sodium reabsorption by stimulating epithelial transport. Under normal circumstances, an increase in salt and water in the body causes a reduction in renin release in the kidney leading to a reduction in angiotensin II and natriuresis. Conversely, if salt levels are low, renin release is stimulated and angiotensin II increases to cause an increase in sodium reabsorption. In the event of abnormal angiotensin II levels for the level of salt and volume in the body, blood pressure will increase with abnormal sodium and water reabsorption, leading to a shift in the pressure–natriuresis relationship. Similarly, if total body volume is perceived incorrectly in the body and thus angiotensin II levels do not respond appropriately, increases in blood pressure will also occur [19]. Angiotensin II also contributes to increasing blood pressure through contractions of the microvasculature.

There is an interaction between sex hormones and the RAS. Plasma renin activity (PRA) has been shown to be 27% higher in men than women regardless of age or ethnic heritage [18]. In castrated normotensive rats, there is a linear relationship between serum testosterone and PRA when rats are treated chronically (2 weeks) with increasing doses of testosterone [18]. Male SHR have higher PRA than females, testosterone treatment of ovariectomized females causes increases in PRA, and PRA decreases with castration in males. In SHR, chronic blockade of the RAS with the angiotensin converting enzyme (ACE) inhibitor, enalapril, results in normalization of the blood pressure regardless of sex, thus removing the sex difference in blood pressure. In addition, enalapril also prevents increase in blood pressure associated with testosterone supplementation in ovariectomized females [18]. These data suggest that the RAS is necessary for androgens to increase blood pressure and also suggest that androgens may increase blood pressure in SHR by activating the RAS.

The mechanisms by which androgens activate the RAS are not clear, but data from two groups have independently shown in SHR and normotensive WKY that castration decreases and chronic testosterone increases renal angiotensinogen mRNA [18]. Chronically increased renal angiotensinogen could increase renal tissue angiotensin II if renin enzyme is not working at V_{max}, which has been reported in both humans and rats.

To support this hypothesis, studies in transgenic mice have demonstrated that an increase in copy number of the angiotensinogen gene causes increase in their blood pressure [18].

One mechanism by which androgens may impact the RAS is by having an effect on synthesis of angiotensin II receptors in the kidney. To date there have been no studies to determine if androgens affect the angiotensin II receptor numbers or affinity. However, Nickenig and colleagues reported that ovariectomy of normotensive rats results in an increase in AT1 receptor number in the aorta [17]. Angiotensin AT2 receptor subtypes have been associated with vasodilation, perhaps through release of nitric oxide. So it is also possible that androgens could reduce the number of AT2 receptors and thereby increase blood pressure.

Androgens could also cause increases in blood pressure by upregulation of aldosterone receptor expression. Angiotensin II stimulates the production of aldosterone, which is responsible for increasing sodium reabsorption in the distal nephron (Fig. 2). It is possible then that androgens could increase sodium reabsorption by increasing angiotensin II to increase aldosterone, or androgens could have a direct effect on aldosterone receptor upregulation. There is evidence to suggest that this may be the case, but this theory is controversial. Miller and colleagues found higher blood pressure and aldosterone levels in men than in women [15], and Schunkert et al. [23], found a positive correlation between dehydroepiandrosterone sulfate (a metabolite of testosterone), aldosterone levels and blood pressure in a population of hypertensive men. However, Kau and colleagues reported that testosterone replacement in castrated male rats decreased corticotropin-stimulated aldosterone release [10].

Estrogens also interact with the RAS (see Fig. 3). Estradiol is important in stimulating angiotensinogen production by the liver. This effect alone should increase blood pressure by causing an increase in renin activity and thus an increase in angiotensin II. However, the increase in renin release with estradiol is offset by estradiol-mediated downregulation of ACE which converts angiotensin I (the product of renin enzyme) to angiotensin II. In addition, as mentioned above, since ovariectomy has been shown to cause an upregulation of AT1 receptors, it is likely that estradiol also modulates the action of angiotensin II by downregulating the receptor responsible for sodium reabsorption and vasoconstriction. Whether estradiol has an effect on AT2 receptors has not been determined.

RAS, sex and renal injury. Angiotensin II contributes to the progression of glomerular injury in male SHR. In aging male SHR, their blood pressure was controlled to

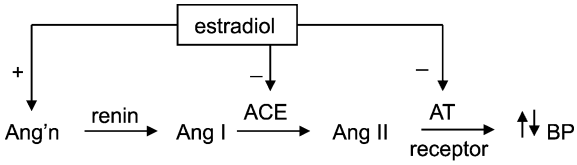

Fig. 3. Mechanisms by which estradiol can modulate the renin–angiotensin system (RAS). Estradiol can increase the synthesis of angiotensinogen (Ang'n) in the liver. This should lead to an increase in blood pressure if all other factors are unchanged. However, estradiol reduces the synthesis and activity of ACE. This will result in a reduction in angiotensin II and thus decrease blood pressure. Estradiol can also downregulate the concentration of AT-1 receptors, which would also contribute a reduction in blood pressure.

normotensive levels by chronic treatment of either triple therapy (reserpine, hydralazine, and hydrochlorothiazide) or ACE inhibitor, enalapril, and kidney morphology was assessed [5,6]. Despite the fact that the blood pressures were similar, the kidneys from rats receiving triple therapy showed glomerular lesions consistent with SHR in which blood pressure was not controlled. In contrast, with enalapril treatment and similar blood pressure as with triple therapy, the glomerular lesions were prevented in the aging SHR. Since enalapril inhibits ACEs and, therefore, formation of angiotensin II, these data suggest that glomerular injury in aging SHR males is mediated by the RAS, independent of the hypertension. In addition, castration completely prevents glomerular sclerosis in aged male SHR [7]. It is possible then that androgens could promote renal injury by a hemodynamic mechanism in which androgens stimulate angiotensin II production leading to an increase in sodium reabsorption in the proximal nephron with a concomitant reduction in the level of sodium reaching the macula densa and resulting in a decrease in glomerular afferent resistance (compared to females) allowing higher glomerular capillary pressure. Angiotensin II will also cause an increase in glomerular efferent resistance that would contribute to higher glomerular capillary pressure. Studies have established that increased glomerular capillary pressure is associated with renal injury. Furthermore, increased glomerular capillary pressure increases cytokine expression. For example, the remnant kidney in uninephrectomized male SHR that also exhibited increased glomerular capillary pressures, showed upregulation of transforming growth factor-beta (TGF-β) and platelet derived growth factor (PDGF)-A and -B chains [24].

To model effects of increased physical pressure on glomerular injury, cultured glomerular mesangial cells are subjected to cyclic strain by stretch and relaxation. (Mesangial cells are grown from primary cultures of glomeruli.) Mesangial cells are the cell type in glomeruli that are responsible for the production of extracellular matrix and cytokines leading to glomerular injury. Stretch of mesangial cells alone causes accumulation of matrix collagen types I, III, and IV, laminin and fibronectin, increases in tissue inhibitors of matrix metalloproteases (TIMPs), reductions in matrix metalloprotease activity and increased TGF-β and TGF-β receptors [2,5]. Stretch of mesangial cells also increases synthesis of angiotensin (AT)-1 receptors and angiotensinogen [2], suggesting the presence of a local RAS in mesangial cells. Few studies have looked at direct effects of angiotensin II on cyclically stretched mesangial cells to mimic glomerular pressure. In unstretched mesangial cells, angiotensin II directly stimulates production of TGF-β and activates transcription factors, NF-κB and AP-1. Furthermore, angiotensin II upregulates Sp1 transcription activity and mediates plasminogen-activator inhibitor type-1 (PAI-1) gene expression. It is not clear whether angiotensin II would potentiate the upregulation of cytokines, transcription factors, and growth hormones in stretched mesangial cells and thus in glomeruli subjected to increased capillary pressure. However, it is clear that all the factors that are upregulated in mesangial cells in response to physical forces and angiotensin II have not been identified. Thus androgens and estrogens that modulate angiotensin II, could potentiate or ameliorate the effects of angiotensin II on mesangial cells [12]. Furthermore, because the sex steroids are also transcription factors, they may also participate in synthesis or inhibition of synthesis of cytokines and thus acting in synergy with angiotensin II.

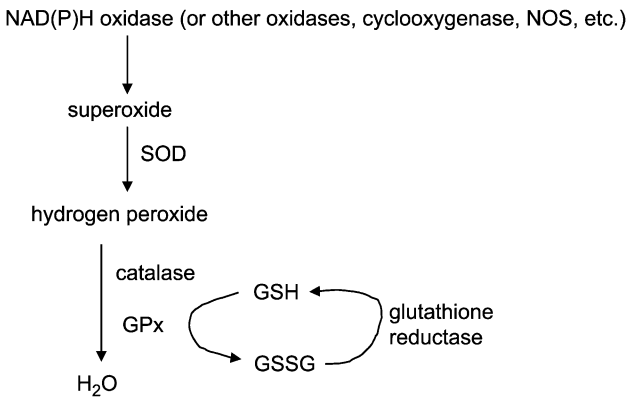

Fig. 4. Antioxidant pathways to neutralize superoxide radicals in the cell. NAD(P)H oxidase, as well as other enzymes, produces superoxide. Superoxide is normally converted to hydrogen peroxide by superoxide dismutase. Hydrogen peroxide is converted to water by catalase and glutathione peroxidase (GPx). In the process, glutathione peroxidase oxidizes glutathione (GSH) to GSSG. Oxidized glutathione (GSSG) is then reduced again to GSH by glutathione reductase. SOD, superoxide dismutase; GPx, glutathione peroxidase; GSH, reduced glutathione; GSSG, oxidized glutathione.

Oxidative stress. There may be sex differences in production and removal of reactive oxygen species that could impact renal injury [18,19]. As shown in Fig. 4, metabolism of reactive oxygen species involves various enzymes. First, superoxide is produced by cyclooxygenase, nitric oxide synthase (discussed later), and various other oxidases. One of the most important oxidases in production of superoxide is NAD(P)H oxidase. NAD(P)H oxidase is an enzyme that produces superoxide when its five subunits assemble at the plasma membrane of cells. Under normal physiological conditions, superoxide is dismutated by superoxide dismutase to hydrogen peroxide. Superoxide can also complex with nitric oxide, another free radical, to produce peroxynitrite, which will be discussed below. Hydrogen peroxide is then hydrolyzed to water by catalase and glutathione peroxidase and reduced glutathione (GSH). Glutathione is oxidized in the process (GSSG). Glutathione reductase then reduces GSSG back to GSH. Glutathione is one of the most important antioxidants in the cell.

The role that nitric oxide synthase plays in oxidative stress should also be mentioned, because it is possible that nitric oxide may play a role in the sex differences in renal function and injury. For the purposes of this chapter, the endothelial nitric oxide synthase will be discussed. However, inducible and neuronal nitric oxide synthases are also present in the kidney and may contribute to the sex difference in renal function and injury. The nitric oxide synthases convert L-arginine to nitric oxide, a free radical, and citrulline. NO is a potent vasoconstrictor and when its synthesis is reduced experimentally, hypertension results. Endothelial nitric oxide synthase requires various cofactors, such as calcium, calmodulin and tetrahydrobiopterin (BH4) (see Fig. 5). Under oxidative conditions in cells, tetrahydrobiopterin can be oxidized to dihydrobiopterin (BH2). Under these conditions, endothelial nitric oxide synthase will produce superoxide, rather than nitric oxide. So under oxidative conditions, levels of NO can be reduced by two methods: NO

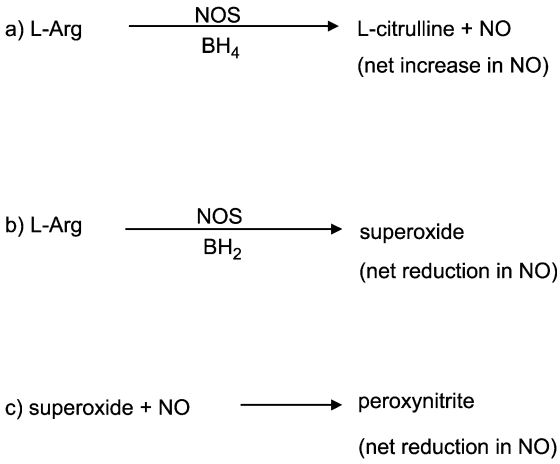

Fig. 5. Nitric oxide (NO) concentrations can be modulated by at least three mechanisms: (a) under normal conditions, nitric oxide synthase converts L-arginine (L-Arg) to L-citrulline and NO. This requires the cofactor tetrahydrobiopterin (BH4). If the cell is in a state of oxidation, tetrahydrobiopterin is oxidized to dihydrobiopterin (BH2). When this occurs, nitric oxide synthase produces superoxide rather than NO, leading to a reduction in NO concentration. NO can also be scavenged by superoxide to produce peroxynitrite. This would result in a reduction in NO that is available to cause vasodilation. L-Arg, L-arginine; NOS, nitric oxide synthase; BH4, tetrahydrobiopterin; NO, nitric oxide; BH2, dihydrobiopterin.

could combine with superoxide to produce peroxynitrite, and NO production could be reduced due to production of superoxide by nitric oxide synthase. Both of these factors could result in an increase in renal vasoconstriction and thereby impact blood pressure and renal function. Finally, NO influences the RAS since a reduction in NO can stimulate renin release [22,26].

Thermodynamically speaking, in the cell the reaction of nitric oxide and superoxide is preferential since the rate of reaction is more rapid than the reaction rate of superoxide and its scavenger, superoxide dismutase [18,19]. When superoxide interacts with nitric oxide, peroxynitrite, one of the most potent oxidative compounds known, is formed [18]. Although peroxynitrite itself is a vasodilator, tachyphylaxis occurs at peroxynitrite concentrations of 3 μM, which is subthreshold as a vasodilator in coronary circulation, and prevents further response not only to its own vasodilator actions, but also causes a long-lasting reduction in response to other vasodilators, such as some prostaglandins and prostacyclin [18,19]. Furthermore, Kooy and Lewis reported that following tachyphylaxis, continued peroxynitrite infusion in rats, increased blood pressure and renal vascular resistance [11]. Therefore, not only will quenching of NO by superoxide increase the vascular tone but also the increase in peroxynitrite could potentiate this effect by causing tachyphylaxis to residual NO.

Peroxynitrite, by virtue of its potent oxidative ability, can produce oxidation of lipids and produce other products that have vasoconstrictive actions (see Fig. 6). As mentioned above, angiotensin II infusion increases F2-isoprostanes in the plasma [18,19]. The F2-isoprostanes, which are prostaglandin F2-like compounds produced by non-enzymatic free radical-induced peroxidation of arachidonic acid, are thought to be

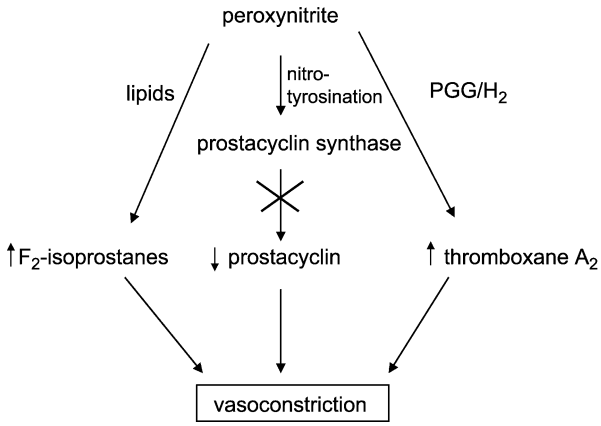

Fig. 6. Mechanisms by which peroxynitrite could cause vasoconstriction. Peroxynitrite can oxidize cellular membrane lipids and arachidonic acid to produce F2-isoprostanes, which are potent renal vasoconstrictors. Peroxynitrite can nitrityrosinate proteins and cause them to become inactive. Peroxynitrite nitrotyrosinates prostacyclin synthase thus reducing the synthesis of vasodilator prostacyclin and causing net vasoconstriction. Peroxynitrite can also produce an increase in thromboxane A2 by increasing prostaglandins G and H2. Thromboxane is also a vasoconstrictor. PGG, prostaglandins G and H.

produced by peroxynitrite [18,19]. Infusion of 8-*iso*-PGF2α (F2-isoprostane) causes renal vasoconstriction with reductions in GFR, mainly by increasing afferent resistance, and at higher doses raises blood pressure [18]. Co-infusion of F2-isoprostane and angiotensin II also potentiate the vasoconstrictor effect of angiotensin II [18].

Peroxynitrite is also capable of reducing concentrations of other vasodilators and increasing vasoconstrictors. Peroxynitrite causes nitrotyrosination of prostacyclin synthase leading to inactivation of the enzyme [27], which will result in a reduction in vasodilator prostacyclin. Peroxynitrite stimulates production of thromboxane A2, another vasoconstrictor. The biological activity of both F2-isoprostanes and thromboxane A2 are mediated via the thromboxane receptor, and testosterone treatment of aortic vascular smooth muscle cells increases thromboxane receptor number [18]. Thus while reactive oxygen species themselves can potentially cause damage to the kidney, the components produced by the oxidative stress cascade may also contribute to renal injury.

Oxidative stress and sex differences in hypertension. Oxidative stress is higher in healthy young men than age-matched women, as measured by higher levels of plasma F2-isoprostanes and thiobarbituric acid reactive substances (TBARS) [18]. Similarly, in a study of men and women, aged 19–78, sex was the only predictor of oxidative stress (plasma F2-isoprostanes), with men having greater oxidative stress than women [18]. When hydrogen peroxide was measured in essential hypertensive individuals, men had higher levels than women, and the level of hydrogen peroxide was positively correlated with PRA and negatively correlated with cardiac contractility and renal function. These data suggest that male sex and the RAS both affect oxidative stress in essential hypertensives. One other possibility is that androgens themselves may cause oxidative stress directly.

RAS, oxidative stress and sex in hypertension. Both supraphysiological and physiological doses of angiotensin II can cause oxidative stress. For example, large

doses of angiotensin II (0.7 mg/kg/d s.c. by minipump) increased blood pressure and superoxide levels in aortic segments of rats, while infusion of norepinephrine, which resulted in a similar increase in blood pressure as angiotensin II, had no effect on superoxide levels [18,19]. Increased superoxide levels could also be normalized by treatment with losartan, an angiotensin II receptor antagonist, or with liposomes containing superoxide dismutase. These data suggested that infusion of angiotensin II at very high doses was capable of inducing oxidative stress independent of elevated blood pressure. It may not be surprising that high doses of angiotensin II could cause oxidative stress since it is such a powerful vasoconstrictor. However, even low doses of angiotensin II (10 ng/kg/min, for 14 days) slowly increased blood pressure and caused increases in plasma F2-isoprostanes [19].

RAS, oxidative stress and sex in renal injury. As mentioned above angiotensin II is capable of causing oxidative stress, and the mechanism is mediated by upregulation of subunits of NADPH oxidase to produce superoxide [19]. Oxidative stress has been shown to activate transcription factors and growth factors, although most studies have been performed in isolated cellular cultures. For example, in cultured renal proximal tubular cells, oxidative stress activated AP-1 and caused synthesis of a heparin-binding epidermal growth factor [21]. Oxidative stress produced by hydrogen peroxide also activates AP-1 in mesangial cells. NF-κB is another nuclear transcription factor implicated in renal injury but mainly in diseases associated with inflammatory processes. For example, NF-κB is upregulated in rat mesangial cells by cytokines, TNF-α and IL-1 [1]. Antioxidants block NF-κB binding in vitro in proximal tubule cells in culture. There have been a few reports in which kidney tissue was evaluated for either AP-1 or NF-κB. For example, NF-κB was shown by Muller and colleagues to be upregulated in double transgenic rats containing human renin and angiotensinogen genes, in which angiotensin II levels are elevated [16]. These investigators found that antioxidants were capable of inhibiting NF-κB binding in heart and kidney, and the life of the rats was prolonged. NF-κB binding has been shown to be upregulated in the kidney with normal aging due to downregulation of IκBα, the endogenous inhibitor of NF-κB. However, it is not clear whether hypertensive renal injury, which is a chronic disease, will be accompanied by sustained increases in transcription factors. Apoptosis may also play a role in the glomerular sclerosis mechanism of hypertensive glomerular injury and oxidant stress is involved in stimulation of apoptosis [9]. Therefore, since androgens and estrogens are capable of controlling the RAS and the RAS produces oxidative stress, it is possible that the effect of sex steroids on renal injury is mediated indirectly via the effects of the RAS on oxidative stress which in turn affects signal transduction.

Schematic diagrams of possible mechanisms responsible for sex differences in hypertension and renal function. The schematic diagram in Fig. 7 is a composite of the hypotheses by which sex differences in hypertension could be mediated. Androgens could promote an increase in blood pressure in males by stimulating renin activity and increasing angiotensin II formation. This would directly stimulate sodium reabsorption, blunt pressure–natriuresis and increase blood pressure. Androgens may stimulate renin release by reducing GFR, directly stimulating sodium reabsorption and thus decreasing delivery of sodium to the macula densa. Alternatively, renin activity (and thus angiotensin II) could also be increased if androgens cause a chronic increase in renal angiotensinogen and renin

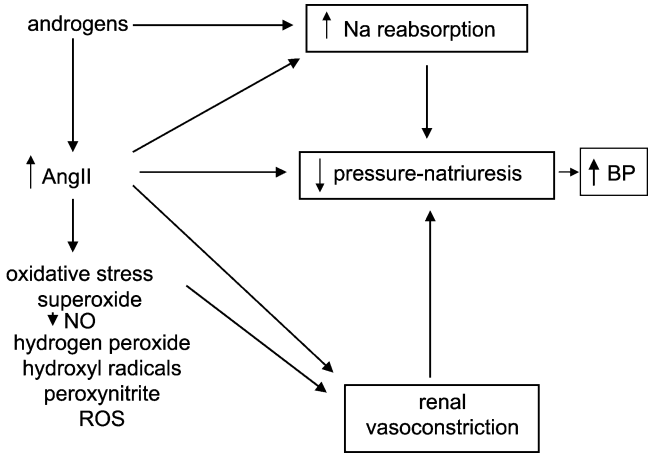

Fig. 7. Schematic diagram showing the possible interactions between androgens, angiotensin II (Ang II) and oxidative stress to cause an increase in blood pressure. As previously shown in Fig. 2, androgens could either directly or indirectly (via Ang II) increase sodium reabsorption. Ang II would directly increase oxidative stress with increases in superoxide, hydrogen peroxide hydroxyl radicals and other reactive oxygen species (ROS). Superoxide would combine with NO to produce peroxynitrite thus causing a net reduction in bioavailability of NO to cause vasodilation. Peroxynitrite could also increase other vasoconstrictors and decrease other vasodilators via its action on plasma lipids, prostaglandins and enzymes. The combination of an increase in sodium reabsorption and renal vasoconstriction would both shift the pressure–natriuresis relationship to the right and cause a sustained increase in blood pressure.

enzyme is working below its V_{max}. Androgens may affect the number and affinity of receptors for angiotensin II affecting sodium reabsorption and/or renal vasoconstriction. In addition, the increase in angiotensin II will cause renal vasoconstriction and may cause an increase in aldosterone, which would increase distal nephron sodium reabsorption. Angiotensin II would also cause an increase in superoxide. With an increase in superoxide, quenching of NO would occur, which also leads to vasoconstriction. Renin release could be further stimulated by the superoxide-mediated reduction in NO. The combination of superoxide and NO produces peroxynitrite. Peroxynitrite oxidizes arachidonic acid to produce renal vasoconstrictor F2-isoprostanes. F2-isoprostanes, mediated by thromboxane receptors, which may themselves be upregulated by androgens, cause vasoconstriction directly, but also indirectly by potentiating the vasoconstrictor actions of angiotensin II.

Peroxynitrite could also inhibit prostacyclin synthesis and increase thromboxane A2 synthesis. Changes in both of these eicosanoids would cause renal vasoconstriction. The combination of increased sodium reabsorption and renal vasoconstriction will shift the pressure–natriuresis relationship to the right and increase blood pressure.

Fig. 8 describes the mechanisms by which sex differences in renal function and hypertensive renal injury could occur. Androgens could promote renal injury by both hemodynamic and non-hemodynamic mechanisms primarily mediated via angiotensin II. One possible scenario is that of a hemodynamic mechanism in which androgens themselves or androgen-mediated increases in angiotensin II cause an increase in proximal tubule sodium reabsorption, leading to a reduction in sodium reaching the macula densa.

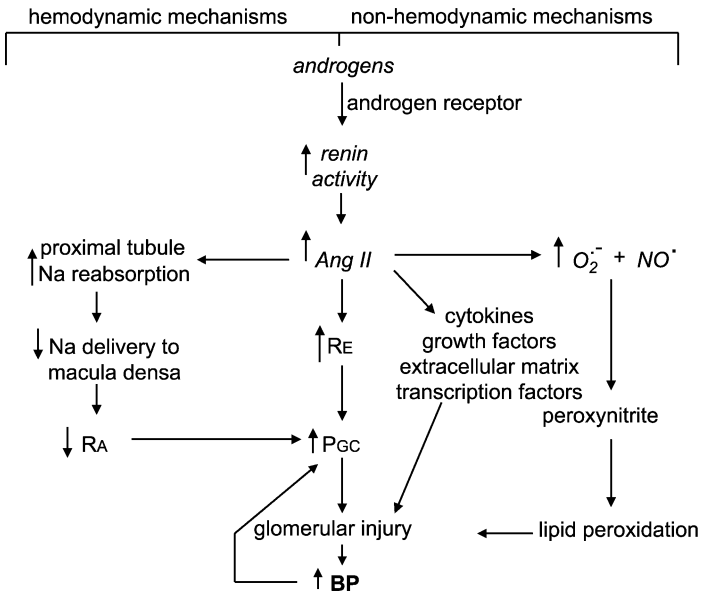

Fig. 8. Schematic diagram showing hemodynamic and non-hemodynamic mechanisms by which androgens, angiotensin II (Ang II) and oxidative stress could interact to cause renal injury. Via a hemodynamic mechanism, androgens could increase Ang II, causing an increase in proximal sodium reabsorption, which would reduce the sodium delivery to the macula densa. By tubular glomerular feedback, the reduction in sodium would be sensed and cause a reduction in afferent arteriolar resistance in an attempt to increase GFR, but would also increase glomerular capillary pressure (PGC). An increase in Ang II would also directly increase efferent arteriolar resistance, leading to further increase in PGC and decrease in GFR. An increase in PGC causes glomerular injury. By non-hemodynamic mechanisms, androgens could again increase Ang II that would cause an upregulation of inflammatory cytokines, growth factors and transcription factors leading to increases in extracellular matrix in the mesangium and fibrosis and glomerular sclerosis. Ang II could also stimulate synthesis of superoxide, which would quench NO leading to an increase in peroxynitrite. Peroxynitrite would oxidize plasma lipids, leading to lipid peroxidation and glomerular capillary injury. Glomerular injury results in increases in blood pressure. Increases in blood pressure would cause further increases in glomerular capillary pressure and renal injury, setting up a vicious cycle. Ang II, angiotensin II; RA, afferent arteriolar resistance; RE, efferent arteriolar resistance; PGC, glomerular capillary pressure; $O_2^{\cdot-}$, superoxide; NO, nitric oxide.

By the tubular glomerular feedback mechanism, this reduction in sodium at the macula densa would lead to a decrease in afferent resistance, allowing the higher blood pressure in males to be transduced to the glomerulus, increasing glomerular capillary pressure and causing glomerular injury. Angiotensin II would also increase glomerular capillary pressure further by increasing efferent resistance. It is possible that androgen-mediated increases in angiotensin II cause an increase in cytokines and an increase in the components responsible for synthesis of extracellular matrix, which would cause renal injury. This would be an example of a non-hemodynamic mechanism. Another non-hemodynamic mechanism would be if androgen-mediated increases in angiotensin II cause oxidative stress and produce superoxide that would combine with NO to produce peroxynitrite which would cause lipid peroxidation and glomerular injury. Further oxidative stress has been shown to

cause upregulation of transcription factors, such as AP-1 and NF-κB, that would also lead to glomerular injury. Glomerular injury, of course, would result in an increase in blood pressure which would lead to further increases in glomerular capillary pressure.

2. Future directions

There have been many hypotheses put forth in this chapter regarding why the kidneys of men cause hypertension and develop renal disease more frequently than women. Needless to say, much research is needed to determine if these hypotheses are correct. Just a few of the questions remaining to be answered are

1. Do sex hormones have a direct effect on sodium reabsorption by the kidney? It is well known that androgens can stimulate angiotensin II to increase sodium reabsorption, but whether androgens cause sodium reabsorption and estrogens produce sodium loss is not known.
2. What are the differences between the two models of hypertension, the SHR and the Dahl salt-sensitive rat, that causes estrogen to be protective of renal function in the Dahl rats and loss of estrogen to be protective in the SHR? There are numerous studies, mainly in vitro, that show that estrogen is a vasodilator, an antioxidant, and an anticarcinogenic. Why then does HT not protect women from cardiovascular aging? By studying the female SHR and Dahl salt-sensitive rats, it may be possible to determine the differences in the roles of ovarian hormones in controlling blood pressure and with subsequent study of these mechanisms in women to possibly identify a subset that would benefit from HT.
3. Do sex hormones, by virtue of their actions as transcription factors, upregulate or inhibit upregulation of proteins known to be deleterious to the kidney, such as certain cytokines? Little is known about the role of sex steroids in signal transduction, although estradiol has been studied more extensively than androgens.
4. Do sex hormones directly cause or protect against oxidative stress in the kidney or is the effect of sex hormones on oxidative stress due to the interaction of sex hormones and the RAS?

These seem to be straight-forward questions, but the answers are difficult to obtain and will keep many investigators working for years.

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Influence of sex hormones on the neuromuscular junction

Gary C. Sieck and Carlos B. Mantilla

1. Introduction

The neuromuscular junction is the final element in the neural control of skeletal muscle contraction. As such, the neuromuscular junction serves as the gateway for the bi-directional communication between motoneurons and muscle fibers. A key feature of neuromotor control is the functional match that exists between the properties of a motoneuron and the muscle fibers it innervates. Certainly, it is obvious that there are sex differences in muscle mass and performance. However, there has been surprisingly little attention paid to sex differences in neuromuscular transmission. Most studies have focused on the influence of sex hormones, and even these are primarily limited to the influence of anabolic–androgenic steroids (e.g. testosterone propionate, testosterone acetate or dihydro-epiandosterone). A review of the existing literature reveals few studies exploring the presence of estrogen and/or progesterone receptors at the neuromuscular junction or effects of ovarian steroids on neuromuscular transmission. Those studies that have been performed are inconclusive; thus, this represents a major deficit in our understanding of sex-based differences in the physiology of neuromuscular transmission.

Most studies examining the effects of anabolic–androgenic steroids on the neuromuscular junction have focused primarily on sexually dimorphic muscles (e.g. the levator ani and bulbocavernosus), which develop as a result of the influence of androgenic steroids during embryonic and early postnatal development. Anabolic–androgenic steroids also influence the maturation of the specific motoneurons that innervate sexually dimorphic muscles. Since trophic interactions are bi-directional between motoneurons and muscle fibers, it is unclear whether the effects of anabolic–androgenic steroids are exerted on muscle fibers, motoneurons or both. There is a vast literature on sexually dimorphic muscles and their innervation that has been extrapolated to other skeletal muscles present in both sexes, but involved in sexually dimorphic behaviors associated with mating. For example, studies of forelimb muscles of male bullfrogs that grow in size and strength during the mating season to facilitate prolonged grasping of the female during intercourse and skeletal muscles involved in avian song

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behavior specialized for courtship. However, there is also a pervasive literature on the general influence of sex hormones on skeletal muscles, and the impact of sex hormones on non-sexual motor function. During major athletic events such as the Olympics, we all become more aware of the impact of sex hormones when athletes misuse anabolic–androgenic steroids to enhance athletic performance. Yet, the impact of sex hormones is not only restricted to abuse, but also can shape and define overall sex differences in neuromotor control. The following chapter explores the impact of anabolic–androgenic steroids on pre- and postsynaptic elements of the neuromuscular junction.

2. The neuromuscular junction

As mentioned above, the neuromuscular junction is the connection between motoneurons and muscle fibers; thus, neuromuscular junctions have pre- and postsynaptic elements, which are physically part of motoneurons and muscle fibers, respectively. Together a motoneuron and the muscle fibers it innervates comprise a motor unit, which is the final common output of neuromotor control of skeletal muscles. The impact of anabolic–androgenic steroids on the neuromuscular junction can reflect influences on either motoneurons and/or muscle fibers. Such influences can alter the transmission of neural signals to muscle fibers affecting force generation and fatigue. Since motor units vary in their physiological properties, the influence of anabolic–androgenic steroids may depend on motor unit type. Thus, to better understand the impact of anabolic–androgenic steroids on the neuromuscular junction it is necessary to first review motor unit physiology.

Both motoneurons and skeletal muscle fibers express androgen receptors [9,19]; therefore, it is reasonable to suspect an influence of anabolic–androgenic steroids on both pre- and postsynaptic elements of the neuromuscular junction and on neuromuscular transmission. As mentioned above, very little attention has been paid to the presence of estrogen and/or progesterone receptors at the neuromuscular junction or to the influence of ovarian steroids on neuromuscular transmission.

Maintenance of synaptic efficacy may be a driving force behind neuromuscular junction remodeling under any condition, including alterations in sex hormone levels. Plasticity at the neuromuscular junction induced by anabolic–androgenic steroids may reflect direct effects on pre- and/or postsynaptic elements via androgen receptors, or such effects may be indirect by affecting motoneuron activity and/or the expression of other trophic influences. At the presynaptic terminal, anabolic–androgenic steroids may directly or indirectly influence the regulation of synaptic vesicle release, vesicle cycling and/or quantal content of neurotransmitter. Postsynaptically, anabolic–androgenic steroids may directly or indirectly influence cholinergic receptor density and/or the open probability of receptors.

3. Differences in neuromuscular junctions across motor unit types

Each motor unit comprises a motoneuron and the group of muscle fibers it innervates. Motor units exhibit great diversity in their mechanical, energetic and fatigue properties,

and the types of motor units in a skeletal muscle are critically important in determining the overall functional capacity of the muscle in accomplishing specific motor behaviors. Structural and functional diversity is evident at each level of the motor unit, including motoneurons, neuromuscular junctions and muscle fibers [8].

Motor units are categorized into four types based on mechanical and fatigue properties of muscle fibers: (1) slow-twitch, fatigue resistant (type S), (2) fast-twitch, fatigue resistant (type FR), (3) fast-twitch, fatigue-intermediate (type FInt) and (4) fast-twitch, fatigable (type FF) [8] (Fig. 1). Correspondingly, muscle fibers are also classified into four types, whether based on histochemical staining patterns or myosin heavy chain (MHC) isoform composition: (1) type I (MHC_{Slow}), (2) type IIa (MHC_{2A}), (3) type IIb (MHC_{2B}), and (4) type IIx (MHC_{2X}) [20].

Among motoneurons innervating a given muscle, there can be considerable heterogeneity in somal and dendritic morphology, which contributes to differences in motoneuron electrophysiological properties and motor unit recruitment. Intrinsic electrophysiological properties of motoneurons depend on motoneuron size and motor unit type such that type S motor units generally have motoneurons with the highest input resistance, lowest rheobase and slowest axonal conduction velocities. In contrast, type FF

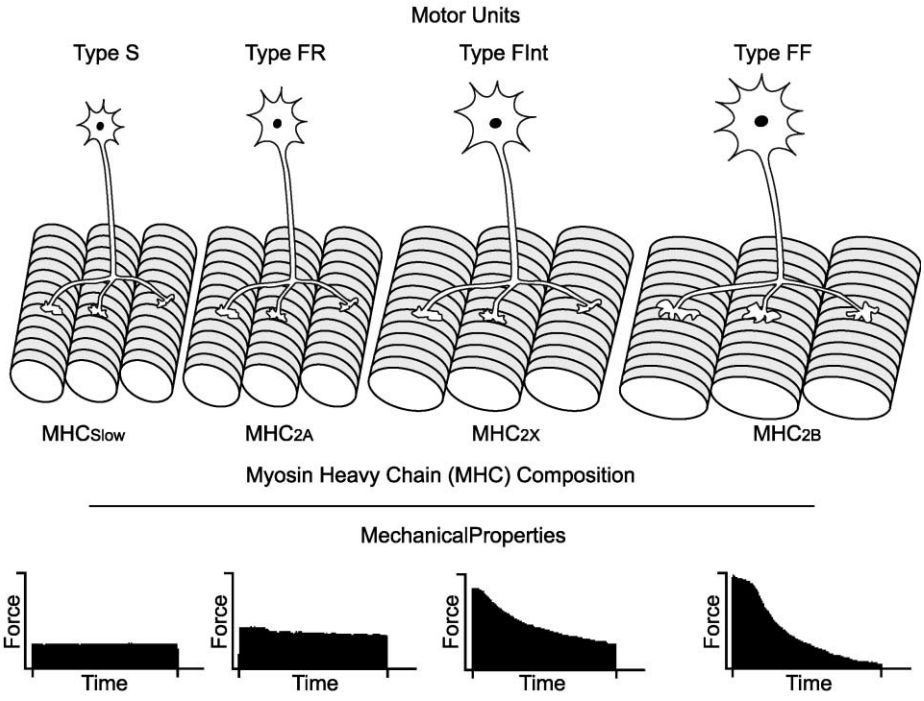

Fig. 1. Motor unit classification is based on mechanical and fatigue properties of muscle fibers (slow-twitch – type S, fast-twitch fatigue resistant – type FR, fast-twitch fatigue intermediate – type FInt, and fast-twitch fatigable – type FF motor units). Muscle fibers comprising motor units display consistent myosin heavy chain (MHC) isoform expression (type S – MHC_{Slow}, type FR – MHC_{2A}, type FInt – MHC_{2X} and type FF – MHC_{2B}).

motor units have motoneurons that are the largest (lowest input resistance) and least excitable (highest rheobase) and display the fastest axonal conduction velocities [8].

Sherrington [15] first proposed that force generation by skeletal muscles is achieved through the orderly recruitment of motor units. The Size Principle proposed by Henneman [10] correlated the recruitment of motor units with the size-related intrinsic electrophysiological properties of motoneurons. Accordingly, smaller motoneurons with smaller axons and slower conduction velocities have lower membrane capacitance, higher input resistance and lower rheobase, and thus are recruited first for a given level of synaptic input. Henneman's "size-principle" is also consistent with the recruitment order of different motor unit types, such that type S and FR motor units are recruited first while type FInt and FF motor units are recruited later [8].

Neuromuscular junctions also exhibit marked differences in structure and function across motor unit types (Fig. 2) [18]. For example, neuromuscular junctions at type IIx and IIb fibers are larger and far more structurally complex at both pre- and postsynaptic elements compared to neuromuscular junctions at type I and IIa muscle fibers [18]. Other differences also exist across neuromuscular junctions of different motor unit types. For example, at type IIx and IIb muscle fibers, presynaptic terminals contain more mitochondria than at type I and IIa fibers. Postsynaptic folding is also more complex at type IIx and IIb fiber neuromuscular junctions. At type I and IIa muscle fibers, mitochondria, rough endoplasmic reticulum, and myonuclei are interposed between the motor endplate and the contractile apparatus.

These morphological differences across motor unit neuromuscular junctions are reflected by differences in electrophysiological properties of neuromuscular transmission.

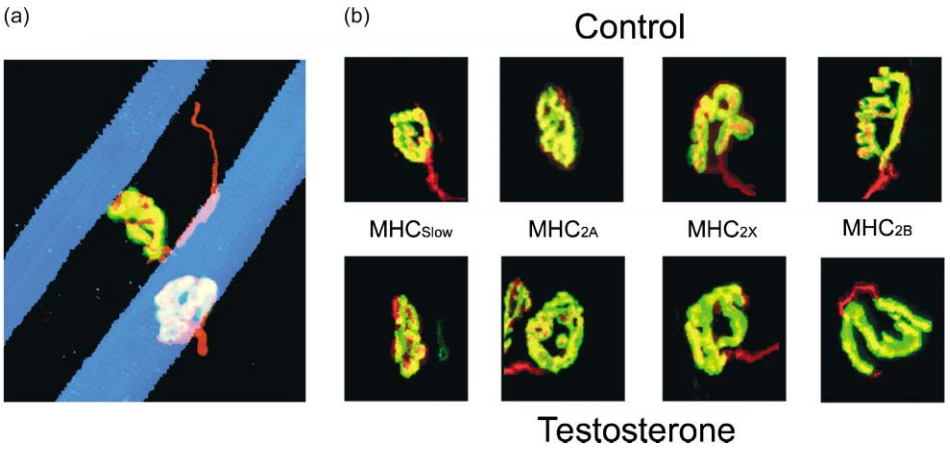

Fig. 2. Morphological differences in neuromuscular junctions at type-identified motor units in the adult rat diaphragm muscle. (a) Triple-labeled confocal image of pre- and postsynaptic elements of neuromuscular junctions (axon nerve terminal shown in red, cholinergic receptors shown in green and MHC_{Slow} isoform expression shown in blue). (b) Note differences in complexity, size and extent of overlap across motor unit types. Also note that treatment with testosterone propionate for four weeks resulted in expansion of neuromuscular junctions at fast motor unit types.

For example, excitatory post-synaptic potential amplitude is larger at type I and IIa muscle fibers [8]. Differences in the safety factor for neuromuscular transmission (defined as the ratio of endplate potential amplitude to muscle fiber activation threshold) also exist across motor unit types [11]. The safety factor for neuromuscular transmission at rat soleus muscle fibers (predominantly type I fibers) is lower than that for type IIb fibers of the rat extensor digitorum longus muscle. However, with repetitive stimulation the safety factor for neuromuscular transmission remains more stable at type I fibers of the soleus compared to type IIb fibers of the extensor digitorum longus muscle. Accordingly, neuromuscular transmission failure depends on the rate of nerve stimulation, and varies across motor unit types, such that FF motor units are far more susceptible to neuromuscular transmission failure compared to type S or FR motor units [12].

It appears that motoneurons exert a predominant influence on the contractile and metabolic properties of the muscle fibers they innervate [7,8]. When innervation of fast and slow muscles is switched, the reinnervated muscle assumes the properties of the newly established innervation, i.e. fast muscles become slow and conversely slow muscles become fast [7]. Within a motor unit, all muscle fibers display similar contractile protein expression and similar metabolic enzyme properties [20]. Not surprisingly, denervation markedly affects muscle fiber properties, which also support a predominant influence of motoneurons. However, as with cross-innervation and motor unit studies, the effects of denervation do not distinguish whether the influence of motoneurons derives from the pattern of activity, which varies across motor unit types, or specific motoneuron-derived trophic influences. It is possible that the post-denervation muscle fiber phenotype may depend on the embryological lineage of muscle fibers, reflecting genetically determined patterns of motor unit differentiation.

4. Influence of androgen hormones on motoneurons and presynaptic elements of the neuromuscular junction

The effect of sex hormones on the motoneuron and presynaptic elements of the neuromuscular junction may be direct, acting through androgen receptors, or indirect, through an effect on motoneuron activity, transmitter production or release, and/or expression of other trophic factors (Fig. 3).

Motoneuron morphology and function. The direct influence of anabolic–androgenic steroids on the morphology and function of motoneurons is poorly understood. Clearly, at sexually dimorphic muscles, the influence of sex hormones on motoneuron survival is evident, but this effect may be indirect, reflecting complex interactions between motoneurons and muscle fibers. For example, anabolic–androgenic steroids may influence the expression of trophic factors produced by the motoneuron and/or muscle fibers that in turn determine motoneuron survival. Since both motoneurons and muscle fibers are potential targets of anabolic–androgenic steroids, an influence on one will affect the other indirectly as a result of neuron-target cell interactions. Therefore, an androgenic effect at the motoneuron (either direct or indirect) will indirectly influence muscle fiber development and conversely an androgenic effect on muscle fibers will indirectly affect motoneurons. Such complex interactions are difficult to dissect experimentally. However,

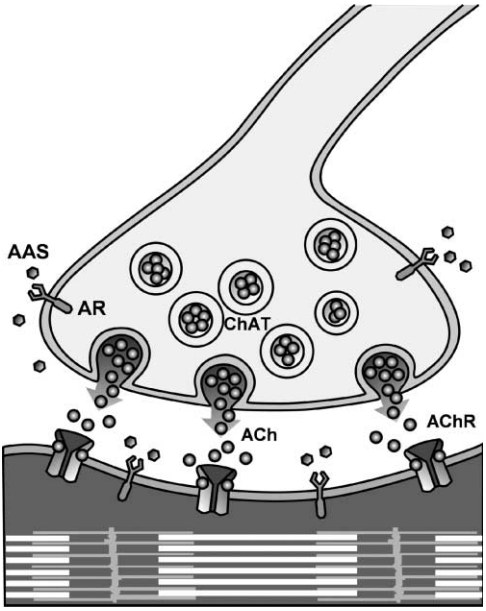

Fig. 3. Schematic demonstrating possible pre- and postsynaptic targets of anabolic–androgenic steroids (AAS) on synaptic efficacy. AAS may activate presynaptic androgen receptors (AR) and modulate neurotransmitter (acetylcholine – ACh) release and/or synthesis via choline acetyl transferase (ChAT). AAS may also exert postsynaptic effects including alterations in ACh receptor (AChR) number and function, as well as effects on contractile protein expression. All of these effects may be fiber type specific.

any effect on motoneuron morphology will also affect recruitment and thus activity patterns, which may also indirectly affect neuromuscular junction structure and function.

The effects of androgenic steroids on motor units may depend on the age and specific muscle groups examined. For example, during development, androgens regulate survival of motoneurons of sexually dimorphic muscles such as the bulbocavernosus muscle by acting on muscle fibers, rather than motoneurons themselves, since only muscle fibers express androgen receptors at this time [6]. However, in the adult rat, motoneurons innervating the bulbocavernosus muscle also contain androgen receptors, which locally mediate androgen effects on somal size [25]. Exogenous testosterone treatment increases bulbocavernosus motoneuron somal size, whereas castration decreases motoneuron size, an effect that is reversible by testosterone treatment. Conversely, in the adult rat, androgen effects on bulbocavernosus muscle fibers mediate upstream effects on the length of motoneuron dendrites. In the adult rat, denervation of the bulbocavernosus muscle results in a downregulation of androgen receptors at motoneurons, which is restored only by reinnervation of muscle fibers [16]. These results clearly illustrate that complex interactions between motoneurons and muscle fiber target cells can influence anabolic–androgenic steroid effects. Whether anabolic–androgenic steroids also exert effects on

motoneuron-target cell interactions in non-sexually dimorphic motor systems is currently unknown. However, as mentioned above, androgen receptors are found at both muscle fibers and motoneurons of several non-sexually dimorphic muscles. Therefore, such an effect would not be unexpected (see also Chapter 14).

Synthesis of neurotransmitters. Anabolic–androgenic steroids exert trophic influences on motoneurons that resemble those of other trophic factors, such as neurotrophins, cytokines and transforming-growth factors. There is evidence for interactions between these trophic substances, which may complicate interpretation of the direct actions of anabolic–androgenic steroids on motoneuron survival and plasticity. Interactions may occur through changes in the expression of receptors for anabolic steroids and/or other trophic factors. In addition, downstream interactions between the signaling cascades initiated by trophic substances and anabolic steroids may occur. These potential interactions are difficult to dissect experimentally.

Anabolic–androgenic steroids also influence neurotransmitter synthesis in motoneurons, and thus may regulate transmitter release at the presynaptic terminal and neuromuscular transmission. Exogenous treatment with testosterone propionate in adult male rats increases the expression of choline acetyltransferase (ChAT) mRNA in motoneurons throughout the spinal cord [3]. Assuming that the levels of ChAT mRNA in motoneurons reflect greater ChAT protein levels at the presynaptic terminal, the capacity to synthesize acetylcholine (ACh) would increase following testosterone treatment. In agreement, it has been shown that ChAT activity in the levator ani muscle of adult male rats (comprising predominantly type IIb fibers) corresponds with serum testosterone levels and parallels changes in motoneuron ChAT mRNA expression. In the soleus muscle, ChAT activity is reduced following castration of male rats, but is unaffected by testosterone treatment. It is possible that the effects of anabolic–androgenic steroids on motoneuron ChAT mRNA expression and presynaptic ChAT activity depend on motor unit type, but this possibility has never been explored. Alternatively, it is also possible that anabolic–androgenic steroids influence ChAT mRNA expression and presynaptic ChAT activity selectively in sexually dimorphic muscles, but this seems unlikely.

Given the motoneuron-target cell interactions discussed above, an influence of anabolic–androgenic steroids on the morphology of neuromuscular junctions at sexually dimorphic muscles is to be expected. Several studies have demonstrated such effects. For example, following castration or testosterone treatment, the bulbocavernosus muscle either atrophies or hypertrophies, and neuromuscular junction morphology changes accordingly [1]. These observations do not clearly demonstrate a direct cause and effect relationship between androgenic steroids and neuromuscular junction structure. In the rat diaphragm muscle, which is not sexually dimorphic, testosterone treatment results in an expansion of pre- and postsynaptic elements of neuromuscular junctions at fibers expressing MHC_{2A}, MHC_{2X}, and MHC_{2B} isoforms without changes in muscle fiber size. In addition, the extent of overlap between pre- and postsynaptic elements of neuromuscular junctions improves at fibers expressing MHC_{2X} and MHC_{2B} isoforms following testosterone treatment. These testosterone-induced changes in neuromuscular junction morphology are accompanied by an apparent improvement in neuromuscular transmission. Susceptibility to neuromuscular transmission failure during repeated stimulation is lessened following testosterone treatment [4] (Fig. 4).

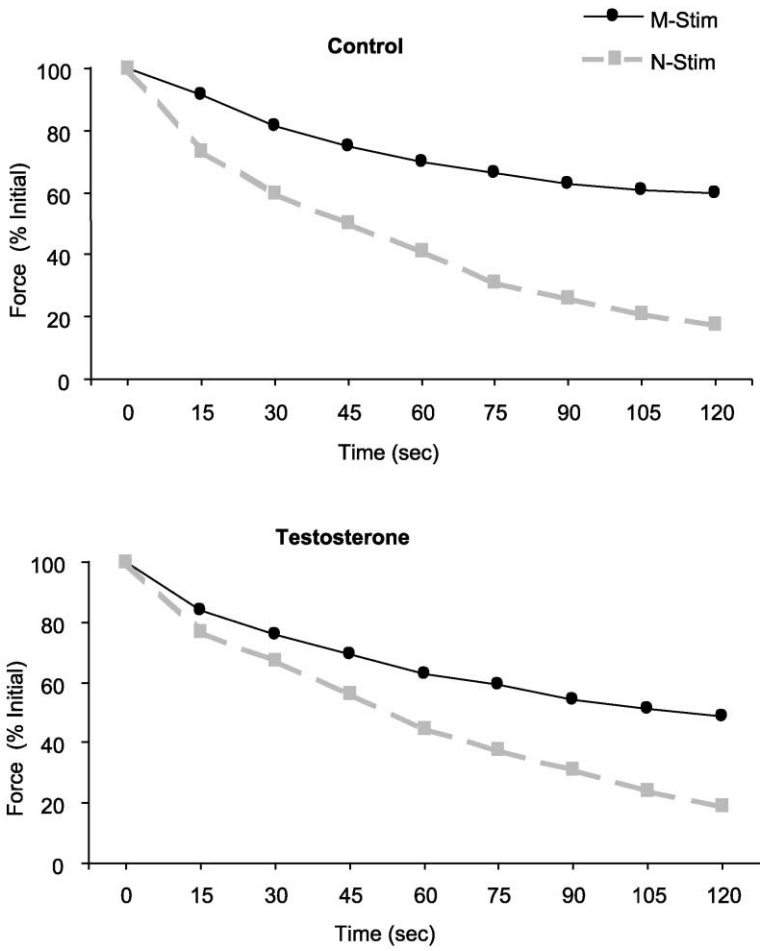

Fig. 4. Effects of testosterone treatment on neuromuscular transmission failure in adult rat diaphragm muscle. Neuromuscular transmission failure was estimated by comparing isometric force elicited by repetitive phrenic nerve stimulation (N; 40 Hz in 330 ms duration trains repeated each for 2 min) vs. direct muscle stimulation (M) superimposed every 15 s. The difference between force generated by nerve (N) vs. direct muscle (M) stimulation was reduced following four weeks of testosterone propionate treatment indicating improved neuromuscular transmission.

The effect of anabolic–androgenic steroids on presynaptic vesicle cycling and release is currently unknown. In sexually dimorphic muscles such as the bulbocavernosus muscle of the rat, there is some evidence that suggests an increase in miniature end-plate potential amplitude with no change in quantal content as evidenced by unaltered end-plate potential amplitude [21]. However, no specific measurements have been performed of the readily releasable or reserve pools of synaptic vesicles at the presynaptic terminal of neuromuscular junctions, either following exogenous treatment with anabolic–androgenic steroids or following castration. As described above, the increase in ChAT

following anabolic–androgenic steroid treatment would suggest an increase in neurotransmitter content, but how this is reflected at the presynaptic terminal remains to be thoroughly explored. At the present time, there is no clear picture of the role of anabolic–androgenic steroids in regulating the presynaptic contribution to neuromuscular transmission.

5. Influence of androgenic hormones on muscle fibers and postsynaptic elements of the neuromuscular junction

Influences of sex hormones on muscle fibers and postsynaptic elements of the neuromuscular junction may be direct or indirect, affecting the expression of cholinergic receptors, the size of muscle fibers (and therefore their excitability), and/or factors influencing excitation–contraction coupling. Obviously, anabolic–androgenic steroids exert a trophic influence on the size of sexually dimorphic muscle fibers, which may affect their excitability via indirect effects on intrinsic electrophysiological properties. However, there is no information available concerning the effects of anabolic–androgenic steroids on factors that influence skeletal muscle membrane excitability (e.g. Na^+ and K^+ ion channel density) or excitation–contraction coupling (e.g. dihydropyridine receptor channels, sarcoplasmic reticulum volume density, ryanodine receptor channel expression).

The effect of anabolic–androgenic steroids on non-sexually dimorphic muscles is even less clear. In combination with resistance training, the trophic effect of anabolic–androgenic steroids on non-sexually dimorphic muscles is enhanced [23]. However, there are a number of studies indicating minimal effects [24]. There also have been a number of studies that have explored effects of anabolic–androgenic steroids on the expression of contractile properties and muscle fiber type. For example, nandrolone treatment induced hypertrophy of diaphragm muscle fibers and improved isometric and isotonic contractile function, especially in type IIx fibers (expressing MHC_{2X}) [14]. In sexually dimorphic muscles, anabolic–androgenic steroids are necessary for expression of fast phenotypes; and, in fact, there may be a critical window during early development during which anabolic–androgenic steroid influences determine muscle phenotype [13]. This is not the case for non-sexually dimorphic muscles. Yet, the effect of anabolic–androgenic steroids on contractile protein expression in non-sexually dimorphic muscles may also be more selective with respect to fiber type without frank phenotypic transition [17]. Regardless, a fiber type-specific effect may be important, since, as described above, the morphology and physiology of neuromuscular junctions vary considerably across fiber types (Fig. 2).

There is evidence that in sexually dimorphic muscles anabolic–androgenic steroids influence the density of nicotinic acetylcholine receptors at the motor endplate [5]. Such an effect would explain the changes in miniature endplate potential amplitude observed in castrated vs. testosterone-treated sexually dimorphic muscles of adult rats [21]. There may also be an effect of anabolic–androgenic steroids on the channel kinetics of nicotinic acetylcholine receptors [2]. Together these results would suggest that anabolic–androgenic steroids enhance the postsynaptic response to neurotransmitter release and thus improve neuromuscular transmission. Whether such effects are present in non-sexually dimorphic

muscles remains unexplored. However, as mentioned above, there is evidence that testosterone treatment results in expansion of motor endplates at type IIx and IIb muscle fibers of the rat diaphragm muscle. In addition, neuromuscular transmission is improved following testosterone treatment of the diaphragm muscle [4].

6. Androgens and muscle fatigue

Muscle fatigue may relate to neuromuscular transmission failure, failure in excitation–contraction coupling or inadequate metabolic support of contraction. A number of studies have reported an improvement in muscle fatigue resistance following anabolic–androgenic steroid treatment [22]. Several potential mechanisms may underlie such an improvement in fatigue resistance, including effects on fiber type. In this respect, the observation of a relatively selective trophic effect of anabolic–androgenic steroids on fast fiber types would seem paradoxical, because these fibers as a group tend to be more fatigable. In the rat diaphragm muscle, fibers expressing MHC_{2X} (type IIx fibers) are the most susceptible to neuromuscular transmission failure and this accounts for a substantial portion of the overall susceptibility of the diaphragm muscle to fatigue during repeated nerve stimulation. Following testosterone treatment, susceptibility to neuromuscular transmission failure is greatly reduced in these fibers, lowering their overall fatigability [4] (Fig. 4). Similar effects of testosterone treatment were observed for diaphragm muscle fibers expressing MHC_{Slow} and MHC_{2A}, but to a much lesser extent. These results are consistent with phenotypic selectivity in the effect of anabolic–androgenic steroids at the neuromuscular junction. However, the mechanisms underlying this selectivity are unknown. Among several possibilities are potential differences in androgen receptor density across fiber types or differences in the effect of anabolic–androgenic steroids on the signaling pathways involved in cholinergic receptor expression.

7. Summary

Motor units in mammalian skeletal muscle are classified based on metabolic and functional properties. The neuromuscular junctions at slow and fast fiber types display remarkable differences in structure and synaptic efficacy. Androgen receptors are present in both motoneurons and muscle fibers, although expression may vary during development. The effects of anabolic–androgenic steroids have been well-characterized in sexually dimorphic muscles. Anabolic–androgenic steroids are clearly necessary for the survival of motoneurons of sexually dimorphic muscles such as the rat bulbocavernosus muscle. Exogenous treatment with anabolic–androgenic steroids can lead to improved synaptic efficacy at neuromuscular junctions by facilitating synaptic vesicle cycling, increasing neurotransmitter synthesis and/or increasing ACh receptor expression. Concurrently, anabolic–androgenic steroids can lead to muscle fiber hypertrophy, which if anything would decrease efficacy of neuromuscular transmission. All of these effects show motor unit type specificity and are enhanced by concomitant resistance training in non-sexually dimorphic muscles.

8. Future directions

The presence of estrogen and/or progesterone receptors and the possible influence of ovarian steroids on neuromuscular junction structure and function should be thoroughly explored in future studies. This may underlie sex differences in neuromuscular transmission that are likely to be present yet have been poorly characterized. Similarly, the influence of anabolic–androgenic steroids on synaptic efficacy at neuromuscular junctions of non-sexually dimorphic muscles needs additional study. Since androgen receptors are present both at motoneurons and muscle fibers, the relative impact of anabolic–androgenic steroids at these two targets needs to be elucidated. In this respect, motor unit specific effects of anabolic–androgenic steroids in mixed muscles also deserve further exploration. Interactions of sex hormones with aging, genetic sex, exercise and environmental conditions (e.g. high altitude, microgravity, oxidative stress) could also be explored in depth. A number of new techniques and experimental tools are now available to address these important issues in sex-based physiology.

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Sex and hormonal influences on skeletal muscle; differentiation and contractile mechanisms

Marybeth Brown

1. Introduction

Asking the question of whether there are sex differences in skeletal muscle seems at first glance to be almost counter-intuitive. Obviously, men have more muscle mass and are stronger than women but in reality essential questions related to muscle function have not been well addressed. Only in the last decade has it occurred to investigators to examine men and women simultaneously in clinical trials. Consequently, there is a remarkable dearth of knowledge concerning appropriate methods to assess muscle function, treat muscle diseases, optimally train athletes, and design treatment programs for patients.

It is entirely probable that there are subtle differences between men and women that at first glance seem irrelevant. With the advent of newer techniques to study skeletal muscle and a heightened awareness of the need to identify sex differences in other systems (e.g. cardiovascular), there is a high likelihood that sex differences in skeletal muscle will be recognized as well. Some important male/female distinctions have been identified but there is a great deal of controversy concerning a number of issues. It is the goal of this chapter to highlight what is known about sex differences in skeletal muscle function throughout the life span.

2. Skeletal muscle in the young

Until puberty, muscle mass is essentially the same for boys and girls, when expressed per unit of height. An example of a study demonstrating this point was performed by Kanehisa et al. [8] who utilized B-mode ultrasound to examine cross-sectional areas of appendicular muscle in 245 males and 275 females ranging in age from 6 to 60 years. Their findings indicate that up to the age of 13 years the differences between sexes were non-existent. Thereafter, the average value for females as a percentage of that for males, was 67% for forearm muscle mass, 55% for upper arm, 77% for lower leg and 73% in the thigh. These values are uncorrected for height or body mass but even when muscle mass is

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corrected for height and weight, similar findings are observed. When boys enter puberty there is a tendency for upper body musculature to increase to a larger extent than lower body mass.

Longitudinal studies of monozygotic twins indicate that a different pattern of genetic expression for muscle growth is present in boys vs. girls. Multivariate analyses of circumference measures of the upper arm, forearm, calf, and thigh in the twins reveal that mass changes with puberty in boys are different from those in girls. For example, in girls 10–13 years of age, two common genetic factors governing the changes in muscle mass at upper and lower extremity sites were apparent. For boys between 10 and 12 years, only one genetic factor governed the proportion of muscle mass at each of the four sites. Thus, current findings suggest that circumference changes with growth and maturation are genetically determined. Moreover, genetic influence accounts for ~95% of the variance in each phenotype although the genetic architecture underlying the variation in limb circumference changes is not identical in both genders. It is tempting to ascribe the changes in boys and girls with puberty to purely genetic expression, however, environmental influences likely have some impact but few of the potential environmental influences have been systematically studied: e.g. nutrient density, physical activity. Gene mapping will be required to validate sex-specific changes in muscle mass with maturation.

Unquestionably, the large increase in testosterone in boys has a huge influence on promoting an increase in muscle mass, primarily through the IGF-1 pathway. Less clear is the role of estrogen as a mediator of change in muscle quantity or quality during maturation in girls. When maturing girls and boys are evaluated with regard to muscle performance, clear differences emerge. Boys steadily improve their standing broad jump performance between the ages of 10 and 17 with a plateauing of performance noted between 17 and 18 years. In girls, there is a distinct improvement in broad jump between 10 and 13 years with a smaller increase up to 16 years. These changes in performance are tied to the increase in muscle mass and strength gain that occurs during that time but sex dissimilarities in performance are not due simply to a difference in muscle mass.

Major points of this section are

1. Muscle mass and strength are the same in boys and girls until puberty.
2. After puberty boys have ~1/3 more upper extremity muscle mass than girls and ~1/4 more leg mass.
3. The pattern of genetic expression for muscle growth is different for boys and girls.
4. Gender performance differences are not due exclusively to differences in muscle mass.

3. Skeletal muscle in adults

Muscle mass. As noted, there is a substantial sex difference in total muscle mass whether expressed in absolute terms, per body weight or height. Men have ~1/3 more mass in their upper extremities and shoulders than women and ~1/4 more muscle mass in the lower extremities. Even when examining individual muscle fibers from biopsy material, there is a significant difference in fiber area between men and women. A typical cross-sectional area (composite of all three major fiber types I, IIa and IIb) for sedentary young men is ~4500 to 5000 μm^2 whereas typical cross-sectional areas for young

sedentary women range between 3500 to 4000 μm^2. With training both men and women can double their fiber sizes. It is not uncommon to find fiber areas of 12,000–13,000 μm^2 in highly trained men (Olympic lifters) but it is rare to find fibers larger than 7000 μm^2 in women. Whether the differences in the upper limit of hypertrophy are the consequence of hormonal influence or due to genetic expression is unclear.

Whether strength is comparable per unit area of muscle mass for men and women has been the subject of intense research. Over the years there have been studies which support and studies which refute the hypothesis that men are inherently stronger than women regardless of muscle mass scaling. Recent techniques for imaging total muscle mass, for determining fiber pennation, and quantifying intramuscular fat have provided evidence to indicate that on a specific unit of muscle mass basis, there are no sex differences. Details regarding metabolites are given in the anaerobic performance section below.

One interesting finding that emerged from studying weight-lifters is that muscle strength and height are related by a common factor and that there are absolute height maximums for both sexes. Above a certain height the ratio of weight lifted to cross-sectional area of muscle mass declines. Ratios in women were a constant fraction of ratios in men.

Major points of this section are

1. Men have bigger muscle fibers than women, regardless of fiber type.
2. Fiber size for each fiber type differs for men and women
   Men: IIa > I > IIb
   Women: I > IIa > IIb.
3. Men and women are able to double their fiber sizes with resistance training.
4. Strength per unit of muscle mass is the same for men and women.

Fiber-type distribution. The literature is surprisingly incomplete on this topic. Most of the early studies of fiber type distribution and training were performed on men and only recently has there been an interest within the research community on potential sex differences in fiber type. As noted, obvious fiber area differences exist and there are studies which suggest that fiber type differences also exist and studies which do not support a sex difference in fiber type distribution. Given the superior aerobic performance capability of women it seems logical that fiber type, a higher proportion of type I and IIa fibers, would be one explanation for this phenomenon but to date there is little evidence to support this hypothesis.

The most methodical examination of fiber type differences in humans was performed by Staron and colleagues [15,16] who collected biopsies from nearly 100 men and 55 women. All of the biopsy material was taken from the vastus lateralis portion of the quadriceps, which clearly leaves ample opportunity for the examination of other muscles in future study. Staron et al. used routine ATPase histochemistry to identify fiber types I, Ic, IIc, IIa, IIab, IIb and myosin heavy chain content using electrophoresis on all the samples from the men and on 26 samples for women. No differences in major fiber type distribution were observed for men vs. women. However, the percentage of area occupied by each fiber type differed for men and women. As expected, fiber areas were higher for men in all three major fiber types, I, IIa, and IIb. For men, fiber areas were highest in the IIa fiber type while for women, type I fibers were largest. Therefore, there were sex differences in

the proportion of area occupied by each fiber type. For men, the area occupied by each fiber type was represented as IIa > I > IIb. For women, the percentage area occupied by fiber type was represented by I > IIa > IIb. Thus, the possibility exists that women have more fatigue resistance or higher long-term aerobic capacity than men, because type I fibers occupy a higher proportion of the total muscle mass in women. Staron's data were obtained for inactive men and women, however, and there is the possibility that sex differences would be greater in trained athletes. It is also possible that sex differences are masked by the enormous inter-individual variability that exists normally, as just within the quadriceps, the distribution of type I fibers ranged from 16 to 95%. Values for myosin heavy chain mirror the fiber type area data with no sex differences observed.

One other muscle group that has been examined reasonably well for fiber type differences is the erector spinae because of the enormous incidence of back pain in our society. As in the quadriceps, sex-related fiber type differences were non-existent and the percentage area occupied by each fiber type favored type I for men and women but with a higher value (62 vs. 76%) for women [10]. Thus, there is no evidence to suggest that fiber type is responsible for sex differences in performance (or back pain). The greater area occupied by the more endurant type I fibers in women is probably related to the better long-term aerobic performance capacity in women.

Although a little peripheral to the topic at hand, one hormone that profoundly influences fiber type distribution (specifically myosin heavy chain isoform) is thyroid T3. All members of the myosin heavy chain multigene family respond to T3 but mode of response is determined in a highly muscle type specific way. Thyroid tumors are not uncommon and hypo and hyperthyroidism markedly alter fiber type composition in both men and women. Alterations in circadian rhythm wreak havoc with T3 levels fluctuating enormously. Circumstances such as 90 min "days" in the space station, extremely long winter nights as is Alaska or Antarctica, and high levels of stress have been known to markedly transform fiber type which are associated with complaints of muscle fatigue.

Major points of this section are

1. There do not seem to be gender-related fiber type distribution differences.
2. The proportion of total area occupied by one particular fiber type differs for men and women
   Women: type I
   Men: type IIa.
3. The inter-individual variability in fiber type distribution is enormous.

Aerobic performance. It has long been observed that there is a sex difference in the ability to perform long-term aerobic activity. Women have the ability to sustain high intensity aerobic exercise longer than men. For example, if men and women exercise on a cycle ergometer at the same relative intensity such as 65% of $VO_{2\,max}$, women will exercise longer than men and have a lower respiratory exchange ratio, greater lipid peroxidation and glycogen sparing, and lower protein utilization. As discussed in the previous section, part of the better aerobic performance capacity in women vs. men is likely due to the percentage of muscle area occupied by type I fibers.

Because women oxidize more fat during submaximal exercise, there is relative sparing of muscle glycogen. Part of the difference relates to circulating levels of the

hormone estrogen. Male rats treated with estrogen improve their exercise times over non-treated males and use less muscle glycogen on an identical protocol. The cellular mechanisms and factors underlying these findings are likely multifactorial and at present are not well understood. Some of the factors that have been hypothesized as having relevance are sex differences in distribution and quantity of adipose storage sites, lipoprotein lipase (LPL) activity, muscle triglyceride content, and mitochondrial CPT1 activity.

Major points of this section are

1. Women are more endurant than men when exercising at the same relative intensity.
2. Greater endurance in women may be due to glycogen sparing, a higher proportion of type I muscle fibers, estrogen, basic differences in energy metabolism, or other factors.

Anaerobic performance. Regardless of how the numbers are expressed, in absolute terms, using a ratio of strength to body mass, or using allometric models, men are stronger than women. When all variables are considered such as muscle pennation angle (which differs for males and females), fascicle length, fiber length and fiber area, the ultimate reason for the difference in strength between sexes is muscle mass. When physical activity is accounted for, the strength per unit of cross-sectional area for the muscles of interest (e.g. biceps, quadriceps femoris) is the same for men and women.

When athletes are compared to non-athletes there is often a difference in force per cross-sectional area of muscle. This is not surprising given the lack of muscle activation, particularly at a high intensity, in the general population. This difference in force/area is eliminated through exercise, usually before there is an increase in actual muscle size, and is attributed to neural adaptation. Neural activation is a very powerful stimulus for change in muscle performance (see Chapter 13).

There is a robust literature describing the magnitude and rate of strength change in response to strength training, mostly in men, but there have been enough studies done on women to indicate that there is little sex difference with overload exercise. Given more muscle mass to begin with, men gain, in absolute terms, more total mass with strengthening than women. In relative terms, the increase in muscle mass and fiber size (hypertrophy) in response to training seems to be quite similar. Men lift substantially more than women whether comparing bench press, leg press or the "clean and jerk" suggesting that perhaps men generate more power per unit of muscle than women. This hypothesis has not been supported by single fiber studies or when force or torque output is normalized to volume of muscle mass using MRI or other imaging technique. What is apparent, however, is that men have longer lever arms which enable them to develop more torque (torque = force × distance) than women.

Using microarray technology, it is now possible to examine changes in gene expression prior to and following initiation of exercise programs. For example, men and women were trained 1–2 h/day, 3 × a week for 9 weeks [13]. In biopsies of quadriceps femoris before and after, training of 179 expressed genes, 136 were expressed higher (> 1.7-fold) in men than women. After strength training, only 28 of these genes were still significantly different between muscles from men and women with all but five of these genes being more highly expressed in men. The biological basis for these differences is

unknown as no specific patterns of genes emerged as being consistently differentially expressed between men and women, which should provide ample research opportunity in the future. It would be expected that some of the sex differences observed are due to hormonal influences.

Major points of this section are

1. Men are stronger than women due to larger muscle mass.
2. Both sexes respond to training with a comparable increase in strength but the genetic expression changes that occur differs for men and women.

Muscle injury. Muscle injury is one of the few areas of study where male/female differences have been examined and mechanisms of action examined to some degree. Most of the work to date has been with animals but human clinical trials are in progress. Essentially, when a muscle injury is produced (e.g. by local injection of toxic substance or downhill running) a cascade of inflammatory events is initiated. Blood levels of creatine kinase (CK) are used commonly as an indicator of muscle damage. It is believed that CK is released from the site of muscle injury due to membrane damage. Male rats have higher CK values than female rats in response to the same injury. Moreover, if male rats are injected with estrogen, their CK values become comparable to those of female rats. Human females also have lower CK values compared with males after exercise-induced muscle injury. These studies suggest that estrogen has the ability to stabilize the muscle membrane.

When damaged skeletal muscle is examined with an electron microscope, there is evidence of greater ultrastructural damage in males and a greater loss of proteins desmin and dystrophin that stabilize the sarcomere. Estrogen suppresses release of heat shock protein (HSP) 70, which is released with muscle damage. In addition, estrogen inhibits leukocyte (specifically neutrophils, macrophases ED1 and ED2) infiltration at sites of damage, which may reduce cytokine-induced damage to adjacent tissue. These findings have been observed in male rats supplemented with estrogen and in female rats indicating that differences in response to muscle damage are influenced hormonally rather than genetically. Hypothetically, an attenuated response to injury should facilitate the repair response (satellite cell activation), which has clear implications for muscle healing with aging. It is well documented that older adults repair muscle injury more slowly than young adults and perhaps estrogen, at least in women, is one factor influencing the rate of muscle repair. Estrogen receptors are present on membrane of skeletal muscle and via these receptors estrogen is known to influence membrane fluidity and function. (A more comprehensive review may be found in Ref. [17].)

Major points of this section are

1. With muscle injury, males have higher CK values and more muscle damage than females.
2. Male rats injected with estrogen have less muscle damage than male rats without estrogen.
3. Estrogen stabilizes the muscle membrane, suppresses the release of HSP-70 and limits the extent of postinjury damage.

4. Menopause and andropause

One of the most contentious topics in the literature in the past decade concerns the role of estrogen for the maintenance of muscle mass with menopause. Phillips et al. [11] reported that women who were on hormone therapy (HT) during menopause maintained thumb adductor muscle mass and specific strength whereas women who were not on hormone lost significant mass and strength. Subsequently, Poehlman and colleagues [12] reported findings for 38 middle-aged women who were followed longitudinally for 6 years. The 18 women who became menopausal during that interval had an accelerated loss of fat-free mass (underwater weighing method) with a concomitant increase of 2.5 kg in fat mass compared to the women who remained pre-menopausal. Also noted was a reduction in postmenopausal women of spontaneous physical activity, from 416 to 289 kcal/day so it is difficult to separate direct effects of hormonal influence alone from that which would occur with changes in activity.

However, other studies do not support a protective effect of estrogen on muscle mass during menopause. For example, Bassey et al. [2] examined grip strength and leg extensor power for more than 2000 women who were between 44 and 55 years. Women were grouped according to reported history of menopausal status (cycling regularly or irregularly), on HT, or cessation of cycling. There were no differences in strength between groups and menstrual status was not associated with any of the variance in muscle measurement. Hormone replacement was not ergogenic as suggested previously. Seeley et al. [14] examined hip abductor, triceps extensor and hand grip strength, balance, gait speed, and self-reported disability in 9704 women 65 years and older. Women who had never been on HT, those who had been on HT and those currently on HT did not differ in any of the measures. If estrogen ever had a protective effect on skeletal muscle it was not apparent from the data provided on the women in this study. More recently, Hagburg et al. [7] determined that aerobic training intensity is associated with lower levels of body fat in postmenopausal women and higher values for lean mass (predominately muscle). Age, years postmenopausal, dietary intake and HT status did not correlate with any of the body composition values. Work from our laboratory examining the combined effects of exercise and HT also suggests that estrogen does little to augment lean mass in older women. Women in their 60s were randomized into a non-exercise group or an exercise group without HT or with HT. Women performed a variety of activities designed to generate relatively high ground reaction forces (e.g. stair climbing). After 11 months lower extremity fat-free mass was evaluated using dual energy X-ray absorptiometry (DEXA). Both exercise groups gained significant strength and fat-free mass but differences between women with and without HT could not be discerned.

Recent work in our laboratory using female rats has yielded some interesting relationships between hormonal status and activity which indicates an indirect role of estrogen on skeletal muscle mass. Ovariectomy (OVX) to rats results in an immediate weight gain, increasing body mass by a little over 20% in 1 month. This weight-gain occurs without any increase in food intake. Occurring concomitantly however, is a marked decrease in spontaneous activity following OVX. OVX rats that are in cages with exercise wheels all their lives decrease their nightly running distance from an average of 13 km/day prior to OVX to $\sim$6 km/day afterwards which may explain some of the weight-gain

associated with the reduction in estrogen. Weight-gain in OVX rats can be prevented by reducing food intake by 20%. These findings suggest that estrogen may influence physical activity behavior. Perhaps postmenopausal women simply are less physically active and therefore more susceptible to muscle decline secondary to inactivity.

In men, there is a concomitant decline in muscle mass and testosterone beginning in middle age and continuing through the remainder of life. A cause and effect relationship cannot be assigned (clinical trials are in progress) but it is hypothesized that middle-aged and older men with low testosterone values may benefit from testosterone therapy. Evidence to support this hypothesis comes from clinical trials of hypogonadal men who show highly significant increases in muscle mass and strength within a month when their serum levels of testosterone are normalized. Total RNA from muscle biopsies taken from these hypogonadal men indicate that testosterone increased mRNA concentrations of insulin-like growth factor (IGF-1) and decreased mRNA concentrations of IGF binding protein-4. Thus, testosterone probably enhances skeletal muscle mass and strength in men by stimulating the intramuscular IGF-1 system.

Removal of the gonads in mature male rats has a small effect on muscle mass but markedly reduces specific muscle force (peak force/muscle mass). One month following gonadectomy specific tension is reduced by $\sim$25% in the soleus, a postural muscle and between 8 and 22% in other locomotor and antigravity muscles. This variable effect of testosterone reduction on muscle contractile function suggests the possibility that the concentration of hormone receptors differs muscle to muscle depending on fiber type, functional demand or other factors not yet unrecognized. Alternately, testosterone may have variable genomic effects depending upon muscle usage, fiber type or other unidentified factors. It appears that contractile protein is particularly affected with low testosterone levels and genes like myostatin are a logical place to begin for answering the unknowns of muscle function and male hormone influences.

Major points of this section are

1. Studies do and do not support an estrogenic effect on muscle mass in menopausal women.
2. Exercise is more likely to influence muscle mass and strength in aging men and women than hormone status.
3. Low testosterone values are associated with weakness, particularly in old age.

5. Aging

With aging, skeletal muscle mass is lost in men and women at a rate of about 1% per year, after the third decade. Thus, by the mid-80s, most men and women have lost about half of their strength. Given that men have 25–35% more muscle mass to begin with, it is clear that women are more at risk for loss of independence with aging than men, due to the reduction in strength that occurs secondary to the loss in muscle mass. This decline in muscle mass is termed *sarcopenia*, referring to an age-associated wasting of muscle tissue. Under "normal" circumstances men and women lose approximately half of their muscle mass and strength by the mid-80s but it should be borne in mind that if disease such as osteoarthritis is present, there is potential for additional muscle loss. Many older adults

also experience bouts of extreme inactivity or bed rest associated with other diseases (i.e. an episode of congestive heart failure or pneumonia) which exacerbates the decline in muscle quantity and quality.

Cross-sectional and longitudinal study suggest that men have a greater loss in lower extremity muscle mass and strength with aging than women, particularly for eccentric contractions. Losses in lower body strength for men begin earlier than those for women, typically in the 30s as opposed to the 50s for women. Part of the sex difference is due to an increase in lower extremity weight for women during middle age, which most likely increases the demand on lower extremity musculature. Unquestionably, life style is a potent determinant of muscle mass and strength at all ages and the differential rate of decline for men vs. women may be a reflection of lifestyle. Also associated with the decline in muscle mass in men is a decline in free testosterone, cardiovascular disease and declines in IGF-1. For women, only total fat mass and physical activity are significantly associated with muscle mass. Even though there is a concomitant decline in sex hormones with age that parallels the decline in muscle mass, there is not a strong correlation between the change in hormone status and muscle mass in women whereas the correlation between the decline in testosterone and loss in muscle mass and strength in men is significant. Although evidence suggests that men lose more muscle mass than women, there may be differing rates of loss at specific sites, which suggests that perhaps the mechanisms underlying age-related losses in skeletal muscle differ for men and women.

Also noted with aging is a decline in the synthesis of myosin heavy chain. Since myosin comprises ~30% of all the muscle protein present, a decline in the synthesis rate of this protein is likely to cause a decline in total muscle mass over time and compromise the ability of the skeletal muscle to remodel. Whether there are sex differences in the rate of decline in myosin synthesis is not known.

The relationship between muscle mass and strength is obvious. If synthesis rate, particularly of myosin heavy chain, declines with age, the ultimate reflection of this decline is a change in muscle strength. There is no question of whether strength is lost with age. However, factors contributing to strength loss remain controversial. One measure of muscle strength is specific force or the amount of force produced per unit of muscle mass. Some investigators have observed a loss in muscle force that exceeds the decline in muscle mass whereas other researchers have not. In our laboratory, for example, in aged rats, a loss in specific force for the soleus and extensor digitorum longus was observed, but not for the plantaris or peroneus longus. Recently Frontera and associates [6] compared the specific force of the whole vastus lateralis portion of the quadriceps muscle and individual fibers from the same muscle in older men and women. Their methods included obtaining muscle strength measures of the quadriceps, CT scans of the thigh to obtain vastus lateralis cross-sectional area, and biopsies to get single fibers from the vastus. There were no differences in strength between young and older individuals after adjusting for whole muscle cross-sectional area. Single fibers from older men, howevers, had lower specific force values than fibers from young men regardless of fiber size. Types I and IIa fibers from older men were stronger than the same fibers from older women even after adjusting for fiber size. These findings suggest that the intrinsic ability of muscle to generate force in old age is lower. One possible reason for this reduction in force is a change in excitation–contraction coupling. Delbono et al. [3] demonstrated that in aging quadriceps, there was

a reduced availability of calcium to initiate contraction. The other possible limiters of muscle force include compromise in energy supply but there is no evidence to indicate that aging alters the energetics of skeletal muscle. It is possible that the age-related reduction in synthesis rate of myosin protein could delay the replacement of dysfunctional proteins, also affecting specific force. Thus, experiments with whole muscle and single fibers provide different insights into muscle function and seem to indicate that the intrinsic ability of old muscle to generate force is reduced and that the reduction in force is due to more than atrophy.

Another controversial area of investigation concerns a possible decline in oxidative metabolism in aging muscle. In cytochrome oxidase deficient muscle fibers in aging muscle there is an accumulation of mitochondrial DNA deletions. Recently, mitochondrial DNA point mutations as well as deletions were observed in 7 of 14 biopsies by Fayet et al. [4] taken from older individuals between 69 and 82 years. Accumulation of point deletions and mutations is likely to be associated with impairment of mitochondrial function in individual muscle cells. Whether there are enough cells with disrupted mitochondrial function in each whole muscle to alter oxidative metabolism is not clear. Sex differences in regard to oxidative metabolism have not been examined systematically.

Although outside the scope of this chapter, it warrants mention that bone mass and skeletal muscle mass are tightly coupled throughout life in both men and women (see Chapter 16). Thus, muscle atrophy and loss is associated with a concomitant decline in quantity of bone, which has obvious implications for osteoporosis in later years. Also associated with total quality of muscle mass is maximal oxygen uptake or aerobic capacity. Maximal aerobic capacity determines how fast and long (duration) one can bike ride, run, ski or walk comfortably. With the reduction in muscle mass that occurs with age, maximal aerobic capacity is affected accordingly which is one reason for the loss of endurance with old age and for the slowing that occurs.

As would be expected, men and women who are active throughout the course of a lifetime show less muscle wasting or sarcopenia than inactive individuals. Even though exercise may delay by a decade or more the age-related declines typically observed, exercise does not prevent age-related changes in skeletal muscle. An illustration of this point may be seen in Fig. 1. Muscle biopsies were taken in my laboratory from men ranging in age from 18 to 82 years, all of whom were elite Olympic or Power lifters. These men had trained a minimum of 6 years, the average length of training was 15 years. All of the subjects were prominent in the lifting field in that they had placed in state or national championships. One was Junior Olympic champion and one subject was ranked ninth in the world in his weight class. Each man had a biopsy taken from the vastus lateralis portion of the quadriceps. Biopsies were stained with ATPase to determine fiber type and areas of each fiber type were determined. Since the IIa fibers were present to the greatest extent, representing 75% of all fibers in the cross-sections, the data for IIa fibers are presented in Fig. 1. Note that the cross-sectional area of IIa fibers decreased after the fourth decade, reaching a fiber size that was half of what young lifters exhibited, in spite of continued rigorous training for many years. Interestingly, fiber area for the oldest lifter at 82 years of age, was comparable to the fiber areas observed for untrained men in their 20s. Thus, even elite lifters experience an age-related reduction in fiber size and a concomitant loss in fiber number as well. Although data for female lifters are sparse, current records for women in

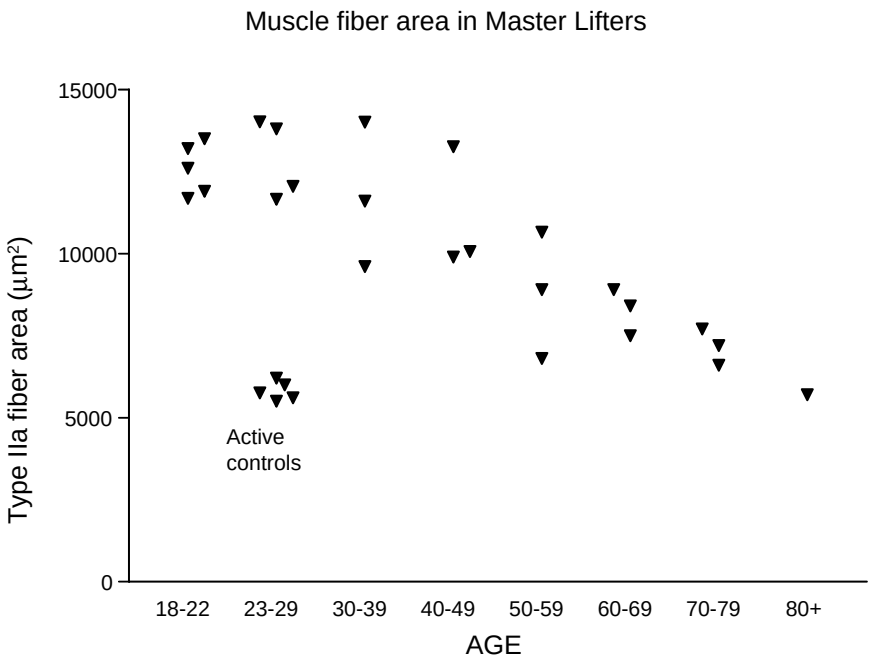

Fig. 1. Average vastus lateralis fiber area for Olympic and Power lifters at various ages. Data for five young active controls (non-competitive athletes, mean age 23 years) are shown for comparison.

their respective age-group (e.g. 45–49 years) reveal the same pattern of decline in lifting ability as men, suggesting that similar aging mechanisms, which result in a loss in fiber number and a reduction in existing fiber size, are operating. More data on this population are needed as women in the menopausal phase of life tend to have a steeper decline in their lifting ability than at any other point in their career, further adding to the controversy concerning the role of estrogen in the maintenance of muscle mass.

Data from Klitgaard et al. [9] also support an age-related decline in muscle mass in very active older men that is sport specific. This group of investigators examined biopsy material from the biceps brachii and vastus lateralis portion of the quadriceps from men who were inactive (controls), swimmers, runners and weight-lifters. Swimmers and runners had fiber areas that were comparable to the inactive controls whereas the weight-lifters had muscle fiber areas comparable to young untrained men. Other benefits of exercise such as cardiovascular fitness were not analyzed in this study. It would appear that only strength training exercise can delay the onset of muscle atrophy. Whether lifting can influence the loss of muscle fibers with aging is unknown.

One of the landmark studies of the past decade showed that older untrained skeletal muscle is as responsive to an exercise stimulus as young muscle. Old men and women are capable of gaining strength and lean body mass with a program of weight-training at ~70–80% of one repetition maximum (1RM: absolute amount of weight that can be

lifted once). Fiatarone et al. [5] recruited very elderly (mean age 86 years) subjects from a nursing home environment underwent training for the quadriceps muscles for 8 weeks [5]. A remarkable 200% increase in strength was observed which translated into functional improvements such as walking up stairs, casting aside ambulation devices, and better gait speed. Since this initial study, several other strength-training studies have been published, all of which demonstrate that older adults are capable of the same relative increases in muscle strength as young men and women. More importantly, the increases in strength were accompanied by concomitant increases in lean body mass and an upregulation of protein synthesis [18]. It would appear that the process of muscle contractile protein accumulation is intact even in old age. Sex-specific differences in the rate of muscle protein synthesis were not apparent. Strength increases, maximum voluntary isometric and isokinetic torque at 60°/s, for men were greater than those for women, however.

Recent study by Bamman et al. [1] has challenged the concept that older men and women adapt comparably to a strength-training stimulus (2003). In a small study, nine men and five women (61–77 years) participated in a 26 week long progressive resistance training protocol consisting of exercises such as leg press, lat pull down and seated rowing. When strength increases were expressed as improvement relative to pre-training values, women lagged behind the men after 25 days and remained behind for the duration of the study. Overall, men showed about an 82% increased in strength as opposed to a ~58% increase for women. Differences in strength were associated with differences in fiber area of the vastus lateralis with men showing a 29, 41 and 43% increases in fiber area in type I, IIa and IIb fibers, respectively, with women showing a 7, 6 and 5% change. These sex differences could not be attributed to varying levels of circulating IGF-1, testosterone or DHEA-S. Whether the women in this study were trained as rigorously as the men was not addressed. Whether there were injuries or other soft-tissue problems, particularly among the women, that would have influenced strength gains also was not addressed and additional studies are needed to confirm these results.

Major points of this section are

1. Muscle mass and strength are lost at ~1% per year starting in the 20s. Men lose more leg strength than women.
2. The rate of muscle protein synthesis declines with age and is associated with the loss in muscle mass.
3. Exercise can attenuate but not prevent age-related decline in muscle mass and strength.
4. Men and women of all ages can increase strength with an appropriate exercise stimulus.
5. Older women may not show the same increase in strength with training as men.

6. Future directions

Much remains to be learned concerning appropriate methods to assess muscle function, treatment of muscle diseases, optimal conditions to train athletes, and design of treatment programs for patients. Future studies should include experiments to investigate the direct influence of hormones on skeletal muscle including a more in-depth analysis of the

distribution of fiber type in muscles from men and women, the role of nutritional supplementation in aerobic muscle performance, and the biological basis for differences in expression patterns of genes and muscles between men and women. Using genomic techniques of microarray analysis, it will be possible to identify sex differences in gene expression and the influence of hormones on their expression. Using molecular and genetic analysis it will be possible to determine whether there is a difference in the rate of decline of myosynthesis and oxidative metabolism between men and women. With an aging population, it will also be important to identify mechanisms of action of estrogen influencing behavior and decline in physical activity as it relates to sarcopenia with age in women. There is substantial opportunity to examine sex related mechanisms of strength change in an aging population including neural mediated changes, satellite cell activation, and expression of selected myogenic regulatory factors. Completely unknown is whether there are differences in any factors mentioned in this chapter with regard to ethnicity in males or females.

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Sex-based differences in substrate metabolism

Tracy Horton and Barry Braun

1. Introduction

In the last 10 years, important differences in mechanisms controlling the storage and utilization of energy yielding substrates between men and women have been identified. Such differences in metabolism have implications for sex-specific etiology and treatment of diseases, especially diseases that have a strong metabolic basis such as obesity, Type 2 diabetes, and coronary heart disease. Biological sex (XX vs XY) and/or the prevailing sex-steroid environment could play direct roles in mediating these male–female differences in metabolism. In addition, these sex-based factors could interact with a number of other neuro-endocrine factors. Alone or in combination, these mediators can impact metabolic pathways at several key points, and affect substrate availability (via effects on substrate storage), substrate mobilization from body tissue stores, substrate uptake at the site of utilization and, within the cell itself, substrate trafficking between storage, oxidation and/or re-cycling (Fig. 1).

Before focusing on the sex-based differences in substrate metabolism, it is useful to consider areas in which metabolic regulation is qualitatively similar between men and women.

2. Metabolic regulation common to men and women

In the *broad* sense, substrate selection is not sex-specific under a variety of physiologic situations. In the resting, post-absorptive state, there are no physiologically relevant differences between men and women with respect to energy expended per unit fat-free mass [45]. Men and women do not differ in whole-body non-protein respiratory exchange ratio when compared at rest, indicating no differences in the percent of energy derived from carbohydrate or fat utilization [25]. Women have been observed to oxidize less protein at rest compared to men [47] but the proportion of resting energy expenditure derived from protein oxidation is generally minor in adequately-nourished humans.

As the length of time from the most recent meal continues, and the body experiences an increasing energy deficit, blood concentrations of catecholamines, glucagon, growth

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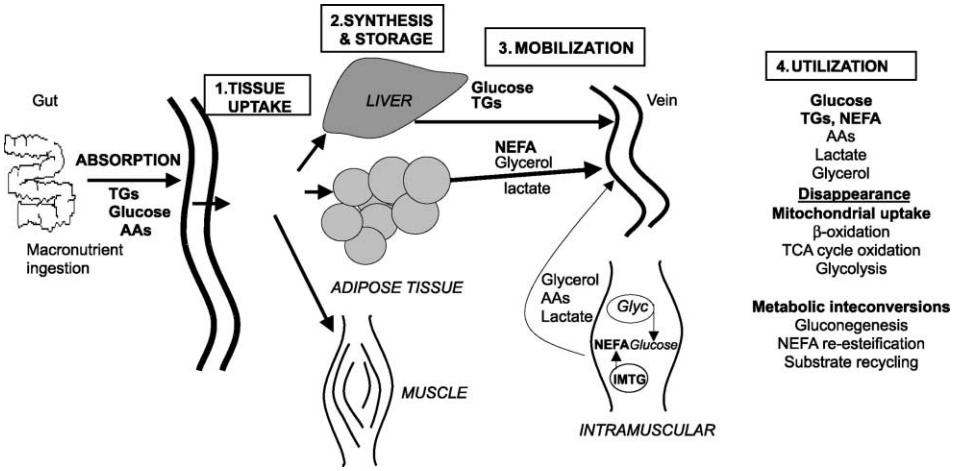

Fig. 1. (1) *Tissue uptake*. Absorbed nutrients are taken up from the circulation into the liver, adipose tissue or muscle, via active or passive mechanisms. (2) *Synthesis and storage*. Under anabolic conditions, nutrients taken up into tissues are predominantly used for the synthesis of macromolecules (e.g. proteins) and storage (e.g. glycogen, lipid droplets). (3) *Mobilization*. Stored nutrients (potential substrates) are mobilized from the different tissues in response to a variety of catabolic stimuli. These can remain within the tissue (for further metabolism) or are delivered into the circulation for distribution to other organs/sites. (4) *Utilization*. Substrates delivered to the organs/tissues, or remaining within the organ/tissue itself, are utilized to provide energy (disappearance) or undergo metabolic conversion into other molecules or are recycled into their original form (futile cycling). At each of these points, there is a potential for sex-based differences in nutrient/substrate metabolism.

hormone, and cortisol increase. These neuro-endocrine changes, or the counter-regulatory response, are similar to those observed under other conditions in which glucose homeostasis is "compromised", for example, during exercise or insulin induced hypoglycemia. With prolonged fasting and/or exercise, insulin levels also decline below the usual post-absorptive baseline. The exact onset and magnitude of these multiple hormonal changes depends to a large extent on the duration of fasting, the intensity and duration of exercise, and the degree of hypoglycemia. Acting in concert, these endocrine changes create a catabolic hormonal environment resulting in fuel mobilization. Lipolysis is increased, supplying tissues with non-esterified free-fatty acids (NEFA) for use as a fuel in the face of diminishing blood glucose. To try to minimize the fall in blood glucose, endogenous glucose production is stimulated. During prolonged fasting and/or hypoglycemia there is predominant utilization of circulating substrates, especially NEFA with blood glucose largely consumed by the nervous system. With exercise, there is greater mobilization and utilization of intramuscular fuels (lipid and glycogen) and nearly all of the blood glucose made available by hepatic (and to a lesser extent renal) glycogenolysis and gluconeogenesis is selectively shunted to the working muscle. In all scenarios, hepatic production of ketones rises, providing an alternative fuel source that economizes glucose use, particularly during fasting and hypoglycemia. As a consequence

of these hormonally driven substrate changes, there is an increase in whole body lipid oxidation and a decrease in carbohydrate (CHO) oxidation.

In contrast, after a meal the catabolic hormonal environment is reversed. The several-fold rise in plasma insulin post-prandially promotes net protein retention and blood glucose uptake and storage (as glycogen) in insulin-responsive tissues. Lipid absorption results in an elevation in circulating lipoprotein triglycerides (TG), which are cleared and stored in peripheral tissues as a result of hydrolysis by lipoprotein lipase (LPL). At the whole-body level, there is a shift to greater CHO oxidation and less fat oxidation. Both the amount of energy consumed, and the macronutrient composition of a meal, will affect the extent of the insulin, glucose and TG excursions, as will the timing of a meal in relation to the preceding meal.

3. Metabolic perturbations as instructive examples

Although qualitatively similar, there are some important quantitative (relative or absolute) sex differences in substrate metabolism. These sex differences are particularly apparent under conditions that "perturb" energy and substrate homeostasis away from the resting, post-absorptive state. Quantitative differences include the utilization of the major energy sources as well as their storage and mobilization from different tissues. This may, in some instances, result in sex-specific metabolic aberrations. The nature of these sex differences in substrate metabolism, along with the direct and indirect role of the prevailing sex hormone environment (estrogen, progesterone, testosterone) and neuro-endocrine response in regulating these differences, will be discussed below.

3.1. Anabolic condition or response to a meal

In the acute post-prandial period (~6 h), the most striking sex difference is the significantly greater circulating TG levels in men compared to women [25]. This is observed despite similar glucose and insulin excursions. Although men tend to have greater fasting TGs than women, and fasting TG concentrations are significantly correlated with the post-prandial TG response, this alone does not explain the greater post-prandial lipemia in men. For instance, men with fasted TG levels similar to women, still demonstrate greater post-prandial lipemia [2,27]. Potential reasons for the lower post-prandial TG excursion in women include a slower rate of lipid absorption from the gut, greater suppression of hepatic very-low-density lipoprotein TG secretion, and/or greater tissue clearance of TG. Few data exist regarding the first two factors. With respect to TG clearance, a greater post-prandial limb (leg) uptake and/or clearance of TG by women has been observed [25,27]. These results might partially explain the greater resting intramyocellular triacylglycerol (IMCL) concentrations observed in women vs men [43]. No data has directly compared the acute post-prandial uptake of TG across an adipose tissue bed in men vs women. Nevertheless, it appears likely that a greater tissue clearance of TG is one factor contributing to the sex difference in the post-prandial lipemia. Observations on post-prandial metabolism and the disposition of dietary lipid may also provide some insight into the obvious sex differences in body fat patterning.

After a meal, men take up more TG into the splanchnic region of the body [35], suggesting greater storage in visceral adipose tissue. By contrast, 24 h post-prandially, women accumulate more meal-derived lipid in subcutaneous adipose tissue relative to men [41].

3.2. Catabolic conditions

Fasting and hypoglycemia. Sex-based differences in metabolism have been observed under conditions of extended (60–86 h) fasting. Women demonstrate a greater increase in circulating NEFA and ketone bodies, and a greater decrease in blood glucose, compared to men [9]. In addition, women demonstrate a greater fall in the rate of endogenous glucose appearance and glucose utilization. When decreases in blood glucose are small, for example, after 22 h of fasting, sex differences in glucose kinetics are not observed [34]. Shorter term fasting also results in a smaller percentage increase in lipolysis in women compared to men [34] mainly due to the greater rates of lipolysis in women. Under conditions of insulin-induced hypoglycemia, endogenous glucose production and glucose utilization are also significantly lower in women compared to men [13]. Under these conditions, higher glycerol and NEFA concentrations are observed in women, again suggesting higher rates of lipolysis. These data, therefore, suggest that only when there is overt hypoglycemia (extended fasting or insulin-induced) do women have a greater release of NEFA for utilization and conversion to ketones, as well as a greater decline in the rate of glucose production and utilization, compared to men.

Exercise. During exercise, a metabolic stress experienced by many in everyday life, normal weight, lean women utilize proportionally less carbohydrate and more lipid compared to normal weight, lean men. Such observations have been made during exercise of mild to moderately high intensity (40–70% $VO_{2\ max}$) when careful attention has been paid to rigorous study design [3]. Such design considerations include controlling for preceding diet and activity, matching men and women for habitual activity patterns, studying women in the same phase of their menstrual cycle and using a large enough sample size (>6 subjects per gender group). It is noteworthy that sex differences in whole-body substrate utilization are not observed in very elite athletes [39]. This finding is likely related to the menstrual dysfunction commonly observed in elite female athletes, including depression of ovarian hormones (even in the presence of eumenorrhea) that probably minimizes sex-based differences. Due to the obligate exponential increase in muscle glycogen utilization as exercise intensity increases to $>75\%$ $VO_{2\ max}$, any substantive use of other fuel sources is squelched and the sex "gap" in carbohydrate utilization is no longer apparent. Protein oxidation is less important in terms of contributing to the total energy expended during exercise ($<5\%$ unless exercise is very prolonged or individuals are in negative energy balance). Therefore, although in one study men were shown to oxidize significantly more protein than women, the absolute difference in protein oxidation was small (3% vs 2% of total exercise energy expenditure) and may have minimal physiological significance [38].

Sex-differences in the sources of lipid and carbohydrate utilized during exercise. Lipid oxidized during exercise is derived from circulating NEFA, IMCL and to a lesser extent, circulating TGs (at least in the over-night fasted state). Carbohydrate is derived from blood

glucose and muscle glycogen. The proportion of these different sources utilized varies with exercise intensity and duration (Fig. 2).

Recent cross-sectional data strongly suggest that with moderate exercise, women utilize more IMCL and less circulating lipids than men, independent of training status [43]. This is consistent with the observation of greater exercise induced whole-body lipolysis in women vs men [7]. It is possible that the greater utilization of IMCL in women vs men is due to their greater initial (resting) IMCL stores [43] or other factors as will be discussed below.

With respect to the sources of CHO utilized during exercise, it appears that women rely less on muscle glycogen as a fuel source than men, at least when significant sex differences in whole-body CHO are observed [43]. Consequently, blood glucose utilization is not different between the sexes, and may actually be higher in women in terms of the relative contribution to total CHO oxidation [7]. Such differences in blood glucose and glycogen utilization are again not apparent in elite men vs women athletes [39]. Interestingly, the metabolic clearance rate of glucose during exercise has been shown to be lower in women as compared to men [7]. This result suggests that for a given plasma glucose concentration, contraction mediated (non-insulin dependent), glucose uptake may be less in skeletal muscle of women relative to men.

Exercise training: matching of energy intake to expenditure. In response to exercise training, some interesting sex differences have been observed with respect to energy balance when subjects were allowed to eat ad libitum with no stipulation to maintain or lose weight. Compared to normal weight men, who lose significant amounts of body weight and body fat after long-term exercise training (>24 weeks), normal weight women

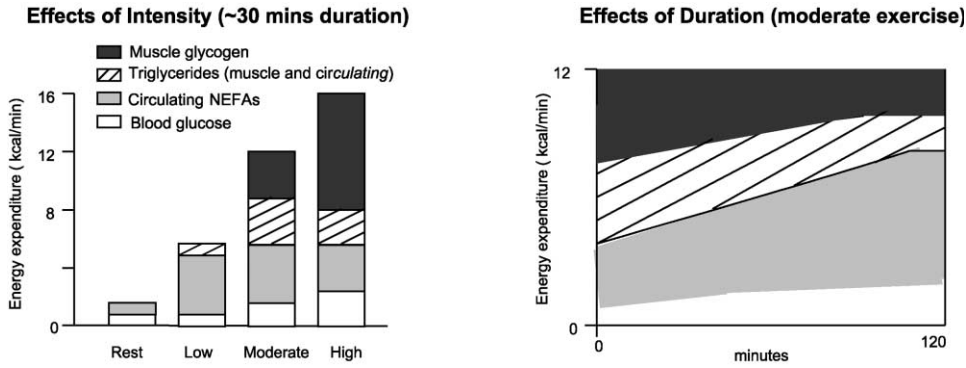

Fig. 2. *Effects of intensity.* As exercise intensity increases, the proportion of energy derived from carbohydrate increases, whereas the proportion of energy derived from lipid decreases. Although the relative contribution of lipid to energy production decreases with increasing exercise intensity, the absolute quantity (e.g. grams per minute) of lipid oxidized does not vary much because total energy expenditure rises proportionally. Muscle derived substrates (lipid and glycogen), especially muscle glycogen, contribute proportionally more to the total energy expended as exercise intensity increases. Conversely, circulating substrates (glucose and NEFAs) contribute proportionally less as exercise intensity increases. *Effects of duration (moderate exercise).* Overall, as exercise continues there is an increased utilization of lipid as a fuel source and a decreased utilization of carbohydrate. Circulating substrates become more important as fuel sources, and in particular circulating NEFAs. Muscle-derived substrate sources become less important, as time goes by, due to their gradual depletion.

show much smaller decreases in body weight and/or body fat [33]. These results suggest that women (normal weight at least) better defend their body energy stores, by adjusting energy intake to meet increased energy requirements, when undertaking an exercise training program. It is possible that the sex differences in the metabolic response to exercise may, in some way, be translated into sex differences in food intake and body weight regulation with exercise training. Sensing, integration, and transduction of energy balance via the adipose and hypothalamic neuro-endocrine signaling pathways (e.g. insulin, leptin, NPY, the orexins, etc.) are almost certainly involved. Although few clear patterns have emerged, circulating leptin concentrations do appear to be higher in women than in men, even after accounting for male–female differences in adiposity [30]. In one tightly controlled study, perturbations in energy balance, not exercise per se, were responsible for alterations in circulating leptin in young women [23]. It will be interesting to see if the same is true in men. Given the critical relationship between energy balance and reproductive status the existence of sex-based differences in the neuro-endocrine response to the energy signal seems likely and will be a promising area for future research. This also may have implications for the development of sex-specific pharmacological and/or behavioral weight control strategies.

3.3. Extreme metabolic perturbation: the special case of pregnancy

From an evolutionary perspective, the necessity for female mammals to provide 100% of the energy required by a developing fetus has likely been a major factor in molding their metabolic priorities with respect to substrate storage and utilization. It can be argued (albeit teleologically), that since a growing fetus requires a "diet" composed mainly of glucose, the conservation of carbohydrate by maternal tissues facilitates the delivery of glucose to the fetus and is a selective advantage (although, taken too far, an excess of resistance to insulin-stimulated glucose uptake in maternal tissues underlies gestational diabetes). A general glucose sparing effect, greatly exaggerated during pregnancy, may be especially manifest in the non-pregnant female whenever fuel and/or glucose supply is limited and/or energy output increased, i.e. during times of metabolic stress. In this context, the increased body fat in women (compared with men) is often viewed as a biologic necessity. Because the body has a limited capacity to store glucose, the subcutaneous stores of adipose tissue can provide an alternative (maternal) fuel source during pregnancy, sparing glucose for the fetus (should the need arise). Indeed, it is generally accepted that the typical body fat content of a woman in her reproductive years is sufficient to ensure the successful outcome of pregnancy and lactation even if the availability of food is limited.

4. Hormonal factors and sex differences in metabolism

4.1. Sex-steroid hormones

Animal studies. In animals, estrogen promotes lipolysis and increases fatty acid availability, while decreasing the rate of gluconeogenesis and sparing glycogen use in

muscle and liver [12]. The addition of progesterone has been reported to antagonize the lipolytic effects of estrogen and reduce fatty acid availability [12]. Conversely, the addition of progesterone appears to accentuate the carbohydrate-sparing actions of estrogen by decreasing hepatic glycogenolysis [12]. Estrogen upregulates mitochondrial enzymes favoring fat oxidation whereas progesterone opposes these actions [5].

Human studies. In human studies, the natural variations in estrogen and progesterone across the menstrual cycle have been used to provide insight into the role of the ovarian hormones in mediating sex-based differences in substrate metabolism. Under normal, resting conditions, there are no differences in the rates of whole-body glucose or NEFA turnover, or oxidation, when the follicular and luteal phases of the menstrual cycle are compared. Measures of amino acid kinetics suggest that whole-body protein oxidation is increased in the luteal vs follicular phase of the menstrual cycle [32] due to the elevation in progesterone. Again, the magnitude of these differences is small thus the physiological significance unclear. Overall, the effect of menstrual cycle variation in sex-steroids on lipid and carbohydrate utilization may not become apparent unless metabolic homeostasis is perturbed [3]. The majority of studies in this area have addressed the response to exercise.

Exercise substrate metabolism: menstrual cycle effects. In a number of exercise studies, substrate metabolism has been measured in the early follicular (low estrogen and progesterone) and/or mid-follicular (MF; elevated estrogen, low progesterone) and mid-luteal (ML; elevated estrogen and progesterone) phases of the menstrual cycle. In well controlled studies with an adequate sample size, no significant differences in whole-body substrate oxidation or glucose kinetics have been observed with moderate exercise cycle (50% $VO_{2\ max}$, 45–90 min) across these phases of the menstrual [26]. With higher intensity exercise (70% $VO_{2\ max}$, 90 min), when demands for endogenous glucose production and utilization are greater, carbohydrate oxidation and glucose utilization are reduced in the ML vs MF phase [4]. In the latter study, cycle phase differences in CHO oxidation and glucose kinetics disappeared when the subjects consumed a glucose drink during exercise which maintained blood glucose [4] and enabled higher rates of glucose utilization. These data suggest that the greater the requirement for maintaining blood glucose via endogenous glucose production, the larger will be the "carbohydrate-sparing" effect of elevated estrogen and/or progesterone. This is likely due to the ovarian hormones restraining hepatic glycogenolysis and/or gluconeogenesis.

Exercise substrate metabolism: effects of sex-steroid administration. The relatively subtle (and highly variable) changes in ovarian hormones during the normal menstrual cycle may not be sufficient to induce significant changes in substrate utilization unless metabolic (glucose), homeostasis is seriously perturbed (e.g. when energy expenditure and glucose flux are greatly elevated during higher intensity exercise). To better control the sex hormonal environment and tease apart the independent actions of estrogen and progesterone, researchers have used exogenous hormone treatment in both men and women. In one study, 4 months of combined oral contraceptive (OCP) use significantly decreased exercise rates of glucose production and utilization. This was observed, however, in both the inactive and high dose phases of a triphasic OCP [44], therefore, it is unclear as to whether or not these changes were due to chronic effects of OCP use or other confounding factors such as changes in body weight, body composition and potentially

activity level over the 4 months. Administration of estradiol to amenorrheic women, or to men, decreases blood glucose production and utilization during exercise [6]. In one study using a gonadotrophic releasing hormone antagonist to suppress normal hormone production in women, replacement with estrogen decreased CHO oxidation, glucose production and utilization, and decreased estimated muscle glycogen utilization [12]. Although estrogen plus progesterone did not affect substrate oxidation, there was a decrease in blood glucose utilization but a compensatory increase in muscle glycogen utilization. The antagonistic effects of progesterone on estrogen mediated muscle glycogen metabolism may have partly been due to the progesterone levels which were much higher than those generally observed during the mid-luteal phase of the menstrual cycle, creating a predominantly androgenic environment. Indeed, normal males treated with estrogen do not decrease exercise glycogen utilization or CHO oxidation, only glucose flux [6]. Hence, an androgenic environment (progesterone or testosterone) appears to attenuate the glycogen sparing effect of estrogen. As circulating catecholamine levels were not different between the hormone supplemented conditions, these data suggest androgenic compounds could increase the responsiveness to catecholamine induced glycogenolysis. Alternatively effects could be indirect. In the study by D'Eon [12] there was a negative correlation between circulating NEFA levels and estimated glycogen utilization, with the estrogen only treatment significantly raising NEFAs compared to the estrogen plus progesterone. An alternative hypothesis, therefore, is that progesterone (or testosterone) may impair estrogen-enhanced lipolysis and thus indirectly increase the requirement for muscle glycogen utilization.

It is not known whether the sex-steroids affect the balance between utilization of circulating NEFA vs IMCL or circulating TGs during exercise. No data are currently available in humans, but animal studies do suggest such an effect. In male rats, estrogen supplementation increases the ratio of muscle LPL activity to adipose tissue LPL activity during exercise [18]. This response would be expected to redirect circulating TG to muscle for oxidation. Whole body fat oxidation was also increased after estrogen administration, implying that free fatty acids derived from circulating TGs could, indeed, have been oxidized. This novel finding using "female" sex hormones in males, suggests that estradiol may impact utilization of a lipid source previously considered unimportant during exercise.

Other metabolic pertubations and the affects of sex-steroids. Fewer unambiguous data are available to explain how sex-steroids mediate the sex differences in response to fasting and hypoglycemia. No effect of menstrual cycle phase on the metabolic response to a 22 h fast was observed in one study although OCP use did decrease glucose turnover in conjunction with higher FFA levels, supporting a glucose sparing effect of the sex-steroids [10]. A recent study on the counter-regulatory response to insulin-induced hypoglycemia found that estrogen supplementation in post-menopausal women decreased glucose production compared to age matched men or unsupplemented post-menopausal women [42]. Thus, estrogen likely plays a role in determining the sex-specific response to conditions where glucose homeostasis is compromised.

With respect to post-prandial metabolism, there is also a paucity of data in terms of the effects of the sex-steroids. Nevertheless, post-menopausal women have elevated post-prandial TGs compared to pre-menopausal women (age matched) thus losing their

pre-menopausal "gender protection" against post-prandial lipemia [46]. Treatment of post-menopausal women with estrogen reduces the post-prandial increase in meal-derived (chylomicron) TGs [40]. Testosterone supplementation or replacement in men, however, does not alter post-prandial lipemia [24]. These data strongly support a role for the ovarian hormones, especially estrogen, in directing post-prandial TG metabolism. This is not altogether surprising given the well-established effects of estrogens and OCPs on hepatic triglyceride metabolism. Whether estrogen effects tissue TG clearance directly is currently unknown.

Testosterone. It is possible that the low level of testosterone in females (at least relative to males) facilitates facets of the observed sex differences in substrate metabolism. Other than the points raised above, little data is available to address this question. Careful study of the effects of testosterone on the glycogenolytic and lipolytic pathways will likely yield provocative and potentially important new data.

4.2. Sympathetic nervous system and catecholamines

Whole-body response. In response to a metabolic stress (e.g. exercise, insulin-induced hypoglycemia, hypoxia and cold exposure), activation of the sympathetic nervous system causes increased secretion of the catecholamines, epinephrine and norepinephrine. In general, blood levels of epinephrine, and in some instances norepinephrine, do not rise as much in women as they do in men [3,13]. In women, skeletal muscle sympathetic nerve activity (MSNA) is less than in men during exercise or hypoglycemia [14,43]. During insulin-induced hypoglycemia, the level of blood glucose required to elicit a significant catecholamine response is the same in men and women, but the magnitude of the response is reduced in women [14]. Thus, it is unlikely that sex differences in the catecholamine response to metabolic stress are due to differences in the "threshold" at which a particular stimulus causes sympathetic activation. Despite both a lower catecholamine response, and a lower muscle sympathetic activation during metabolic stress, women have similar or greater lipolysis [7,9,13] but lower rates of glucose production and utilization compared to men [7,9,13]. Of significance, is a recent study that demonstrated estrogen supplementation decreased the epinephrine and MSNA response to insulin-induced hypoglycemia in post-menopausal women when compared to age matched non-supplemented women and men [42]. This suggests a role for estrogen in mediating the sex-based differences in sympathetic activation in response to metabolic stress.

These data suggest that women may be more sensitive to the lipolytic action of catecholamines, while maintaining a similar level of sensitivity to glycogenolysis and possibly glucose production. The lower catecholamine response may facilitate a decreased reliance on blood glucose and/or muscle glycogen, i.e. glucose conservation. The mechanism by which the lipolytic response to catecholamines is enhanced in females, under these conditions, is unclear. Some insight into this issue can be gained from studies in subcutaneous adipose tissue performed in vivo and in vitro.

Subcutaneous adipose tissue. The microdialysis technique has been used to assess the lipolytic response to acute exercise in men and women. This technique allows metabolites

to be sampled in the usual hormonal environment [1]. Although both sexes exhibited a greater lipolytic response to exercise in abdominal vs gluteal adipose tissue, the effect in the abdominal region was much more pronounced in women than in men [1]. These results support the hypothesis that lipolytic sensitivity to catecholamines is higher in women, and also suggests that the difference is manifested specifically in abdominal fat tissue. Data also imply that the receptor-mediated mechanisms regulating subcutaneous adipose tissue lipolysis differ between men and women. Lipolysis is increased by β-adrenergic receptor activation and reduced by α-adrenergic receptor stimulation. Catecholamines, especially epinephrine, stimulate lipolysis mainly via β-adrenergic mechanisms but are also α-adrenergic agonists, especially when circulating concentrations are relatively low. The balance between β-adrenergic stimulation and α-adrenergic inhibition determines the overall rate of lipolysis at a tissue. Pharmacologic blockade of β-receptors reduces abdominal adipose tissue lipolysis during exercise in both men and women whereas α-adrenergic blockade increases lipolysis in men only [1]. The need to "overcome" α-adrenergic opposition to β-adrenergic stimulation of lipolysis may help to explain why men have higher catecholamine levels during exercise and other stress situations, but similar or lower rates of lipolysis compared to women.

Adipose tissue biopsies for the study of in vitro subcutaneous adipose tissue lipolysis. Using this technique, a longitudinal exercise training study reported a sex-specific effect in gluteal adipocytes. Although no sex difference was observed in gluteal adipocyte lipolysis in response to epinephrine at baseline, similar to what was observed with acute exercise and the microdialysis studies, chronic exercise training induced a lesser increase in epinephrine stimulated gluteal adipocyte lipolysis in women compared to men [15]. Somewhat contradictory to the microdialysis data, however, a cross-sectional study observed no difference in epinephrine stimulated lipolysis in abdominal adipocytes from trained and untrained, men and women [11]. The limitation with the in vitro approach is that it removes the target cells from the normal circulating hormonal and substrate milieau and so may explain differences in the results between the two methodological approaches. Nevertheless, both trained men and women were shown to have higher abdominal adipocyte lipolytic sensitivity to epinephrine compared to untrained controls [11]. This contrasts to the chronic training data in gluteal adipocytes and suggests a sex-specific, and site specific, effect of exercise training on the lipolytic sensitivity of adipose tissue. Further studies are required, however, to more fully characterize these sex differences in adipose tissue lipolysis in different anatomical sites as well as the response to other lipolytic/antilipolytic stimuli.

Sex-steroid interactions with catecholamines. Sex-steroid hormones may also serve to regulate substrate metabolism via indirect influences on catecholamine action. Relative to men, higher levels of estrogen during exercise in women [3] could act synergistically with sympathetic activation, possibly by enhancing cell signaling that stimulates activity of hormone sensitive lipase. Although purely speculative, subcutaneous adipocytes, especially abdominal adipocytes, could have cell surface estrogen receptors that are not present (or present in much lower density) in other adipose tissue beds and thus selectively enhance lipolysis in this region. Alternatively, IMCL may be more sensitive to the lipolytic actions of catecholamines in females. It is assumed that the mechanism of

epinephrine stimulated lipolysis of IMCL and peripheral adipose tissue is similar, but, if and how the ovarian hormones modify this action is not yet known.

From the data presented above, it might be expected that estrogen replacement therapy would increase the rate of lipolysis, measured at rest, in post-menopausal women but the one study to date does not support that idea [29]. Furthermore, when compared under resting conditions, blood glycerol concentrations and NEFA release in response to administration of epinephrine *or* norepinephrine is similar in men and pre-menopausal women [28,48]. However, the usual feed back loop between the two areas of the sympathetic nervous system is broken under these controlled experimental conditions. In the intact physiological system, the combination of epinephrine and norepinephrine acting in concert may mediate the sex difference in substrate flux. Or, as described above, sex differences in catecholamine action may only be observed when there is an increase in energy requirements (exercise) or when blood glucose is compromised (hypoglycemia).

Although results from controlled studies of single hormones are vital to understanding how the system is regulated, all researchers recognize that there is an interaction between the different endocrine hormones and that studying one in isolation will not fully reflect the complete physiologic mechanism.

4.3. Other regulatory hormones

Other regulatory hormones that could contribute to the sex-based differences in substrate flux in response to metabolic stress are glucagon, cortisol, growth hormone and thyroid hormone. For example, changes in glucagon in response to exercise, fasting and hypoglycemia are lower in females relative to males [9,13]. As glucagon increases hepatic glucose production, this observation is concordant with the lower glucose flux rate in females during fasting and hypoglycemia. Glucose flux during exercise was reported in one study to be equal in men and women [7], but more research is necessary to deduce a role for glucagon in mediating sex-based differences in exercise glucose utilization. The effects of the other regulatory hormones are complicated by multiple feedback mechanisms. Cortisol concentrations are higher in females vs males during hyperinsulinemic/hypoglycemia [13], but the opposite may be true during exercise [3] with no clearly discernable sex-based difference during extended fasting [9]. The growth hormone (GH) response to metabolic stress is lower in females vs males (although resting levels are higher in women) [9,13]. Since GH stimulates lipolysis, differences in the absolute circulating concentration are apparently not responsible for increased lipolysis in females, unless there are sex differences in sensitivity to GH and/or an interaction with other factors. No sex-specific differences have been observed in thyroid hormone levels in response to metabolic perturbations, although changes in thyroid hormone usually occur with more chronic vs acute metabolic stress. Once more, these data emphasize the importance of considering interactions between different hormones, and/or other sex-based factors.

4.4. Insulin action

Insulin action at the liver and muscle. There are conflicting data regarding whether or not sex-steroid hormones can affect insulin's action to suppress endogenous glucose production and stimulate glucose uptake. It appears that women may be more sensitive to the action of insulin on skeletal muscle glucose metabolism [36] but this conclusion is far from established. A number of confounding variables complicate the ability to discern whether there are sex differences in insulin action. Body fat content and distribution, lean tissue mass, and acute and chronic exercise patterns, diet composition, energy balance, and hormonal status all need to be controlled for (or at least accounted for when data are interpreted) when making sex comparisons of insulin action. With respect to hormonal status in women, certain data suggest that insulin action is decreased in the ML vs MF phase of the menstrual cycle [16] and that the use of OCPs may also impair insulin action [21]. Data are not completely consistent, however. Although it seems intuitively contradictory given the preceding data, the relative loss of ovarian hormones after menopause seems to reduce insulin sensitivity. This change, however, may be attributed to advancing age per se and/or increases in visceral adiposity. The influence of hormone treatment (HT), using either estrogen or estrogen plus progesterone, on insulin action has also been difficult to discern. Overall, therefore, it appears that a deficiency of the ovarian hormones or a high progesterone/estrogen ratio impairs insulin action. These observations are consistent with results from more invasive studies conducted in rodents.

Lipid-induced insulin resistance. If differences in insulin action between men and women are real, the question arises as to whether or not this is simply a result of the greater visceral and/or upper body adiposity observed in men relative to women. There is a negative correlation between insulin action and upper body obesity (in particular visceral adiposity). Whether this relationship is also true in normal weight individuals is less well established. In addition, a negative correlation between circulating lipids and insulin action is well accepted by most researchers. Elevated circulating TGs and NEFA, and/or elevated IMCL, are associated with insulin resistance and Type 2 diabetes. Recently, it has been reported that lean, healthy, pre-menopausal women have elevated IMCL compared to men [37,43] and women generally have higher fasting NEFA levels than men [37]. Strangely, however, muscle insulin action in women is not lower than it is in men and may actually be higher, as discussed above. The impact of different types of IMCL (oleate vs palmitate vs ceramide), and/or the manner in which IMCL is "packaged" by droplet proteins such as perilipin, likely explain the paradoxical effects of IMCL content on muscle insulin resistance in the sexes. This is an intense area of current research. Furthermore, certain data show that lean, healthy women do not develop peripheral insulin resistance when circulating NEFA levels are artificially elevated using an intralipid plus heparin infusion whereas men do [20], although this has not always been observed. Nevertheless, these provocative data imply some sex-specificity in at least a portion of the pathophysiology of muscle insulin resistance and possibly diabetes.

Insulin action at adipose tissue. Insulin also acts on adipose tissue to enhance glucose uptake and suppress lipolysis. In vitro measures of glucose uptake from subcutaneous (gluteal or abdominal) adipose tissue show that glucose uptake/fat cell is greater in women.

The sex difference disappears when data are expressed per cell surface area, as women can have larger fat cell size [19]. If the fate of glucose taken up by fat cells in women is predominatly the conversion to glycerol for NEFA re-esterification to TG; this may facilitate TG storage in women and partially account for their greater lipid stores relative men. There are indirect data from meal studies suggesting that insulin does not suppress adipose tissue lipolysis in men as efficiently as in women [27], although not all data are in agreement [25]. Sex differences in the trafficking of ingested nutrients to their sites of storage, and the potential interactions between sex-steroid hormones and insulin in this process, requires further investigation

5. Potential sites of regulation

5.1. Body composition

The sex-steroid hormones are instrumental in determining the differences in body composition and fat distribution between men and women. Their effects are mediated mainly via increased expression of certain genes in target tissues which promote characteristic changes in body composition and fat patterning. At puberty in women, the initiation of the cyclical changes in estrogen and progesterone, along with low levels of testosterone, promote subcutaneous fat accumulation in a characteristic female pattern, i.e. gluteal and femoral in preference to the abdominal and more visceral fat deposits in males.

It has been suggested that the greater subcutaneous body fat in women may promote increased lipid utilization, under stress conditions such as exercise, by virtue of a larger pool of available substrate [45]. However, data show no correlation between body fat levels and either resting or exercise lipid oxidation across gender and across a range of body fat levels. Furthermore, data discussed above, suggest that lipid from non-adipose tissue sources, including IMCL or even circulating TG (derived endogenously from liver secretion or exogenously from meal absorption), are more important as exercise fuel sources in women than men. Also, a simple one-to-one correspondence between the size of body fat stores and lipolysis/lipid oxidation is problematic given that men have greater visceral adiposity; and visceral adipose tissue is considered more lipolytically sensitive than subcutaneous adipose stores (abdominal and even more so femoral). Hence, it might be predicted that males would have higher rates of lipolysis during metabolic stress due to their greater visceral fat mass and higher catecholamine response. Visceral adipose tissue, however, comprises only a small proportion of total body fat and to what extent this adipose tissue depot contributes to whole-body lipolytic rates is uncertain.

To allow for these sex-based differences in absolute total and lean body mass it is conventional to express substrate production and utilization rates, relative to the whole-body, lean body mass, or fat mass, or to account for these differences statistically. When data is treated this way, sex differences in substrate utilization with metabolic perturbations persist, suggesting that the ratio of fat to lean tissue does not explain the sex differences in substrate metabolism.

5.2. Blood flow

The apparent sex difference in lipolytic sensitivity could simpy be explained by sex-based differences in adipose tissue blood flow in response to metabolic perturbation. If, under these circumstances, women maintained a higher blood flow to the subcutaneous adipose tissue, contact between circulating catecholamines (or other hormones/factors) and adipocytes would be increased. In addition, enhanced blood flow might allow greater "drainage" of glycerol and FFA into the general circulation. Indeed, estrogen has been shown to increase nitric oxide production and thus vasodilation [8]. Whether this would be specific to adipose tissue perfusion, or a generalized effect on the entire vasculature, is not known.

5.3. Greater reliance on fat: lipolytic "push" or carbohydrate constraint "pull"?

From a mechanistic viewpoint, an unanswered question concerns which of two possible forces drives the shift to greater fat/less carbohydrate use in women. Does the enhanced ability to mobilize and utilize fat as an energy source, in response to metabolic stress, displace carbohydrate oxidation (a lipolytic "push")? Or, does a limited capacity to utilize carbohydrate leave a deficit that needs to be filled by increased fat utilization (carbohydrate constraint "pull")?

Lipolytic "push". As discussed above, it appears that the absolute quantity of fat stores is less important than the ability to access these stores, i.e. lipolytic capacity and lipid mobilization, in terms of sex differences in lipid utilization. Indeed data has been presented illustrating a potential role for catecholamines in mediating sex differences in the lipolytic response to perturbations. As noted above, increased blood flow to peripheral adipose tissue, especially under conditions of metabolic stress, could also increase NEFA removal and delivery to tissues for utilization. At the cell, there also may be other factors favoring lipid utilization. These are illustrated in Fig. 3. The only data available to date suggest that there is no sex difference in CPT-1 activity whereas in one study, females have been reported to have a greater capacity for β-oxidation relative to men [43]. In animal studies, however, estrogen treatment increased CPT-1 and β-hydroxy acyl CoA dehydrogenase activity whereas progesterone antagonized this effect [5]. More work in the area of enzyme regulation and gene expression is required to better understand sex specific regulation of lipid metabolism.

Carbohydrate constraint "pull". An alternative view is that ovarian hormones confer some form of constraint on carbohydrate utilization. Thus, almost by default, cells are obligated to utilize more lipid to satisfy their energy requirements. Consistent with this hypothesis, the operation of a "reverse" Randle cycle was recently postulated. The reverse Randle cycle proposes that glucose availability and metabolism is the primary determinant of the pattern of substrate utilization within tissues as opposed to vice versa that is, the original Randle hypothesis. Hence, the ovarian hormones may exert effects via one or more of the mechanisms illustrated in Fig. 4. Future research is needed to evaluate sex-specific differences in cellular glucose metabolism at these sites.

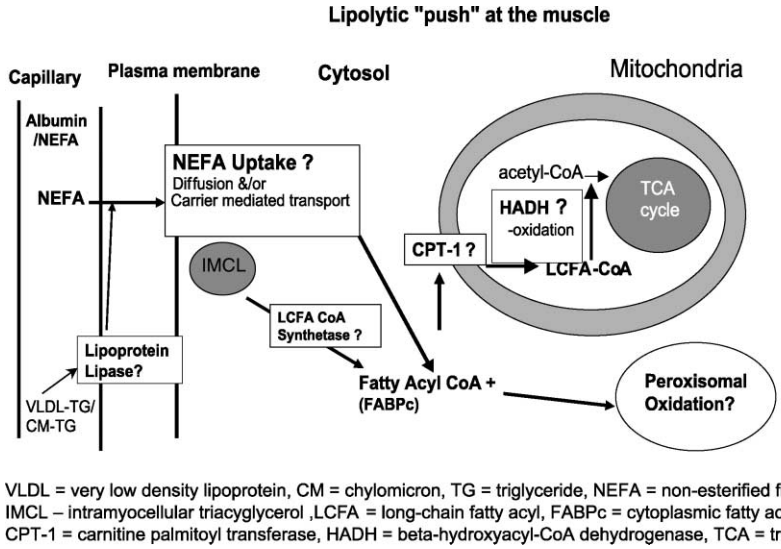

Fig. 3. "?" marks potential sites for sex-based differences in metabolic regulation under the scenario of the lipolytic "push" at the muscle. These include (a) increased release of FFA from circulating TG via LPL action (b) increased ability to take up circulating NEFA (via active and/or passive transport), (c) greater formation of long chain fatty acyl Co-A within cells (higher long-chain CoA synthetase activity) (d) greater transport of long chain fatty acyl Co-A into the mitochondria (greater CPT-1 activity) (e) greater peroxisomal oxidation of NEFAs and/or (f) greater capacity for β-oxidation (greater β-hydroxy acyl CoA dehydrogenase activity).

6. Future directions

Some facet of "femaleness", at least part of which is rooted in endocrine factors, appears to confer a homeostatic advantage in the ability to adapt to changes in metabolic status. This is illustrated by an intriguing study on mice [17] who lacked the peroxisome proliferator-activated receptor alpha (PPARα). PPARα is closely involved in the expression of genes involved in free fatty acid oxidation and peroxisome proliferation. In this study, when male knockout mice were given entoxomir (which blocks CPT-1 activity and thus transport of LCFA CoA into the mitochondria) they accumulated considerable fat in the muscle and liver, became profoundly hypoglycemic and died within 24 h. By contrast, female knockout mice did not accumulate as much lipid or become as hypoglycemic, and 75% survived. Supplementation of male knockout mice with estradiol lowered organ lipid accumulation, led to improved glycemia, and dramatically increased survival rates. It appeared that male mice lacking PPARα and treated with Entoxomir, were unable to utilize FFA and relied exclusively on blood glucose. Similarly treated female mice, apparently by virtue of higher estradiol levels, maintained some capacity to utilize lipid and were seemingly less dependent on glucose as an energy source.

Recently, provocative studies of the insulin signaling pathways have suggested other venues in which females may "better" adapt to metabolic perturbations. Compared with

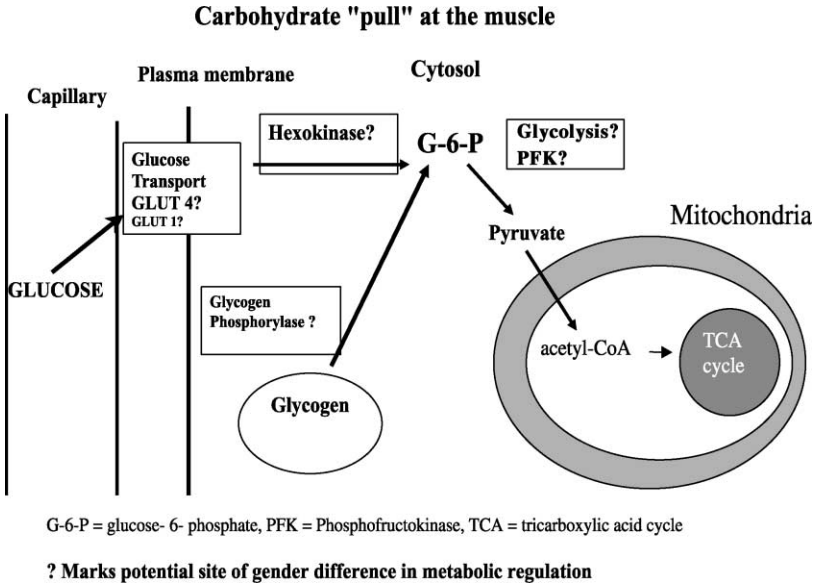

Fig. 4. "?" marks potential sites for sex-based differences in metabolic regulation under the scenario of the carbohydrate "pull" at the muscle. These include (a) lower circulating glucose availability due to lower endogenous glucose production (Ra), (b) lower insulin and/or non-insulin mediated glucose uptake (contraction or non-contraction mediated), (c) reduced glucose phosphorylation within the cell (lower hexokinase and/or glucokinase activity – less likely) (d) decreased glycogen phosphorylase activity and thus decreased glycogen mobilization (e) decreased glycolytic capacity (e.g. lower phosphofructokinase activity) thus lower pyruvate production. The later would lower Malonyl CoA levels which may promote increased lipid utilization.

females, male mice heterozygous for the null alleles of the insulin receptor and insulin-receptor substrates 1 and 2 (in liver, muscle and adipose tissues) developed diabetes at dramatically higher rates [31]. Furthermore, fatty acid-induced insulin resistance in male animals was associated with alterations in cellular fatty acid trafficking and impairment in the proximal insulin signaling pathway [22]. No such changes were observed in female rats. These data suggest that the intracellular metabolism of NEFA may be different in females vs males, which could lead to a decreased accumulation of metabolic mediators that affect insulin signaling.

These studies raise important questions as to the etiology of metabolic disease in men vs women with clinical implications regarding the prevention and treatment of diseases such as obesity, diabetes, and cardiovascular disease. Will sex differences in hormonal regulation of substrate metabolism mandate different treatment (exercise, diet, pharmacological agents) schemes for men and women? Questions such as these require systematic study at both the cellular and whole-organism level. At the cellular level, future work needs to focus on signaling mechanisms by which the sex-steroids exert affects in men vs women. Tissue distribution of the different sex-steroid receptors within cell membranes as well as within the cell need to be identified, along with potential

signaling mechanisms via non-nuclear and nuclear actions. These areas promise to guide a cornucopia of new research in the area of the endocrine regulation of fuel utilization in men and women.

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Sex differences in skeletal development

Lorraine A. Fitzpatrick

1. Introduction

Sex differences in the skeleton begin early in life and impact the growth and maturity of bone. In this chapter, the cellular basis of bone tissue will be reviewed with emphasis on how androgens and estrogens impact these cellular processes in males and females.

2. Bone structure and composition

The skeleton not only provides structural support for the body, it also functions as a reservoir for calcium and phosphate ions. The skeleton participates in calcium homeostasis, a process which is under the influence of sex steroid hormones. Bone consists of an inorganic phase, made up of hydroxyapatite and an organic phase. The organic phase consists of extracellular matrix composed of type I collagen, proteoglycans and noncollagenous proteins. The noncollagenous proteins include osteocalcin, bone sialoprotein, osteopontin, osteonectin and thrombospondin. There are numerous growth factors and cytokines within bone matrix that regulate bone remodeling.

There are three major cell types in bone, osteoblasts, osteocytes, and osteoclasts. There are many other cell types present in the bone marrow which, due to its proximity to bone, play a vital role in the differentiation of bone cells and the regulation of bone modeling.

Osteoblasts. The cell responsible for bone formation and mineralization is the osteoblast. Osteoblasts are derived from mesenchymal stem cells. Although much is known about the mechanisms by which the stem cells become committed to the osteoblast phenotype, all the factors required are not fully understood. The core binding transcription factor (CBFA1) is essential for osteoblast differentiation. Loss of function of CBFA1 in transgenic mice results in a complete lack of ossification of cartilage and heterozygous loss of function causes cleidocranial dysplasia. The fibroblast growth factors (FGFs), transforming growth factor beta (TGFβ), bone morphogenic proteins (BMPs) are other growth factors required along the osteoblast differentiation pathway. Glucocorticoids, estrogen, androgens and 1,25-dihydroxyvitamin D also influence osteoblast differentiation.

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The periosteum and bone marrow are important sources of mesenchymal progenitor cells. FGF and TGFβ are potent mitogens for the periosteal osteoprogenitors in marrow stromal cells. BMPs also have osteoinductive effects and differentiation activities that are dependent on their concentration and the progenitor cell phenotype. Parathyroid hormone (PTH) stimulates the growth of osteoprogenitor population and parathyroid hormone related peptide (PTHrP) functions as a local cytokine regulating cell growth and differentiation during development.

The progression of osteoblast maturation requires sequential activation and suppression of genes that encode varying phenotypic and regulatory proteins. Committed preosteoblasts are recognizable by detectable levels of alkaline phosphatase enzyme activity, an ectoenzyme and early marker of osteoblast phenotype. As the osteoblast differentiates, it becomes capable of the biosynthesis and organization of the bone extracellular matrix. The osteoblasts secretes type I collagen and bone matrix proteins as osteoid, or unmineralized matrix. The collagen fibers mature to support mineral deposition in cell–matrix and cell–cell interactions, facilitated by integrin adherence are important for this process. The accumulated extracellular matrix down regulates the growth of the osteoid seam along the bone surface. The mineralization stage occurs conjointly with expression of markers of mature osteoblasts such as the bone matrix proteins osteocalcin and bone sialoprotein which may regulate the ordered deposition of mineral.

Osteocytes. The osteocyte is a terminally differentiated osteoblast. Osteocytes develop as mineralized osteoid enrobes the surface osteoblasts. Osteocytes are distinguished by numerous cellular extensions of their plasma membrane that lie within the calcified bone matrix. These cytoplasmic projections enable the osteocyte to respond to mechanical and biochemical stimuli and play a central role and initiate appropriate modeling and remodeling responses.

Osteoclasts. Osteoclasts are multinucleated bone resorbing cells derived from hemopoietic precursors of the monocyte/macrophage lineage. Cells are characterized by the presence of a ruffled border and lysosomal enzymes including tartrate resistant acid phosphatase. Hydrogen ions generated by carbonic anhydrase II are delivered across the basal cell membrane by a proton pump to dissolve bone mineral. Lysosomal enzymes including collagenase and cathepsins are released and together degrade bone matrix.

The interactions between the osteoblasts and osteoclasts are essential for bone remodeling. The characterization of the cytokine system involving tumor necrosis factor ligand family members has provided new insights into osteoclast formation, differentiation, action and apoptosis. Receptor activator of NFκB ligand (RANKL) is a member of the tumor necrosis factor ligand family and is produced by bone marrow stromal cells, osteoblasts, chondrocytes, osteoclasts and other cells. RANKL stimulates the differentiation, fusion and survival of osteoclast precursor cells. In vivo, the administration of RANKL to mice results in severe osteoporosis due to increased osteoclast-mediated bone resorption [37]. Mice deficient in RANKL present with severe osteopetrosis from the lack of osteoclasts [35].

The receptor for RANKL is RANK, a transmembrane protein that is a member of the TNF receptor superfamily. RANK is essential for normal osteoclast differentiation and activation. Transgenic mice that overexpress soluble RANK have an osteopetrotic phenotype [30].

Osteoprotegerin (OPG) acts as a soluble decoy receptor and prevents RANKL from binding and activating RANK on the osteoclast surface. In vitro, OPG inhibits the differentiation, survival, and fusion of osteoclast precursors, blocks activation of mature osteoclasts and induces osteoclast apoptosis [29]. Mice with targeted ablation of OPG have osteoporosis due to enhanced osteoclast activity [6]. Overexpression of OPG creates mice with osteopetrosis, in a manner similar to the RANKL and RANK knockout mouse models. Parental administration of OPG to rats increased BMD and prevented ovariectomy-induced bone loss [53].

In the skeleton, these three components, RANKL, RANK and OPG coordinate the actions of the osteoclast. RANK is expressed by osteoclasts and cells of the osteoblast lineage produce RANKL and OPG. Many cytokines and hormones can alter the interactions of RANKL/RANK/OPG and alter osteoclast homeostasis. OPG production is stimulated by multiple factors such as the proinflammatory cytokines such as IL-1 and TNFα, TGFβ, BMP-2 and -7, 1,25-dihydroxyvitamin D, 17-β estradiol and PTH, to name a few. RANKL is also regulated by a myriad of cytokines and hormones including IL-1, IL-6, TNFα, and the steroid hormones.

RANKL and OPG mediate some of the antiresorptive effects of estrogen. Estrogen stimulates the expression of OPG in osteoblasts via transcriptional activation of ERα. Estrogen deficiency in rats results in decreased OPG expression and increased RANKL production; estrogen therapy prevents these effects [40]. Administration of OPG to animals or humans is consistent with the effects at the cellular level and results in suppression of bone turnover.

3. Bone remodeling

Bone is the dynamic tissue, being constantly broken down and rebuilt. The coordinated actions of osteoclasts and osteoblasts are essential in the initiation and completion of the remodeling cycle. Remodeling is a surface phenomena and serves to maintain the mechanical integrity of the adult skeleton and provides a mechanism by which calcium and phosphate ions may be released. Osteoclast activation is the first step in the remodeling sequence. Osteoclasts are activated at specific sites and over a period that has been estimated to last ten days, resorb an area of bone. The defect that is created is filled by osteoblasts that proceed to generate osteoid that then mineralizes. The formation phase of this process is estimated to take approximately three months. In a young adult skeleton, the amounts of bone formed and resorbed are similar. The sites at which bone remodeling occurs are termed basic multicellular units (BMUs) or bone remodeling units. In normal human adults, approximately 20% of cancellous bone surface is undergoing remodeling at any given time.

Many systemic hormones and locally produced cytokines and growth factors regulate bone remodeling. Mechanical stress is also a major determinant of remodeling with the osteocyte as the major mechanosensory bone cell. Many hormones such as thyroid hormone, glucocorticoids and 1,25-dihydroxyvitamin D influence bone modeling. Estrogens and androgens are also important systemic factors during the process of bone

remodeling. The remainder of this chapter will focus on sex steroids and the sex differences that occur in the skeleton.

4. Effect of sex steroids on bone

Sex steroids contribute to skeletal growth, maturation and maintenance in both the male and female skeleton. Both estrogens and androgens are essential for skeletal acquisition and preservation of bone mass. Sex steroids influence the function of osteoblasts and osteoclasts. For example, many growth factors and cytokines are secreted by osteoblasts under the influence of estrogen including TGFβ, TIEG, a TGFβ-inducible gene that inhibits DNA synthesis, and insulin-like growth factor-1. BMP6 mRNA expression increases in response to estrogen in a fetal osteoblast cell line. The regulation of bone matrix protein production by estrogen is complex and there have been multiple conflicting studies that may reflect the differences among osteoblast-like cells in culture. In general, estrogen acts as a mitogen in osteoblast cells and increases the expression of alkaline phosphatase and type I collagen. In some systems where estrogen has an antiproliferative effect, effects on alkaline phosphatase may be variable. Estrogen increases expression of receptors for growth hormone, progesterone and 1,25-dihydroxyvitamin D. PTH responsiveness in osteoblast-like cells is also modulated by estrogen.

Estrogen receptors are present in osteoclasts and the lack of estrogen is associated with an increase in bone resorption. Estrogen affects both osteoclast number and activity. In oophorectomized animal models, there is a marked increase in the proliferation and differentiation of osteoclast precursors and reduced osteoclast apoptosis. In postmenopausal women, increased production of bone-resorbing cytokines such as interleukin-1 (IL-1), granulocyte–macrophage-colony stimulating factor, and TNFα by monocytes occurs after natural or surgical menopause, and these changes are attenuated by the administration of exogenous estrogen.

The presence of the androgen receptor in bone cells has been established [11], but less is known about the actions of androgens in the skeleton. Androgens enhance osteoblast proliferation, consistent with their anabolic actions on the skeleton (for review see Ref. [32]). Variable results have been noted regarding the expression of markers of osteoblast differentiation such as alkaline phosphatase or osteocalcin in response to exposure to androgens. In general, dihydrotestosterone (DHT) enhances expression of alkaline phosphatase, type I collagen, osteocalcin and mineralization of extracellular matrix. DHT and dehydroepiandrosterone (DHEA) increases activity and concentrations of bone-regulating cytokines and growth factors such as IL-6 and TGFβ.

5. Estrogen receptors and bone cells

Estrogen has multiple roles in the skeleton and mediates some actions through the estrogen receptor. Two estrogen receptors have been identified, estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ) (see also Chapter 3). These two receptors exhibit homology in the DNA- and ligand-binding domains and have similar binding affinities. ERα and ERβ differ in the N-terminal regions. Several studies have documented

the presence of ER in osteoblasts. During osteoblast differentiation, there is differential mRNA expression of ERα and ERβ. ERβ increased gradually over time with increasing maturity of osteoblasts [1]. Studies by Lim et al. suggest that both subtypes of the ER are expressed in cancellous bone, but not in cortical bone [39]. In addition, expression is reduced after ovariectomy. ERβ has been localized to the callus of human bone [65], to rat bone [50] and to human growth plate cartilage [49]. Differential expression of ERα and ERβ occur during osteoblast differentiation. To explore the differential expression of ERα and ERβ in bone, Bord et al. [5] evaluated ER expression in human bone. There were no differences in expression related to age or sex. Marked differences in expression were noted in cancellous and cortical bone. ERα was more evident in cortical bone and ERβ was dominant in cancellous bone. Osteocytes in the cortical bone demonstrated intense ERα immunoreactivity, and osteoblasts within osteons and on the periosteal and endosteal surfaces were strongly positive for ERα. In the cancellous bone, low levels of ERα expression were observed in osteocytes and osteoblasts. In cortical bone, most osteocytes expressed little ERβ. Osteoblasts in cortical bone also expressed low levels of ERβ, except on the endosteal surface. In contrast, ERβ expression was strongly present in cancellous bone osteocytes, with intense staining present in osteoblasts apposed to bone spicules within the primary spongiosa and in the mature cancellous bone. Many osteoclasts also stained positively for ERβ. These anatomical-based studies suggest that differential distribution of the ERα and ERβ may result in differential regulation by estrogen at cancellous versus cortical sites in the skeleton.

6. Sex steroids and the skeleton: lessons from animal studies

"Knockout" models for the estrogen receptor have allowed new insights into the role of sex steroids in the skeleton. However, often there are unexpected findings in these models, so that mice that are deficient in an estrogen receptor may not mimic the human phenotype completely.

The ERα knockout mouse has a 20–25% reduction in bone density [12] compared to normal littermates. In the male ERα knockout mouse, there is no skeletal phenotype [70]. In the mice that were deficient in ERα (ERKO) or both ERα and ERβ (DERKO), long bone growth was foreshortened suggesting that the missing ERα was responsible for this defect. In mice lacking ERα, there was a decrease in total body, femoral and spine bone mineral content (BMC). However, cancellous bone was not changed in any of the knockout animals, while cortical bone was reduced in ERKO and DERKO mice. The reduction in cortical bone was due to reduced cross-sectional area due to smaller periosteal and endosteal circumferences [66].

However, the female ERβ knockout (BERKO) has an increase in cross-sectional cortical area resulting in an increase in BMD. Although the lack of estradiol through ovariectomy results in a rapid increase in bone turnover and bone loss, deletion of one or both ERs failed to show the same effect in female mice. The aged BERKO female mice had significantly higher trabecular bone volume due to a less pronounced loss of trabecular bone during adulthood. In these BERKO female mice, there was an increase in the mRNA expression of ERα suggesting that ERβ is involved in the regulation of trabecular bone

during adulthood and ERβ acts are a repressor by counteracting the stimulatory action of ERα on bone formation [69]. This group of investigators suggested that ERβ was involved in both the regulation and maintenance of cancellous bone in female mice [70]. However, Sims et al. concluded that ERβ was essential for the pubertal feminization of cortical bone in female mice. Other investigators have found differing results, with explanations including the different backgrounds of the mice or different compensatory mechanisms being induced to adjust for the missing receptor action. ERβ can antagonize the activity of ERα that leads to complex interactions that are difficult to interpret. In some of the ERα knockouts, for example, a truncated receptor is expressed that effectively alters bone under pharmacological conditions. Studies by Gentile et al. indicate that this may be possible, as high levels of estrogen can overcome the bone loss noted in DERKO animals after ovariectomy [21]. Studies with full knockout mice that do not express any form of the receptor confirms that ERα only regulates bone remodeling in the male mouse [54].

In the ERα deleted mice, circulating levels of estradiol and testosterone were altered [54], a finding not seen in the ERβ deficient mice. To eliminate the role of sex steroids that might compensate for ER deficiency, ERKO, BERKO and DERKO mice were orchidectomized. Total body area bone mineral density (BMD) fell in all groups, but animals with an intact ERα (BERKO or wild type animals) responded to estrogen with maintenance of BMD [41]. These studies confirm that in the male skeleton, ERα is the predominant ER necessary for development and maintenance of the male skeleton.

Another animal model has been utilized to evaluate the role of androgens on the skeleton. The testicular feminized rat has androgen insensitivity due to an inactive androgen receptor. The skeletons in these animals have reduced bone size in terms of femoral length, diameter, and cortical thickness [64]. These studies emphasize the importance of androgen on bone size, usually through regulation of the outer periosteal surface. This finding was confirmed in elegant studies in which ovariectomy in normal female rats results in an increase in periosteal bone formation rate. In contrast, orchidectomy in normal male rats decreases periosteal bone formation [62].

7. Skeleton growth in childhood and adolescence

Infancy is a period of the most rapid skeletal growth, not only in relative terms but also in absolute terms. A newborn infant has a disproportionately large head and limbs are short relative to the rest of the body. At the time from age 3 years to the onset of puberty, children of both sexes grow more slowly without any major systemic fluctuation. For some children, there is a small growth spurt around age 8 years with further deceleration before puberty.

Adolescence is a period of rapid skeletal development and is essential for the attainment of peak bone mass. Many factors are involved in the skeletal development at this age, some of which are programmed genetically and others that are under hormonal influence. Many candidate genes are under evaluation for prediction of peak bone mass in prepubertal children. Lifestyle choices such as calcium intake or physical activity can also alter bone accretion and development.

During puberty, there is a sharp increase in growth rate in both sexes (Fig. 1). Epiphyseal fusion results in the cessation of growth. Several mechanisms result in skeletal

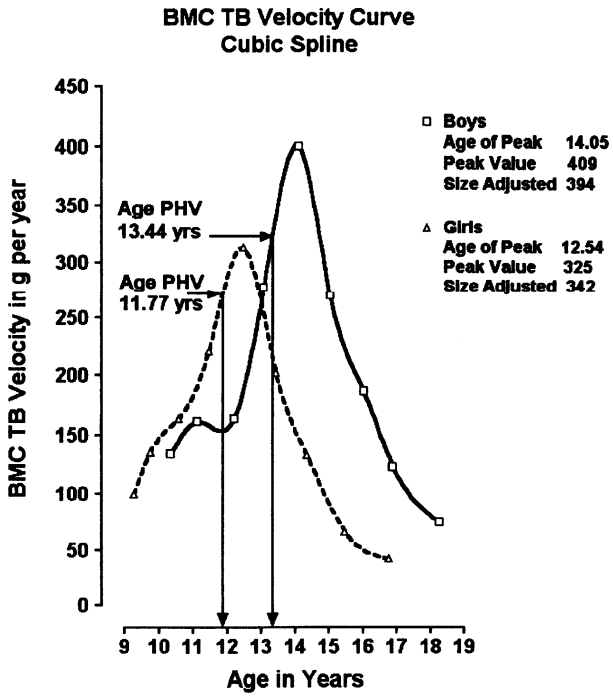

Fig. 1. In a study of 53 girls and 60 boys, physical activity, dietary intakes and anthropometry were assessed every 6 months. Dual-energy X-ray absorptiometry scans were performed of the total body (TB) annually. Distance and velocity curves for height and bone mineral content were fitted for each child at several skeletal sites using a cubic spline procedure. The ages at peak height velocity (PHV) and peak bone mineral content (BMC) were identified. Sex-based differences are evident as the child progresses through puberty.

dimorphism between boys and girls. The peak velocity and duration of growth spurt is less in girls than in boys. This results in a 13 cm mean difference in adult height between men and women. The average age for the start and peak of pubertal growth spurt is younger in girls compared to boys. BMD is similar in boys and girls, but the bones in boys are larger providing distinct biomechanical advantages [16].

As more is learned about the accretion of bone mineral during puberty, peak bone mass has become a focus of attention in the attempt to increase bone mass in children. The rate of mineral accretion is very rapid during puberty and peak bone mineral velocity is higher in boys than in girls. Peak bone mass attainment varies with specific skeletal site. For example, the hip matures at age 16–8 years [45]. In girls, approximately 90% of the total body mineral content is achieved by 16.9 $\pm$ 1.3 years. Ninety-nine percent of total body mineral content is achieved by 26.2 $\pm$ 3.7 years [60].

8. Hormonal determinants of peak bone mass

There is a large body of evidence of the importance of sex steroids in the determination of peak bone mass. The varying amount of estrogens and androgens in the premenopausal,

Table 1
Serum levels of several steroid hormones at menopause

	Premenopausal ovulatory woman	Ovarian conservation	Bilateral oophorectomy
Estradiol (pmol/L),18–110	18 ± 9	18 ± 7	16 ± 8
Estrone (pmol/L)	70 ± 34	69 ± 39	67 ± 35
Androstenedione (nmol/L), 2.9–9.9	1.08 ± 0.39	0.95 ± 0.44	0.99 ± 0.45
Testosterone (nmol/L)	0.56 ± 0.26	0.4 ± 0.2	0.29 ± 0.14

women undergoing hysterectomy with intact ovaries and women undergoing bilateral oophorectomy are listed in Table 1. Adult patients with hypogonadotrophic hypogonadism commonly have bone loss, as a result of inadequate bone mineral accrual during puberty [18]. Adult men with constitutional delay also have decreased bone mass [19]. In young female ballet dancers, those with late menarche and long periods of secondary amenorrhea have a higher incidence of metatarsal stress fracture [67]. Even women who recover from anorexia nervosa during adolescence may have persistent osteopenia [2]. The time during adolescence when the skeleton accrues mineral is a critical time that is sensitive to changes in hormone status.

9. Bone measurement techniques: problems in understanding sex-based differences

Many of the studies that evaluate peak bone mass are fraught with problems. Normative data is usually obtained from cross-sectional studies of adolescents. Techniques by which BMD is measured may vary greatly in precision, methodology, the normative database utilized and determination of absolute density. Pubertal and ethnic differences may not be accounted for in the assessment of BMD. Tanner stage is a major determinant of BMD in girls whereas in boys, weight is a major determinant [34]. Precise, noninvasive methodology to measure bone has improved our understanding and ability to predict fracture rates and measure bone loss. Dual energy X-ray absorptiometry (DXA) is a commonly used technique for assessment of BMC. However, there are some caveats about the technique that make it difficult to assess sex-based differences in bone mass. DXA relies on attenuation or absorption of energy as the X-ray scans the region of interest. Two energy settings are used to distinguish soft from mineralize tissue components. DXA measurements are based on a two-dimensional projection of a three-dimensional structure. As a result, there are three skeletal parameters that must be accounted for: the size of the bone scanned, the bone volume and the mineral density of the bone. The ensuing value is referred to the BMC that can be expressed as BMC per surface area (BMD) when the projected area of the bone is accounted for. However, DXA scans only approximate bone size such that bones with equal mineral density but different cross-sectional dimensions may result in different BMC and BMD values. Correction factors may create additional errors especially since they cannot account for the changes in the size and shape of the bone during growth [46].

Recent evidence suggests that neither cancellous nor cortical bone density differs between men and women [23]. The cross-sectional area of the vertebral column is 11% smaller in prepubertal girls than in prepubertal boys matched for age, height and weight [24]. With growth and maturity, this difference becomes magnified with about a 25% smaller cross-sectional area in vertebrae in women compared to men, even when accounting for body size. Cross-sectional areas of the femur, however, do not differ between males and females when matched for age, height and weight [25]. Body weight appears to be the driving force in determination of cross-sectional and cortical bone area at the femur mid-shaft. It has been proposed that mechanical loads may be responsible for this lack of difference [63].

During the growth of long bones, the mass of bone inside the periosteum increases in proportion to the enlarging column of the whole bone. Volumetric apparent BMD of long bones such as the femur or radius is independent of age and sex in the 7–9 years old age group. At puberty, trabecular apparent BMD increases in both boys and girls; thus growth does not result in more dense bone, but in a larger bone in males. In young adulthood, men and women have the same volumetric BMD but have differences in bone size [52].

Modeling and remodeling of the periosteal and endosteal surfaces result in differences in bone diameter. Sex and racial differences in femoral neck diameter, for example, are greater than the differences in cortical thickness.

Periosteal apposition increases bone width in both sexes during pubertal growth. During puberty, there is an acceleration of periosteal apposition with less endocortical expansion in boys. The end result in boys is an increase in bone diameter, cortical thickening and an increase in the medullary diameter. The accelerated periosteal apposition is likely to be dependent on androgens [73]. In females, periosteal apposition is inhibited by estrogen and endocortical bone formation is stimulated. The result is an increase in cortical thickness and narrowing of the medullary cavity [61].

One reason that women have more fractures than men is due to the fact that the smaller skeleton experiences more architectural damage and is less able to adapt. The larger cross-section area of bone over which mechanical stress can be distributed results in a more stable bone. In men, as the endosteal surface is removed, the reduced trabecular surface of bone is partly compensated for by periosteal bone formation. Although this happens in both sexes, it occurs to a greater extent in men, thus creating bone that is less likely to be damaged by stress.

10. The role of androgens in the female skeleton

In testicular feminization, an inactivating mutation in the androgen receptor results in androgen resistance in XY males. Most of the subjects undergo orchiectomy to avoid testicular cancer and are raised as females. These phenotypic females have reduced BMD [43]. There was a significant decrease in BMD at the spine and hip, especially in those women with poor compliance with estrogen therapy.

In women who have polycystic ovarian syndrome (PCOS), metabolic syndrome is a significant feature. In addition, these women have hyperandrogenism and higher BMD [72]. However, in some studies, levels of testosterone were not correlated with lumbar

spine BMD and correlated with regional muscle mass [15]. Others have suggested the obesity associated with this syndrome may account for the increase in BMD noted. In a study of PCOS women with lean body mass (BMI $< 26\ kg/m^2$), androgen levels were significantly elevated compared to lean controls, but there were no differences between the two groups in total body BMD [26]. Overall, the findings did not establish on the role of androgens in skeletal health in women with PCOS.

Women with amenorrhea may have higher levels of androgens, and escape the detrimental effect of estrogen deficiency on the skeleton [7]. Perimenopausal women with higher androgen levels have slower rates of bone loss than their counterparts with low androgen levels, independent of estrogen status [56].

Observational studies have attempted to correlate serum levels of androgens and BMD in women. The evidence is often conflicting, and different approaches have confounded the literature on the role of androgens in premenopausal and postmenopausal women. In a study by Slemenda et al. [55], pre-, peri- and postmenopausal women were evaluated for the effect of endogenous sex steroids and sex-hormone binding globulin on bone mass and rates of bone loss. Multiple measurements of bone mass were made over 2–8 years. In all women, regardless of menopause status, bone mass was negatively associated with concentrations of sex-hormone binding globulin and positively associated with weight. Bone loss was significantly associated with lower androgen levels in premenopausal women and with lower estrogens and androgens in peri- and postmenopausal women.

The role of androgens in patients with osteoporosis-related fractures also suggests that androgens have a life-long role in the skeleton in women. Longcope et al. measured androgens and estradiol levels in postmenopausal women with vertebral crush fractures. A comparison group consisted of age-matched women who did not have a vertebral fracture. Women with a vertebral fracture had a lower metabolic clearance rates of estrone and testosterone than those women in the control group. The women with fractures also had a decrease in androgen levels compared to the control women [42].

11. Treatment of women with androgens: effect on the skeleton

Attempts to increase BMD in women have included the use of low doses of testosterone, adrenal androgens or synthetic androgens. Most studies have utilized androgens in combination with estrogen with the rationale that combining the anabolic effect of androgen with the anti-resorptive effect of estrogen would provide the greatest increase in BMD. In women, the premenopausal ovary contributes about half of the circulating androgens in women. Peripheral testosterone is produced from conversion of the weaker androgen androstenedione. The adrenal gland produces large quantities of the weaker androgens DHEA and DHEAS which can be converted to androstenedione and then to testosterone or estrogen. Circulating levels of total and free testosterone decline 50% between the third and fifth decade of life [74]. In addition, DHEA and DHEAS levels decline continuously with age. This is due to the age-related decline in adrenal androgen production and loss of the midcycle increase in ovarian testosterone secretion in the late reproductive years. After ovariectomy, both testosterone and androstenedione fall acutely by approximately 50% [31]. In women on a combined oral contraceptive pill or on oral

hormone therapy, there is a decline in circulating free testosterone [44]. The latter is a result of increased sex hormone binding globulin combined with suppression of LH production by the pituitary, and resulting in a lower stimulus for the ovarian stromal production of testosterone.

Beneficial effects of testosterone replacement on BMD in women have been reported. Using a combination of estradiol and testosterone implants in a prospective, 2-year, single-blind randomized trial, women were treated with either estradiol alone or estradiol with testosterone. Women with an intact uterus received cyclical oral progestins for uterine protection. BMD by DEXA of the total body, lumbar spine and hip increased significantly in both treatment groups, but the increase was more rapid in the testosterone-treated women at all sites. In addition, the increase in BMD was significantly greater at all sites in women on the testosterone plus estradiol compared to estradiol alone [13]. In other studies, oral esterified estrogens combined with methyltestosterone suppressed markers of bone resorption, increased markers of bone formation and increased BMD at the spine greater than estrogen alone [51,68]. Synthetic androgens had been used to increase BMD in women, but side effects often limit their use. Nandrolone decanoate has been studied in several trials with positive effects on skeletal metabolism [20,22,48].

There are fewer controlled trials of the effects of adrenal androgens on the skeleton. In a small uncontrolled trial, percutaneous DHEA (10% cream) was administered to postmenopausal women. There was a significant increase in hip BMD at 6 and 12 months compared to baseline values [36]. In women with adrenal insufficiency, replacement of DHEA in a double-blind, placebo-controlled, cross-over trial provided 4 months of DHEA to 24 women, ages 23–59 years. There were no significant differences in markers of bone formation (osteocalcin) or resorption (pyridinoline or deoxypyridinoline crosslinks) [8].

12. The role of estrogen in the male skeleton

Initially, it was thought that androgens played a dominant role in the male skeleton, but several emerging lines of evidence have made it clear that estrogen is essential to the male skeleton. In 1994, a 28-year-old man was described that had a point mutation in the ERα gene, replacing cytosine with thymidine at codon 157. The result was a severely truncated estrogen receptor that was incapable of binding estrogen. The subjects' serum levels of estradiol and estrone were above normal, bound and free testosterone and dihydrotestosterone levels were normal, but LH and FSH were in the castrate range. The patient was extremely tall at 204 cm, had eunuchoid proportions and the epiphyses had not fused. His bone age was 15 years and he was still growing. The BMD as assessed by DXA was more than two standard deviations below average for a 15-year-old male. Large doses of exogenous estrogen resulted in 10-fold higher levels of estradiol, but no skeletal response [58].

In another "experiment of nature", three men with aromatase deficiency have been found with point mutations in exon IX [9,47] or exon V [14] in the aromatase gene. In all three cases, although the mutation was slightly different, there was no aromatase activity and estrogen levels were undetectable. The two adult men with mutations in exon IX had unfused epiphyses, eunuchoid features, genu valgum and a bone age of 15 years. The patient described by Morishima et al. [47] had markedly elevated androgen levels and

reduced BMD. Both men responded to the administration of estrogen [4,9]. In one patient [4,9], the administration of estrogen resulted in a fall in androgens, and LH and FSH levels returned to the normal range. Longitudinal growth ceased and the epiphyses closed with estrogen therapy. BMD increased substantially with an improvement of 20.7, 15.7 and 12.9% in the lumbar spine, femoral neck and forearm over a 3-year period. These cases illustrate an anabolic effect of estrogen on the male skeleton. In addition, these syndromes of estrogen deficiency or resistance in men confirm the essential role of estrogen in the male skeleton. Without estrogen, continued skeletal growth occurred and there was no pubertal growth spurt.

In the testicular feminization syndrome, point mutations in the androgen receptor prevent XY males from responding to androgens. However, they have normal pubertal growth spurts and respond to estrogen [28,71]. Premature skeletal maturation is noted in patients with estrogen-secreting tumors [10]. This suggests that the pubertal growth spurt is under regulation of estrogen in the male skeleton.

13. Studies in adult men: effect of sex steroids on the skeleton

To test the role of estrogen and androgen on the maintenance of bone mass in the adult men, several investigators have evaluated the role of sex steroids in the mature skeleton. In one cross-sectional study, Slemenda et al. [57] found significant correlation with BMD at various sites and estradiol levels in men over 55 years of age; there was an inverse correlation with testosterone levels. In a study of community-dwelling men aged 50–89 years, a statistically significant positive relation was seen between bioavailable estradiol and BMD at all sites [27]. However, these studies could be complicated by the inclusion of men that did not achieve an adequate peak bone mass, and not necessarily reflect the current role of estrogen in the skeletal maintenance. In longitudinal cohort studies, the rates of change in BMD are related to sex steroid levels [33]. In younger men aged 22–39 years, the rate of increase in BMD of the forearm was significantly correlated to serum total and bioavailable estradiol and estrone levels but did not correlate with total or bioavailable testosterone levels. In an older cohort of men aged 60–90 years, the rates of bone loss at the forearm sites, which is largely cortical, were most closely correlated with serum bioavailable estradiol levels. The authors suggest that a threshold of estradiol levels are important in that estradiol levels less than 40 pmol/L may result in bone loss in older men.

14. Clinical trials of sex steroids on the male skeleton

To establish a direct causal relationship between estradiol and the male skeleton, several studies have altered the sex steroid levels in men to assess the effect on markers of bone metabolism. In one study, Falahati-Nini et al. evaluated the relative contribution of estrogen and testosterone in elderly men [17]. Endogenous testosterone and estrogen levels were suppressed with the combination of an aromatase inhibitor and a long-acting GnRH agonist. The men were treated with physiological doses of estrogen, testosterone or the combination and markers of bone formation and resorption were measured. Significant increases in urinary deoxypyridinoline (Dyp) and N-telopeptides of type I collagen (NTx),

markers of bone resorption were prevented with complete replacement of both estrogen and testosterone. The group treated with estrogen alone also almost completely prevented any change in bone resorption markers. The group treated with testosterone alone, however, was less effective in preventing bone resorption. The effects of estradiol on urinary NTx and Dyp were highly significant and accounted for 70% of the effect of sex steroids on bone resorption. Testosterone accounted for only 30% of the effect. In a slightly different trial design, Leder et al. confirmed an independent effect of testosterone on bone resorption [38]. In a slightly longer term study, older men were on 2.0 mg/day of anastrozole, an aromatase inhibitor. The authors concluded that endogenous estrogen derived from aromatization of testosterone plays a role in bone metabolism of older men by limiting the rate of bone resorption [59].

15. Summary and future direction

Sex-based differences in the skeleton is an area of intense research and many new concepts in bone growth and differentiation have developed over the past years. Estrogens and androgens have targeted effects at the cellular and organ levels. Initially, it was thought that estrogen was essential for the female skeleton, with androgens playing a dominant role in the male. Evidence has proved this concept to the contrary, and estrogens are critical to the development of skeletal health in both sexes. Androgens also play an important role in bone and their anabolic nature make them future therapeutic targets. Lastly, sex differences in skeletal size has lead to a better understanding of the mechanical differences in the properties of bone. Additional information on sex-based differences in bone architecture will provide insightful information on the determinants of bone strength.

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Sex differences in steroid-induced synaptic plasticity

Russell D. Romeo and Bruce S. McEwen

1. Introduction

Steroid hormones have a wide range of effects on the central nervous system (CNS). For instance, steroid hormones help shape the CNS during perinatal development (see Chapter 18), and continue to modulate the structure and function of the CNS into adulthood and throughout the life span of the organism. Specifically, steroids can influence such factors as neuronal survival, neurogenesis, neurite outgrowth, synaptogenesis, receptor expression, RNA synthesis, and neuronal excitability.

The structure and function of the male and female CNS differ on several parameters, and most of the structural differences between the male and female CNS are established early in development by the perinatal hormonal milieu. Examples of these structural differences include sexually dimorphic brain nuclei, in which particular cell groups are larger in either males or females. However, other sex differences in the structure and function of the CNS are more subtlety affected by circulating steroid hormone levels in adulthood, and may play important roles in sex-based physiology and pathophysiology.

In this chapter, basic steroid hormone biochemistry and mechanisms of action, and the influence of sex steroids on the sexual differentiation of the CNS during perinatal development will be reviewed. Then, discussion will focus on sex differences in steroid-induced synaptic plasticity in the adult brain and what role sexually differentiated afferents may play in this plasticity. Particular emphasis will be placed on the estrogen-induced increase in dendritic spine density in the CA1 pyramidal cells of the hippocampus. This topic is relevant to the growing body of evidence for the actions of sex hormones outside of the reproductive neuroendocrine axis. It also tells an important and emerging story about the importance of non-genomic as well as genomic molecular actions of estrogens. Finally, based upon the developing view that sex steroids work via multiple mechanisms and interact with neurotransmitters in many regions of the CNS, areas of research will be identified that need to be pursued to further our understanding of how the sex of the organism and steroid hormones interact to influence differences in steroid-induced synaptic plasticity.

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2. Steroid hormone biochemistry and mechanisms of action

In mammals, the precursor to all steroid hormones is cholesterol. Steroid hormones are characterized by their three six-carbon rings and one five-carbon ring. The sex steroids (e.g. androgens and estrogen) are synthesized from cholesterol via enzymatic cleavage. For example, testosterone is produced from a series of enzymatic reactions that convert cholesterol to progesterone, then to testosterone acetate, and finally to testosterone. Testosterone is the precursor of estrogen, such that estrogen is formed by the aromatization of testosterone by the aromatase enzyme. In males, the testes are the major endocrine gland that produce and secrete testosterone, while the ovaries are the major source of estrogen in females. Although testosterone is considered the "male sex steroid" and estrogen the "female sex steroid" it is important to note that testosterone and estrogen are found in both sexes. In fact, estrogen formed locally in the CNS of males by the aromatization of testosterone is largely responsible for the masculinization of the male brain and plays a major role in activating male sexual behavior.

Steroid hormones act on a variety of cell types through many different mechanisms. In the context of this chapter, emphasis is given to how steroid hormones act on neurons, and specifically on how steroids affect synaptic function differently in males and females. Mechanisms of action of sex steroids on neurons are outlined briefly below as the general topic has been discussed in detail in Chapters 3–5.

The conventional mechanism of steroid hormone action consists of four basic steps [17]. First, the hormone travels through the blood stream (or is formed locally) to act on the target cell. Second, the lipophilic hormone then diffuses through the plasma membrane of the neuron and binds to its intracellular receptor located in either the cytoplasm or the nucleus, causing the dissociation of a heat-shock protein that is commonly bound to the unoccupied hormone receptor. These heat-shock proteins are thought to stabilize and, therefore, extend the half-life of the receptors. Third, the hormone–receptor complex then undergoes a conformational change (i.e. change in shape) allowing the hormone-receptor complex to migrate, dimerize (the joining of two hormone–receptor complexes), and bind to particular areas of the DNA known as hormone response elements (HREs). Finally, once the hormone–receptor complex binds to its HRE, transcription of a particular gene is initiated allowing the transcription, or repression, of genes that ultimately produce proteins altering the functioning of the neuron (i.e. upregulation or downregulation of a particular receptor, changing the excitability or morphology of the neuron).

This genomic mechanism of steroid action provides a useful heuristic, but recent advances in understanding of steroid hormone action have uncovered several indirect genomic and non-genomic actions of steroids [10]. For instance, estrogen has been shown to bind to receptors located in the cellular membrane of neurons, which activate second messenger cascades to initiate gene transcription. These indirect genomic effects of estrogen are not mediated through HREs but instead are mediated by such DNA regulatory sites as activator protein 1 (AP-1) and the cAMP response element (CRE). In addition to these indirect genomic effects, steroids can act through non-genomic mechanisms such as directly modulating the permeability of ion channels and acting as anti-oxidants in neurons. Interestingly, steroids may also act at specific local sites in the neuron, which do not involve interactions with the genome. For instance, a recent ultrastructural study in female rats

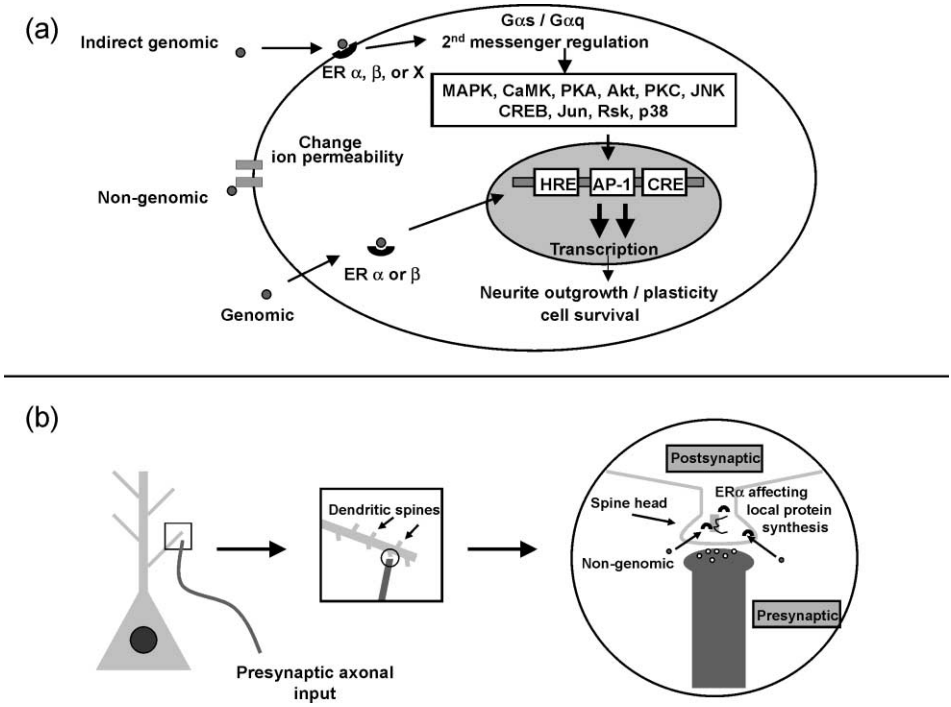

Fig. 1. The various mechanisms of steroid hormone action. (A) The indirect genomic, non-genomic, and genomic actions of estrogen on neurons. Indirect genomic mechanisms include the activation of estrogen receptors (e.g. ERα, β, or X) linked to second messenger cascades that activate gene transcription through DNA binding domains such as AP-1 or CRE, which ultimately alters neuronal function. A non-genomic mechanism of action includes the ability of estrogen to influence ion permeability. In the classic genomic mechanism, estrogen binds to the nuclear form of the ER (e.g. ERα or β) which permits the steroid/receptor complex to translocate to the nucleus. This steroid/receptor complex then binds to the hormone response elements (HREs), which activates transcription of the genome and alters neuronal function. (B) A putative non-genomic effect of estrogen acting through ERα on local protein synthesis in the spine head. This mechanism would presumably allow for the rapid regulation of spine-specific mRNAs and proteins by estrogen.

determined that extranuclear estrogen receptors (ERs) are located in axon terminals and spines of hippocampal CA1 pyramidal cells [12]. Various mRNAs for synaptic proteins and translation machinery have been localized in dendritic spines suggesting that translation of such proteins can occur in the dendrite itself. Thus, estrogen may act directly on the synaptic apparatus and local mRNAs to affect synaptic function and synaptogenesis. These various mechanisms of steroid hormone action are presented schematically in Fig. 1.

3. Sexual differentiation of the CNS

Males and females differ vastly in their reproductive physiology and behavior. Thus, it should not be surprising that the brain areas that mediate these events are different in males

and females as well. Exposure to different hormonal milieus during perinatal development ultimately leads to many of the structural and functional differences observed in the brains of males and females [16]. For instance, male rats experience increases in testosterone production and secretion during both prenatal and early neonatal development, which upon conversion in the brain to estradiol via the aromatase enzyme, organizes the brain in a masculine fashion. This early masculinization of the brain then allows the hormonal stimulation received in adulthood to act on these organized neural pathways to activate the appropriate male physiology and behaviors. In the perinatal female, the estrogen produced by the mother and the prenatal ovary does not masculinize her brain because this estrogen is bound by α-fetoprotein and is unable to cross the blood–brain barrier, ultimately resulting in a feminized brain. Indeed, the feminized brain appears to be the default pattern of development in that a brain that does not receive androgenic and estrogenic stimulation during development results in a feminized brain.

As neuronal cell groups undergo sexual differentiation during development, steroids act both directly on the target neurons and indirectly on their afferents and/or efferents. The anteroventral periventricular nucleus of the hypothalamus (AVPV) and the spinal nucleus of the bulbocavernosus (SNB) are two sexually differentiated nuclei in the CNS. These nuclei provide good examples of how steroids act directly and indirectly on the target neurons during perinatal development, which ultimately results in robust structural and functional differences in the CNS that mediate sexual dimorphisms in physiology and behavior.

The AVPV is a bilateral structure located along the third ventricle in the anterior portion of the ventral hypothalamus. This region is sexually dimorphic in that it is larger in females than males and contains a greater number of dopaminergic and peptidergic neurons in females. It should be noted, however, that males have a greater number of enkephalin-containing neurons compared to females indicating the sexual dimorphism in the AVPV is cell-type specific. This area of the hypothalamus is thought to control the surge of luteinizing hormone (LH) females experience when estrogen levels rise during their estrous (rodents) or menstrual (human) cycle. In males, an LH surge does not occur even in response to high levels of estrogen. However, when males are castrated at birth, an LH surge can be elicited after estrogen treatment in adulthood, and conversely, this response is lost in females when they are treated neonatally with testosterone. Thus, it appears that the neonatal hormonal milieu is responsible for organizing this structure in a masculine or feminine fashion. The AVPV expresses sex steroid receptors suggesting that steroids present during development act directly on these neurons. Indeed, male mice that are genetically engineered to lack estrogen receptor α (ERα) have a larger, feminine AVPV with a greater number of dopaminergic cells compared to the ERα-expressing wild-type male mice. It has also been shown that the sexually dimorphic innervation from the principle nucleus of the bed nucleus of the stria terminalis (BSTp) to the AVPV, which is greater in males than females, is dependent on prior exposure of the AVPV to testosterone during development [6]. For instance, a female AVPV that has been exposed to testosterone neonatally has a large, masculine innervation from the BSTp to the AVPV. Taken together, it appears that the estradiol formed from the aromatized testosterone allows not only for the differentiation of the AVPV but also the eventual sexually dimorphic innervation of this structure.

Sex differences are not just limited to the brain. The sexually dimorphic SNB is composed of motoneurons located in the fifth and sixth lumbar segments of the spinal cord. In humans, this nucleus is known as Onuf's nucleus. These motoneurons innervate the levator ani (LA) and bulbocavernosus (BC) muscles that are attached to the base of the penis and are responsible for penile reflexes, which are essential for successful reproduction. There are more SNB cells in the male than in the female, and the neurons are larger in males. The SNB motoneurons also innervate the external anal sphincter, which are the cells that reside in this nucleus in females. Interestingly, females are born with both the LA and BC muscles (which are attached to the clitoris); however, the lack of androgenic stimulation during neonatal development in females allows these muscles to atrophy. Once these muscles atrophy in the female the SNB motoneurons that innervate them degenerate. Conversely, in males these muscles are spared due to the androgenic stimulation they receive by the perinatal testes. These muscles in turn provide trophic support for the motoneurons, allowing them to survive during development and into adulthood [3]. In addition to these effects on the efferents of the SNB, androgenic steroids also influence the size of the motoneurons in adulthood. The SNB system provides a good example of how steroids act primarily indirectly on the target tissues of neurons that ultimately results in neuronal sexual differentiation.

These two nuclei represent two very robust sex differences in the CNS of males and females. These examples were intended to show that sex steroids can act both directly and indirectly on the nucleus and its targets to influence the sexual differentiation of the CNS. However, there are many differences between the male and female brain which are much more subtle than the two instances mentioned above. These subtle differences and how exposure to steroids during adulthood can alter synaptic plasticity differently in the male and female brain are discussed in Section 4.

4. Sex differences in steroid-induced synaptic plasticity in the adult brain

Neurons receive excitatory input via their dendritic spines, which contain the AMPA- (alpha-amino-3-hydroxy-5-methyl-4 isoxazole propionic acid) and NMDA- (*N*-methyl-D-aspartate) type ionotropic glutamate receptors. Dendritic spines are specialized membraneous protrusions from the dendritic processes, which contain actin and other scaffolding and signaling molecules. These spines come in a variety of shapes and sizes (e.g. filopodium, thin, stubby, mushroom). The morphology of the spine appears to be indicative of its maturity. For instance, spines that are thin or filopodium-like are thought to be immature, while the mushroom-shaped spines may be more mature and developed. How the morphology of the spine dictates its function is relatively uncertain. However, it has been suggested that spine morphology plays a role in calcium compartmentalization, which would have profound effects on such events as intracellular signaling [5].

Sex steroids have been shown to affect the density and morphology of dendritic spines. Similar to the history of identifying sexually dimorphic brain nuclei, the hypothalamus was the first brain area in which sex steroids were shown to influence neuronal processes in a sex-dependent fashion. Specifically, spines located on the dendrites of neurons in the ventromedial nucleus (VMN) and arcuate nucleus (ARC) of the hypothalamus have been

shown to exhibit sex differences in spine density.The VMN is a fundamental component of the neural circuitry that mediates the expression of female sexual behavior. It has been shown that ovariectomized females have significantly fewer VMN synaptic contacts compared to ovariectomized females treated with estrogen [1,4]. Furthermore, females in the proestrous stage of their estrous cycle (i.e. when estrogen levels are relatively high) have a greater spine density than females in diestrous (i.e. when circulating estrogen levels are low) [4]. The spine densities were similar in intact males and females. However, there was a sex difference in that males expressed a greater number of spines after castration compared to males with testes. In contrast to the ability of estrogen to increase spine density in females, estrogen-treated males exhibit a relatively low number of spines, similar to the density observed in intact males. Thus, estrogen has opposite effects on VMN spine density in males and females. Interestingly, when males were treated with a single injection of an aromatase inhibitor on the first day of life, estrogen treatment in adulthood caused an increase in spine density like that observed in females [7].

The ARC plays an important role in the control of gonadotropin secretion from the anterior pituitary. The dendrites of these neurons exhibit a sex difference such that females have twice the number of spines compared to males. Similar to the VMN, androgenic and estrogenic stimulation received early in development appear to sexually differentiate these neurons. Specifically, females that receive testosterone neonatally evince fewer spines than untreated females, while males that are castrated neonatally show a greater number of spines compared to control males [9].

Taken together, these data indicate that estrogen can influence spine density in adulthood. Furthermore, these studies show that the ability of estrogen to modulate spine density on hypothalamic neurons in males and females is sexually differentiated early in development by estrogen aromatized from the testosterone secreted by the neonatal testes. As mentioned earlier, the VMN is a very important nucleus in the control of female mating behavior. Thus, it is not surprising that estrogenic stimulation that activates female mating behavior would also promote spine density, and hence, increase excitatory input to the VMN. Likewise, the greater number of spines, and presumably the greater excitatory input to the female ARC, may help modulate the LH surge that causes ovulation in females.

Recently, there is increased interest on effects of sex steroids on brain areas outside of the hypothalamus and regulation of biological events other than reproductive physiology and behavior [10]. Indeed, steroid receptors are located in many extrahypothalamic regions of the brain such as the cortex and hippocampus and steroids influence such factors as mood and cognition. A growing body of evidence shows that estrogen can significantly improve an organism's ability to learn and remember. The mechanisms by which estrogen mediates these beneficial effects on learning and memory are not well understood. However, a very interesting and emerging story points to the ability of estrogen to affect the structure and function of the hippocampus, an area of the brain implicated in learning and memory (i.e. working memory, place learning, spatial learning, reversal learning, etc.).

Female rats that lack estrogenic stimulation, due to removal of their ovaries, have significantly fewer dendritic spines on the apical dendrites of the CA1 pyramidal cells of the hippocampus compared to females that received estradiol after ovariectomy. The CA1 pyramidal cells of the hippocampus receive large amounts of excitatory input, and appear to play a pivotal role in mediating spatial and explicit memory. These findings were

further confirmed by exploiting the natural fluctuations in estrogen that the female rat undergoes during her 4–5 day estrous cycle. Specifically, brains from females on the day of proestrous, when estrogen levels are at their highest, had a significantly greater number of dendritic spines on their CA1 cells compared to brains from females examined on the day of estrous, when estrogen levels are relatively low. These studies were supplemented by experiments that demonstrated that the increase in spine density was accompanied by an increase in synaptic input to the apical dendrites of the CA1 pyramidal cells. These structural alterations are paralleled by physiological and behavioral changes as well. Specifically, adult female rats experiencing high levels of estrogen show enhanced hippocampal long-term potentiation, a putative electrophysiological correlate of learning and memory, and improved memory retention on a hippocampal-dependent spatial memory task [15,18].

There is a robust sex difference in the magnitude of the response to estrogen in CA1 hippocampal cells, such that in adult male rats, CA1 spine density increases only 15% in response to estrogen treatment, while in females there is a 30% increase with estrogen treatment [7]. Similar to the sex difference in estrogen-induced spine density in the hypothalamus, administration of an aromatase inhibitor to male rats on the day of parturition causes a significant reversal in the potential of the male hippocampus to respond to estrogen treatment in adulthood. Specifically, neonatal treatment with an aromatase inhibitor increases CA1 spine density of castrated, adult males in response to estrogen compared to adults not treated with the aromatase inhibitor. Furthermore, females prenatally exposed to testosterone demonstrate masculinized spatial abilities such as superior performance on a water maze task. It is interesting to note that both aromatase and ERs are present, at least transiently, in the perinatal hippocampus, indicating that steroids can act directly on this developing neural structure.

The mechanisms by which estrogen increases spine formation and synapses in females are not fully understood. Accumulating data suggest estrogen-induced spine formation involves multiple sites including activation of both genomic and non-genomic ER signaling pathways, as summarized in Fig. 2a. The estrogen-induced increase in CA1 spines and synapses is mediated by the nuclear ER. However, CA1 cells are devoid of appreciable levels of nuclear ERs (but do contain some extranuclear ERs). Instead, these effects of estrogen appear to be predominantly mediated trans-synaptically through multiple systems that do possess nuclear ERs [11]. First, estrogen allows for the disinhibition of CA1 cells via reduction of inhibitory input from the GABAergic interneurons, which do express relatively high levels of ER. Second, estrogen induces increases in NMDA receptor levels in the CA1 cells, which when blocked do not allow estrogen to augment CA1 spine density. The ability of estrogen to increase NMDA receptor levels appear to be via increases in cholinergic inputs to the CA1 region of the hippocampus [2]. Interestingly, it has been shown that cholinergic activity in the forebrain can be increased by estrogen treatment in females but not in males [8]. However, males treated neonatally with an aromatase inhibitor and later with estrogen show an increase in forebrain cholinergic activity compared to control males treated with estrogen [8]. Whether estrogen fails to increase spine density in males because estrogen is unable to modulate the inhibitory input or increase cholinergic activity, and thus, NMDA receptor levels is currently unknown (Fig. 2b).

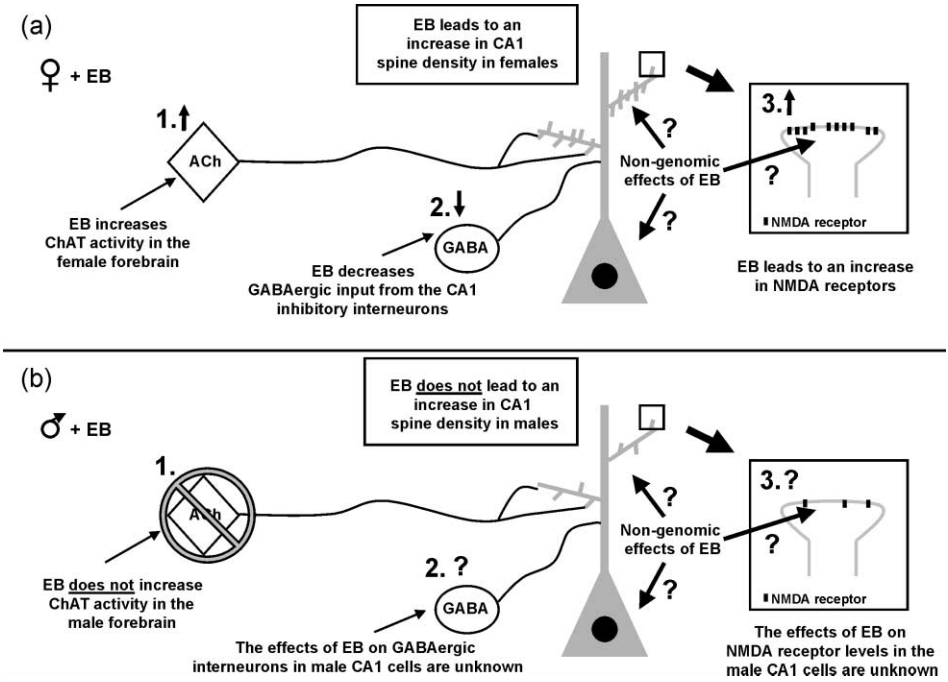

Fig. 2. The effects of estradiol benzoate (EB) on the CA1 pyramidal cells of females (a) and males (b). (a) EB promotes dendritic spine formation in females through a number of mechanisms. (A_1) EB increases acetylcholine (ACh) production in the forebrain of female rats (ChAT, choline acetyltransferase), while EB transiently decreases the inhibitory input produced by the GABAergic interneurons of the CA1 region (A_2). These effects of EB on cholinergic inputs and GABAergic interneurons appear to allow for the increase in NMDA receptors (A_3), which is necessary for the increase in spine density. The possible non-genomic effects of EB on spine formation in females and males are presently unknown. (b) EB fails to increase spine density in males. (B_1) EB does not increase cholinergic expression in the forebrain of males. It is unknown whether EB regulates GABAergic function in CA1 interneurons (B_2) or fails to upregulates NMDA receptors in the dendritic spines of CA1 cells (B_3). The failure of one or all of these mechanisms likely accounts for the inability of EB to increase spine density in CA1 pyramidal cells in the male hippocampus.

In addition to these trans-synaptic effects of estrogen on hippocampal spine formation, extranuclear ERs are located in axon terminals and spines of hippocampal CA1 pyramidal cells [12]. As mentioned earlier, various mRNAs for synaptic proteins and translation machinery have been found in dendritic spines suggesting that translation of such proteins can occur in the spine. Thus, estrogen may act in a nongenomic fashion directly on the synaptic apparatus and local mRNAs to affect spinogenesis. This possibility remains to be tested and is under investigation. The various mechanisms through which estrogen may mediate sex differences in spine density on hippocampal CA1 pyramidal cells are presented schematically in Fig. 2(a) and (b).

Although estrogen in males has been shown to be relatively ineffective in increasing spine density, recent evidence indicates that testosterone can affect spine density in males [14]. Males treated with testosterone have a greater spine density in CA1 hippocampal

neurons compared to castrated males not receiving testosterone. Whether the mechanisms for testosterone-induced spine formation in males are similar to that for estrogen-induced spines in females remains to be determined. A common link may be in the ability of steroids to regulate the cholinergic activity of hippocampal inputs. Specifically, estrogen has been shown to increase cholinergic activity in the forebrain of females, which appears to be responsible for the increase in NMDA receptors necessary for spine growth in females. Estrogen does not affect cholinergic activity in males; however, males treated with testosterone do show greater amounts of cholinergic activity in the forebrain compared to castrated males not receiving testosterone [13]. Thus, it is possible that estrogen in females and testosterone in males increases cholinergic activity in the forebrain that leads to an increase in NMDA receptors on CA1 hippocampal cells, which in turn, allows for the spine growth seen in estrogen-treated females and testosterone-treated males.

Men tend to experience decreases in androgen production and secretion during normal aging along with a concomitant decrease in memory function. Cognitive function is best in aged men with high levels of bioavailable testosterone [19], and working memory is improved in aged men receiving testosterone supplementation. Whether these memory promoting effects of testosterone are through its action on the hippocampus remain to be determined. However, the effect of testosterone on hippocampal spine density and cholinergic activity in the forebrain of males suggests testosterone's cognitive enhancing properties may be working, at least in part, through these mechanisms. Interestingly, males express significant levels of androgen receptors in their CA1 pyramidal cells, suggesting testosterone may influence the hippocampal CA1 cells directly. Additional studies are needed to clarify this possibility.

5. Summary

1. Most of the structural differences between the male and female CNS are established early in development by the perinatal hormonal milieu. However, other sex differences in the structure and function of the CNS are more subtlety affected by circulating steroid hormone levels in adulthood, such as dendritic spine density and morphology.
2. Although the genomic mechanism of steroid hormone action provides a useful model to understand steroid signaling, recent advances in our understanding of steroid signaling have uncovered several indirect genomic and non-genomic actions of steroids. These disparate mechanisms of action may have implications for how steroids affect synaptic plasticity.
3. Neurons receive excitatory input via their dendritic spines, which contain the AMPA- and NMDA-type ionotropic glutamate receptors. Dendritic spines are specialized membraneous protrusions from the dendritic processes, which contain actin and other scaffolding and signaling molecules.
4. Sex steroids have been shown to affect the density and morphology of dendritic spines in both hypothalamic and hippocampal neurons. However, these effects appear to be dependent upon the sex of the organism. For example, estrogen increases the spine density of hypothalamic VMH and ARC neurons and hippocampal CA1 pyramidal cells in females but not males.

5. Estrogen increases spine density of CA1 hippocampal pyramidal cells in females by increases in cholinergic input from the forebrain and decreased inhibition by GABAergic interneurons. The role of ERs in the spine head on local protein synthesis is under investigation. Whether the male hippocampus is unable to respond to estrogen because of the failure of one or all of these mechanisms is currently being studied.
6. Estrogen appears to have beneficial effects on learning and memory and the amelioration of certain neurodegenerative disease such as Alzheimer's disease, which is in part likely through its action on the hippocampus. Thus, males would benefit far less than females from any therapeutic effect of estrogen. Hence, it is imperative to further our understanding of how estrogen may affect spine density and synaptogenesis of males and females differently, as is investigating the effects of SERMs and SARMs on the brains of males and females.
7. The ability of androgens to increase hippocampal spine density and cholinergic activity in the forebrain of males suggests future promise for androgen or SARM replacement therapy for older men experiencing significant decreases in cognitive function.

6. Future directions

The mechanisms by which steroids induce differences in spinogenesis in males and females remain largely unknown. In regard to estrogen-induced spinogenesis, future research needs to advance our current understanding of both the basic mechanisms of estrogen's action in the female hippocampus as well as the sex difference in response to estrogen. For instance, the expression of synaptic proteins that contribute to the estrogen-induced morphological sex difference needs to be characterized. Furthermore, sex differences in the cellular mechanisms (e.g. cholinergic innervation and GABAergic input to the CA1 region and NMDA receptor regulation) that mediate the ability of estrogen to act differently on spine formation in males and females need to be elucidated. Finally, how and when these mechanisms are sexually differentiated and organized during perinatal development need to be established.

As mentioned earlier, increased attention has been focused on the ability of estrogen to mediate beneficial effects on learning and cognition, and recent evidence suggests that estrogen replacement therapy in postmenopausal women may aid in the amelioration of neurodegenerative diseases such as Alzheimer's disease. How estrogen mediates these beneficial effects is not well understood, but it may be through the ability of estrogen to influence the structure and function of the hippocampus, an area of the brain implicated in learning and memory and impaired in patients with Alzheimer's disease. Thus, if the beneficial effects of estrogen on these processes is in part through the effect of estrogen on spine growth and synaptogenesis, then males would benefit far less than females from any therapeutic effect of estrogen. Hence, it is imperative to further our understanding of how estrogen may affect spine density and synaptogenesis of males and females differently. Furthermore, given that testosterone increases hippocampal spine density [14] and cholinergic activity in the forebrain [13] of males it would be interesting to further investigate the possible therapeutic benefits of androgen replacement therapy for men experiencing significant decreases in hormone production and secretion during aging.

In addition to the beneficial effects of estrogen on hippocampal structure and function, selective estrogen receptor modulators (SERMs; e.g. tamoxifen, raloxifene) can affect hippocampal physiology and cholinergic activity in the forebrain. Some SERMs have anti-estrogenic effects in selective peripheral tissues; however, SERMs mimic effects of estrogen in particular brain regions, such as increases in NMDA receptor levels in the hippocampus and increases in cholinergic activity in the basal forebrain. It should be noted that SERMs such as tamoxifen also act as anti-estrogens in certain brain regions (e.g. hypothalamus). As SERMs have fewer side effects than pure estrogen, these compounds may provide a safer and more effective alternative to estrogen therapy. Whether SERMs influence the brains of males and females differently is currently unknown. Furthermore, novel compounds that modulate the androgen receptor known as selective androgen-receptor modulators, or SARMs, may provide benefits to males when estrogen or SERMs are ineffective.

The role that aberrant or inadequate spinogenesis may play in sex-specific physiology and pathophysiologies is currently unexplored. Fewer dendritic spines certainly lead to a less excitable neuron, yet a greater number of spines may not equate to a better functioning neural circuit. Moreover, the exact functional significance of spine shape and number needs to be clarified. Thus, many questions remain unanswered. However, future research will undoubtedly lead to a greater understanding of these phenomenon, which will elucidate the role sex differences play in steroid-induced spine formation and synaptic maintenance during normal physiological function and various pathophysiological states.

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Hormones and the developing brain

Margaret M. McCarthy

1. Introduction

Many people are familiar with the notion that steroids act on the adult brain to influence physiology and behavior. Throughout adulthood, males have relatively constant and high levels of testosterone, critical to spermatogenesis, libido, beard growth and a variety of other characteristics associated with maleness. Females, on the other hand, experience variation in hormones, principally estrogens and progestins, both as a function of, and principle driving force behind, the menstrual cycle, pregnancy and lactation. With advancing age, female reproductive capacity is lost and women enter into the menopause, a life stage characterized by low levels of steroid hormones. But when it comes to hormones and the brain, few people think about the developmental effects. Across numerous species, including humans, the hormonal milieu during the perinatal period is widely divergent between males and females due to high levels of androgen production in males. The primary function of this hormonal surge appears to be to permanently distinguish male from female brains and optimize the capacity for reproductive success for both sexes throughout the lifespan. However, that the effects are permanent smacks of determinism, evoking a healthy level of skepticism when it comes to consideration of the human brain. In attempts to understand the effects of hormones, myriad other variables are either ignored or controlled for when possible. Early experience, environment and epigenetic factors other than hormones are rarely incorporated into studies of sex differences. This myopic approach is not because scientists conducting the studies are unaware of these variables, but rather stems from the sound scientific practice of manipulating only one variable at a time. Nonetheless, problems can arise when generalizations are made from controlled studies in animals to a species as malleable and unpredictable as humans. Thus, a through understanding of the current state-of-the-art is essential to the student of sex-based biology. A goal of this chapter is to begin to provide that understanding as well as instill an appreciation for the fascinating research area of hormonally mediated sex differences in the brain.

2. The developing brain is undifferentiated in regards to sex

As with the gonads, the brain begins life as a bipotential organ, equally capable of assuming either a male or female phenotype. The variable determining the eventual

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gender-specific configuration of the brain is the hormonal milieu experienced during a confined perinatal developmental period. The hormonal milieu in turn is a function of the gonads, which are themselves determined by the genetic sex of the individual, in particular the presence or absence of the SRY gene on the Y chromosome. By constructing the organism in this way, nature has ensured that gonadal sex is in synch with brain sex, a phenomenon referred to as the sexual differentiation of the brain. This chapter will review the current state of understanding of the sexual differentiation of the brain at the molecular, cellular and behavioral level. Data from experimental animals will be interpreted in the context of what is known in humans.

Previous chapters introduced the concept that the fetus is an exquisitely sensitive target for steroid hormones. Circulating gonadal steroids in the adult brain influence neuronal activity, synaptic patterning and gene expression (Chapter 18). Steroids can act in the brain via a variety of mechanisms, including at the membrane, interacting with cognate receptors in the cytoplasm at sites distant from the nucleus or by activating nuclear receptors and altering transcription. The relative amounts and types of steroids in the blood, i.e. estradiol and/or progesterone versus testosterone, determine what the functional outcome will be. However, an additional important, and at times forgotten, component of steroid action in the adult brain is the influence of prior exposure to steroids during development. In a process akin to imprinting, early hormonal exposure will influence the sensitivity as well as character of response to hormones in adulthood.

3. The effect of steroids on the developing brain are organizational and followed by activational effects in adulthood

In 1959, the concept of a sequential action of steroids on the brain was proposed in a seminal paper by Phoenix et al. [13]. Following a lead originating with the work of Frank A. Beach on beagles, these researchers examined the offspring of female guinea pigs that had either been treated with androgen or vehicle during pregnancy. They discovered that the female guinea pigs born to these mothers did not act like females as adults, meaning there was no evidence of an estrous cycle (the rodent equivalent of a menstrual cycle) and the females did not display sexual receptivity when in the presence of a male suitor. Even more striking, if provided with testosterone as adults, these genetic females behaved exactly as genetic males. Similar studies were being conducted on the rat by Charles Barraclough at the same time, with the distinction that in these experiments the females were not treated with androgen until they were born, and the focus was on the impact on reproductive physiology rather than behavior. Two important observations emerged. One was that the rat was a better experimental model than the guinea pig, which is a highly precocial species, unlike humans. The second was that a graduate student named Rodger Gorski involved in these early findings decided to pursue the neuroanatomical correlates of this early hormone action and was the first to report a major sexual dimorphism in the mammalian brain in 1978. In the interest of historical fairness, however, he was not the first person to think of looking for sex differences in the mammalian brain. Studies conducted 10–15 years early by Don Pfaff [12] and the team of Raisman and Field [14] had reported small but significant differences in

the microneuroarchitecture of brains of male and female rats. In fact these differences were so small that they required an electron microscope to be detected, as it was assumed if there were sex differences in the brain they would be subtle and exacting. The discarding of this dogma came from an unlikely source, the canary. If you have ever wondered why the price of a male canary is three times that of a female, your questions are quickly answered when you hear one sing. Only males are capable of the complex and beautiful song that is one of the most beautiful sounds in nature. Reasoning that such a highly refined behavior must have a neuroanatomical underpinning, Fernando Nottebohm and his student at the time, Arthur Arnold, set about discovering the song control nuclei in the canary brain and reported in 1976 that these nuclei were either wholly absent or greatly diminished in female canary brains. By analogy, Roger Gorski decided it would be a good idea to step back and take a more distant look at the rat brain, particularly in those regions controlling sexual behavior, and thus was discovered the sexually dimorphic nucleus (SDN) of the preoptic area. In time, a coalescing of behavioral, endocrine and neurobiological approaches has led to a subdiscipline within neuroscience known as Behavioral Neuroendocrinology. Students of this subdiscipline are dedicated to understanding how hormones influence brain and behavior throughout the lifespan and across a myriad of species, from fish and reptiles through amphibians, birds and mammals including humans.

The organizational/activational hypothesis of hormone action is in essence a framework for the concept that early hormone action exerts permanent (organizational) effects on the neural architecture that is then acted upon in the adult. In most species the early exposure occurs perinatally, meaning around birth. In rats and mice, gestation lasts 20–22 days with litter size being in the order of 6–12. During late gestation, for reasons that are still not understood, there is copious production of testicular androgens leading to a hormonal surge selectively in males. A second surge occurs at birth such that males experience 2–3-fold more androgen exposure than females. In rodents these hormonal surges are the result of a complex interplay between active steroidogenesis, probably from the adrenals and gonads, and changes in metabolism that lead to a build-up of steroids. In primates, there is an activation of the hypothalamic–pituitary–gonadal axis during fetal development in males that must then be actively suppressed post-natally until puberty. Regardless of differences in mechanism, in both species the outcome is the same, a sexually dimorphic exposure to gonadal steroids during a restricted developmental period. The principles of organizational/activational hormone effects are illustrated in Fig. 1.

4. Organizational hormone effects occur during a restricted sensitive period

Sensitive periods are a general phenomenon in developmental neurobiology, referring to an opportunistic window during which events must occur or forever be precluded. An illustration of this familiar to many is the lazy eye. If the good eye is patched when an individual is still a child so that the lazy eye has to work harder, the condition is corrected. If, however, the good eye is not patched until adulthood, the lazy eye cannot be coaxed into compensating. This example actually involves the musculature controlling eye movement but the same is true for the brain's detection of visual stimuli. If kittens

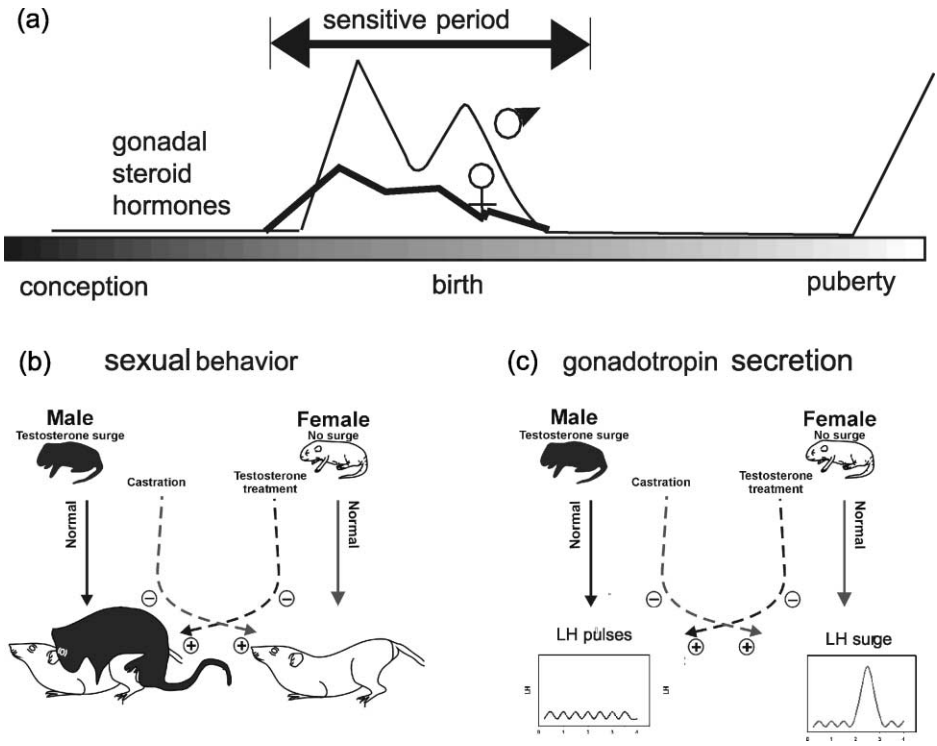

Fig. 1. *The organizational/activational framework for steroid action on the brain*. Early in development, gonadal steroids exert an organizing effect on neural circuits. (a) These effects occur only over a limited period of time referred to as a "sensitive period". After this time point the brain becomes refractory to the organizing influences of steroids. Exposure to steroids during the sensitive period is different in males versus females and is a function of their respective gonads. As a result, the brain is organized distinctly in males versus females. In adulthood, gonadal steroids again act on the brain but the effects are limited by the previous organization and are referred to as activational in that they result in the activation of gender specific behaviors and physiological responses. In rodents, the expression of (b) sexual behavior and (c) the control of gonadotropin secretion from the anterior pituitary are both organized during development and activated in adulthood.

are raised in an environment that consists only of horizontal bars they will forever be incapable of "seeing" vertical bars and vice versa. There are many other examples of sensitive periods that occur at different developmental times and involve both intrinsic and extrinsic stimuli. In the case of sex differences in the brain, the stimulus is intrinsic, being hormones. The sensitive period is operationally defined by the onset when hormonal secretion begins and the termination being that point beyond which extrinsic hormone is ineffective at exerting an organizational effect. Exogenous administration of testosterone to females has revealed different timing for the termination of the sensitive period for distinct endpoints.

In humans, the sensitive period for sex determination (i.e. development of the gonads and accessory duct systems) occurs relatively early, within the first trimester of pregnancy.

This is followed by a period of gonadal activation during the second trimester, leading to elevated androgen in males. This period is often cited as the time when sexual differentiation of the brain occurs but is based on no definitive evidence. At birth, there is a second surge in androgen secretion in males in response to a release of LH from the newborn pituitary [6]. As a result, newborn males have 2- to 3-fold higher levels of testosterone than females for the first day or two of life. Androgen levels then drop and remain relatively low in both sexes until they begin to diverge again at about 12 years of age at which point males again have 2- to 3-fold higher levels, as opposed to the 5-fold higher levels that characterize the adult [8]. This is in part due to a drop in androgen production or increased aromatization by the female adrenal post-puberty. Currently, the precise timing of the critical period for sexual differentiation of numerous parameters in the human remains relatively unknown.

5. Organizational hormone effects result in a masculinized or feminized brain

The neurologic organizational action of hormones is synonymous with sexual differentiation of the brain. The end point of sexual differentiation is either masculinization or feminization. Masculinization refers to a constellation of behavioral and physiological endpoints reviewed below. The sensitive period for each endpoint can vary and may be independent of, or covary with, other endpoints. Masculinization is often considered an active or driven process, as it is dependent upon exposure to androgens and their metabolites. Feminization obviously also refers to a collection of endpoints, some of which may be truly dimorphic from those of males, meaning genuinely opposite, or may be allomorphic. Truly sexually dimorphic traits are those that are mutually exclusive or expressed to extreme in one sex or the other. Examples include maternal behavior, aggression, sexual receptivity, control of gonadotropin secretion and certain food preferences (male rats hate saccharine, females love it). Traits that are allomorphic are those which are different between the two sexes on a population level but continue to show considerable overlap on the individual level. These would be cognitive responses, stress and anxiety and nociception (sensitivity to painful stimuli). In humans, most sex differences are allomorphic and include uniquely human traits such as verbal and spatial skills.

Whether masculinzation and feminization represent opposite ends of a continuum or are discrete but intermingled categories, is a matter of theoretical debate [5]. Masculinization is initiated by a signal, secretion of testicular androgens, whereas feminization is the default pathway. This neatly parallels the process of sex determination in which the male gonad requires an initiating signal originating from the gene SRY in order to override the default pathway of ovarian development. Although brain feminization and ovarian development are both default pathways, this is not synonymous with passive, both are active, complex and poorly understood processes. The lack of a clear initiation signal, contrary to the case in the male, makes feminization a more elusive process to characterize. In the rodent, behavioral studies indicate an additional active process termed defeminization. Feminized animals exhibit the sexual receptive posture known as lordosis in response to males. Masculinized animals exhibit mounting behavior

towards females. But a masculinized animal that has not been defeminized will display mounts towards a female and lordosis in response to a male. Animals with this phenotype have been generated by specific manipulations, such as blocking the expression of the nuclear receptor co-activator SRC-1 during the sensitive period [1], thereby unveiling this developmental process. But its not that such an animal will flip between male and female sexual behaviors on a whim. To fully reveal the phenotype animals must be gonadectomized as adults and treated with the hormonal milieu appropriate to that sex, i.e. estradiol plus progesterone for females and testosterone for males. In other words, the organizational effect of the early manipulation (blocking SRC-1) is only revealed by testing for the activational effect of gonadal hormones in adulthood. Unlike rodents, in primates it is still unresolved as to whether the sensitive period for sexual differentiation is prenatal, thereby precluding the ability to readily manipulate the animal after birth. Thus it is difficult to make strong statements about whether processes such as defeminization occur in humans. Any generalizations are made more difficult by the lack of clear distinction in sexual postures of males and females. This raises another conceptual point. Sexual behavior and sexual preference are not necessarily synonymous. Studies involving steroid manipulation of neonatal rodents to alter their sexual behavior in adulthood, are not models of homosexuality. Homosexuality is the preference for members of the same sex as sex partners. Experiments in animals in which adult sex behavior has been determined by early hormone exposure usually do not include sexual preference as a parameter and when they do, find that the phenotypic sex of the brain is consist with preference for opposite sex partners, regardless of the genetic sex of the animal. Thus, neonatally androgenized females display male sexual behavior as adults and prefer the company of females. Likewise, males that are castrated at birth have a feminized brain and respond appropriately to other males by exhibiting lordosis and again, preferring the company of males as do unaffected females. This is highly consistent with what is emerging regarding our understanding of gender identity in humans, which is that for male identity in particular, the importance of prenatal androgen exposure is paramount and overrides the influence of socialization or rearing environment [19]. There is no perception of gender or body identity in animal models so there is no internal conflict. This does not mean there are no animal models for homosexuality or sexual preference, there are, and they include rodents, ferrets and primates. But this is an emerging and understandably controversial component of sexual differentiation research, a thorough review of which is beyond the scope of this chapter.

6. Aromatization of testosterone to estradiol mediates sexual differentiation of the rodent brain

When exploring the specificity of hormonal effects on the developing brain, researchers were quick to notice that compared to testosterone, estradiol was as effective, and often even more effective, at inducing masculinization. This seemed counterintuitive given that estradiol is equated with femininity, and in fact estradiol must be suppressed for proper development of the testis, while testosterone is the quintessential male hormone. The conundrum was clarified in studies conducted on rats. First, is that fetal and newborn

rodents have high circulating levels of a steroid binding globulin called alpha-fetoprotein, which has a strong affinity for estradiol. This helps to sequester the estradiol of maternal origin in the bloodstream of the fetus, effectively preventing its entry into neurons and other cells. Testosterone, however, is not bound by alpha-fetoprotein and moves freely into neurons. Second, is that aromatase, the p450scc enzyme that converts testosterone to estradiol, is found at very high levels in some regions of the developing brain. Neurons of the hypothalamus, preoptic area and hippocampus exhibit more aromatase activity during the perinatal sensitive period than at any other time in life. About 1 week after birth, levels will drop precipitously and for the rest of life the majority of estradiol synthesis will be in the periphery, mostly the ovary, although neurons continue to locally produce estradiol from testosterone throughout life. Third, is the distribution of estrogen receptors, which is broader and for the most part denser than that of androgen receptors. The ability to locally synthesize estradiol within neurons allows for fine-tuning of steroid hormone differentiation within discrete subregions of the brain. In song birds, the brain has been found capable of synthesizing estradiol de novo, however, this possibility has not been examined in mammalian brains. Subtle differences in the timing and amount of aromatase activity, concentration of estrogen receptors and degradation of estradiol likely contribute additional regional and cellular heterogeneity.

The role of aromatization in primates appears to be much less than that for rodents. Instead, it is the direct action of androgens on neurons that differentiate the brain into a masculine phenotype [19]. There also appears to be far less of a role for early postnatal hormone exposure in primates compared to rodents, but both this and the potential for an important involvement of estradiol in primate brain differentiation remain to be definitively established. Regardless of the specifics of the hormone and the timing of its action, it is clear that gonadal steroids exert profound and permanent effects on the primate brain that are analogous to those seen in rodents.

7. Neuroanatomical sex differences: the sexually dimorphic nucleus of the preoptic area

Once it was established that there is a repertoire of behaviors that are either hormonally dependent (sex behavior, maternal behavior, aggression) or hormonally modulated (anxiety, learning and memory, feeding), the obvious next step was to determine the anatomical substrates mediating these behaviors and whether or not they are sexually dimorphic and/or modulated by hormones.

It is not uncommon for brain structures to be named for some physical attribute. When a small but distinct nucleus within the preoptic area was found to be 5–7 times larger in males than females, the first such sex difference noted, naming it the sexually dimorphic nucleus was an obvious choice. In the rat, the SDN is a collection of large dense and darkly staining cells embedded within the medial preoptic nucleus (MPN) (see Fig. 2). Other than their size and propensity for taking up Nissl stain, there was little to distinguish these cells from the surround until the recent discovery that they contain high levels of the calcium binding protein, calbindin. Neurons of the surrounding preoptic area seem entirely devoid of this particular cellular marker. However, as has been maddeningly characteristic of

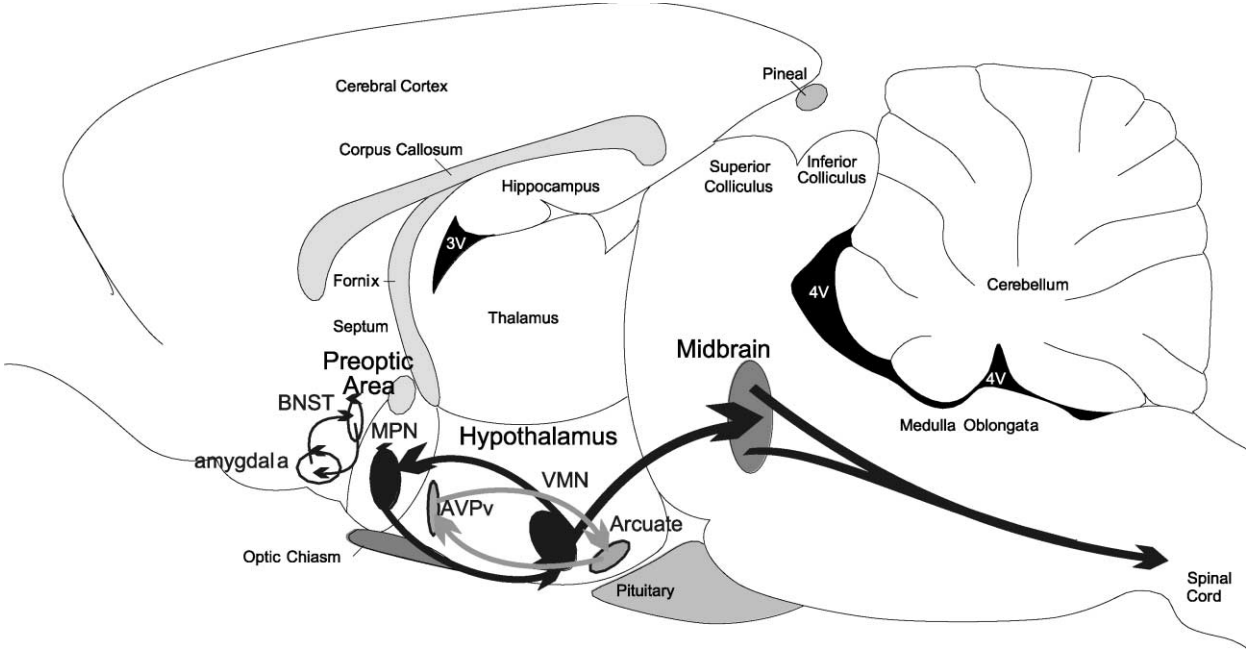

Fig. 2. *Sexually dimorphic neural circuitry*. Many regions of the brain show sex differences in size and synaptic patterning. These regions do not exist in isolation but instead exhibit a high level of reciprocal connections, leading to a sexually dimorphic neural circuitry that underlies gender differences in complex behavioral and physiological responses. The medial preoptic nucleus (MPN) and ventromedial nucleus of the hypothalamus (VMN) are critical areas controlling sexual behavior and have strong reciprocal connections and additional projections to midbrain and the spinal cord. The anteroventroperiventricular nucleus (AVPV) and the arcuate nucleus are both involved in the control of gonadotropin secretion from the anterior pituitary and they project to each other. The bed nucleus of the stria terminalis (BNST) and the amygdala are brain regions involved in detection of olfactory cues originating from the opposite sex and play an important role in reproductive and aggressive behaviors. These two regions also project to each other.

the SDN, there is no established function of either the calbindin expression or the SDN itself. The preoptic area is a major brain region regulating expression of male sexual behavior, but there is no unique role elucidated for the SDN. Analogous structures in other species have also been named the SDN, including humans, but with the exception of the gerbil in which the SDN regulates scent marking, there remains a lack of understanding of precisely why this collection of cells exists.

Nonetheless, the SDN remains a topic of considerable intrigue and serves as a valuable example of many principles of neuroanatomical sex differences. A first one being that variance in size does not necessarily correspond to a variance in function. A second general principle is that most of the neuroanatomical sex differences identified to date involve males being larger than females. The male brain is larger than the female brain, and many gross structures within the brain, such as the hippocampus, are larger in males. Given this generality, exceptions to the rule are always of special interest, and there are some notable cases in which female structures are larger than male, these will be discussed below. A third general principle is that no one sexually dimorphic structure stands alone. Just as the SDN is embedded in the medial POA, the mPOA is also intricately and reciprocally connected with other brain structures that are either themselves dimorphic or constitute part of a neural network controlling sexually dimorphic physiology and behavior (see Fig. 2). Virtually all components of this network have a high concentration of neurons expressing receptors for estrogen and/or androgens. This principle critically relates to a fourth; identifying the mechanism by which steroids modulate neuroanatomical sex differences is complicated by the potential for steroid effects that act directly on the cells of interest as well indirect via hormonal modulation of afferent input. A final principle is that the sex differences in size of particular structures or nuclei appears to occur via a common mechanism, differential naturally occurring cell death or apoptosis. Put quite simply, males and females start out with the same number of neurons in a particular area (meaning there is no sex difference in neurogenesis or migration) and then neurons begin to die in one sex either as a result of insufficient hormone exposure or in response to hormone exposure. This scenario has been established for the SDN, a motor nucleus in the spinal cord controlling the penis called the SNB, a subdivision of the bed nucleus of the stria terminalis (BNSTc) and the hippocampus, all of which are larger in males, and for a nucleus in the hypothalamus that controls the LH-surge (AVPV), and the visual cortex, both of which are larger in females. Exactly how steroids are dictating whether neurons will live or die is unknown and complicated by the fact that the peak of cell death (or survival) appears to be several days after differential hormone exposure. It is further complicated by the fact that parameters affecting cell death appear to be intermingled with factors determining neurochemical phenotype [16], making for a complex mosaic of effects that remains poorly understood.

8. Volumetric sex differences in the human brain

As with rodents, male human brains are larger than females (although not when corrected for body size) and as with rodents, there are many regions that are larger in males

than females. However, unlike the research on experimental animals, studies in humans are fraught with numerous technical as well as political pitfalls. Technically, the greatest challenges originate in the source of the tissue, cadavers. Differences in time from death to collection and fixation combined with variance in sex, age, ethnicity and life history make for a heterogenous sample number under the best of circumstances. Further complicating the field is that any report of sex differences in the human brain receives widespread media attention, complete with circuits of radio and TV talk shows, newspapers and popular magazines. When there is a replication of a study on human sex differences that fails to find an effect, the report is generally met with silence by the media and often, regrettably, with scorn by other researches. Thus any review of morphometric sex differences in the human brain requires a healthy respect for the sources of variation and hidden agendas that can influence both the generation of the data and interpretation of the results. Given all these caveats, when a human brain structure is found to be statistically different between males and females, it suggests a relatively robust effect.

An analog to the SDN was identified and found to be larger in human males than females. A detailed analysis across the lifespan revealed that the degree of difference varies with age, not manifesting until after about 10 years of age, becoming maximal in young adulthood and gradually declining in both sex difference and in size in older adults [18]. In some ways this even more neatly parallels the rat in that the sex difference in SDN volume is not apparent until 5 days of age and is maximal just post-puberty. However, enthusiasm must be tempered by the fact that the sexual activity and life experiences of humans are also going to be dramatically different between 10 years of age and young adulthood.

In the human hypothalamus, rather than a single nucleus, a series of nuclei are sexually dimorphic. These nuclei are referred to as the interstial nuclei of the anterior hypothalamus (INAH) and are numbered 1–4 (INAH-1, -2, -3, -4) with INAH-1 being the original SDN. Although there is no sex difference in INAH-1, both INAH-2 and -3 are larger in males. These nuclei, and other brain structures have also been compared among heterosexual males and females and homosexual males (reviewed in Ref. [4]).

The inconsistencies in reports of sex differences in hypothalamic structures also applies to many early studies on cortical volume, size of the cerebellum and in particular the size and shape of the corpus callosum, the major fiber tract between the two cerebral hemispheres. Like the hypothalamus, all these studies suffered the same limitations of being conducted on post-mortem tissue. Recently there has been an increasing use of magnetic resonance imaging (MRI) to quantify the volume of brain regions in living subjects. This approach offers the great advantage of allowing for a precise matching of the age, socioeconomic status and ethnicity of the male and female subjects. But like all advances, it also has shortcomings. Although the resolution of MRI scanning has increased tremendously in the last decade, the precision of the images is still far from that which is obtained on fixed tissue, and questions such as cell density or cell type cannot be answered with this approach. Smaller brain structures, such as the INAHs, cannot yet be visualized by MRI. Nonetheless, this technique has confirmed sex differences in some adult brain regions, as listed in Table 1.

Table 1
A partial list of sex differences in the human brain

Brain area or structure	Sex difference	Method used	Assessed during development?	Effect size	Reference
INAH-2 and -3	M > F	Post-mortem tissue, Nissl stain	No	Moderate	J. Neurosci. (1989) 9,497
SDN-POA (INAH-1)	M > F	Post-mortem tissue, Nissl stain	Yes; fetus to 93 years	Moderate	Science (1985) 228,1112
BSTc	M > F; sex difference not apparent until adulthood	Post-mortem tissue, immunocyto-chemistry	Yes; fetus to 50 years old	Moderate to large	J. Neurosci. (2002) 22,1027
Anterior commisure	M < F	Post-mortem tissue, camera lucida tracings	No	Small	J. Comp. Neurol. (1991) 312,97
Massa intermedia	M < F	Post-mortem tissue, camera lucida tracings	No	Large	J. Comp. Neurol. (1991) 312,97
Anterior commisure	M < F	Post-mortem tissue, camera lucida tracings	No	Small	Proc. Natl Acad. Sci. (1992) 89,7199
Anterior commisure	M = F	Cross-sectional area	No	No effect	Brain Res. (2002) 936,95
Total cerebrum volume	M > F	MRI	No	Large	Cereb. Cortex (2001) 11,490
Total cortex volume	M < F	MRI	No	Large	Cereb. Cortex (2001) 11,490
Lateral and III ventricles	M > F	MRI		Moderate to large	Cereb. Cortex (2001) 11,490
Hypothalamus	M > F	MRI	No	Moderate	Cereb. Cortex (2001) 11,490
Amygdala	M > F	MRI		Moderate to small	Cereb. Cortex (2001) 11,490
Caudate	M < F	MRI	No	Moderate	Cereb. Cortex (2001) 11,490
Hippocampus	M < F	MRI	No	Small	Cereb. Cortex (2001) 11,490
Total cerebrum volume	M > F	MRI	Yes; 4–18 yrs	Moderate	Prog. Neuro-Psychopharmacol. Biol. Psychiatr. (1997) 21,1185–1201

(*continued*)

Table 1
continued

Brain area or structure	Sex difference	Method used	Assessed during development?	Effect size	Reference
Caudate	M < F	MRI	Yes; 4–18 yrs	Moderate	Prog. Neuro-Psychopharmacol. Biol. Psychiatr. (1997) 21,1185–1201
Putamen	M > F	MRI	Yes; 4–18 yrs	Moderate	Prog. Neuro-Psychopharmacol. Biol. Psychiatr. (1997) 21,1185–1201
Lateral ventricles	M > F; develop more rapidly in males	MRI	Yes; 4–18 yrs	Large	Prog. Neuro-Psychopharmacol. Biol. Psychiatr. (1997) 21,1185–1201
Amygdala	M > F; develop more rapidly in males	MRI	Yes; 4–18 yrs	Moderate	Prog. Neuro-Psychopharmacol. Biol. Psychiatr. (1997) 21,1185–1201
Hippocampus	M < F; develop more rapidly in females	MRI	Yes; 4–18 yrs	Moderate	Prog. Neuro-Psychopharmacol. Biol. Psychiatr. (1997) 21,1185–1201
Cerebellum	M > F	MRI	Yes; ~12 to 80	Moderate	Am. J. Neuroradiol. (2001) 22,1161
Pons	M > F	MRI	Yes; ~12 to 80	Small	Am. J. Neuroradiol. (2001) 22,1161
Posterior temporal cortex (auditory cortex)	M < F; decreased neuron density in males	Post-mortem tissue, Nissl stain	No	Small to moderate	J. Neurosci. (1995) 15,3418–3428
Primary auditory cortex	M < F	Post-mortem tissue, silver stain	No	Small to moderate	Neuroreport (2001) 12,1561

Numerous studies have reported sex differences in the size of various brain regions and structures. This table is a representative survey of studies done in post-mortem tissue using histochemical or tracing methods and those done in live subjects using MRI. Note several studies on the anterior commisure, an intrahemispheral fiber tract. The most recent study found no sex difference. This is also true for studies of the corpus callosum, another intrahemispheral fiber tract that has been reported to be larger, smaller or not different between men and women. Those studies are too numerous and inconclusive to list here. Abbreviations: M: male; F: female; INAH: interstitial nucleus of the anterior hypothalamus; SDN-POA: sexually dimorphic nucleus of the preoptic area; BSTc: bed nucleus of the stria terminals-central subdivision; MRI: magnetic resonance imaging.

9. Sex differences in synaptic patterning

Because they are relatively easy to identify and intuitively of interest, sex differences in the overall size of brain structures have been the focus of much attention. However, an equally profound sex difference is found at the cellular level in many brain regions, this being what is generically referred to as synaptic patterning. Synaptic patterning is the frequency and density of synaptic contacts within a particular brain region. Not all synapses are made equal and they are in three basic types. One is the axosomatic synapse, referring to an axon terminating on the soma (cell body) of another neuron. Second is the axodendritic synapse, when an axon terminates on the dendrite of a target neuron and the last is a specialization of this known as the axodendritic spine synapse. Spines are small protuberances on dendrites that are the site of predominantly excitatory synapses and changes in spine density are a hallmark of synaptic plasticity. Classically, it was thought that the brain was a relatively hard-wired structure, i.e. once connections were made they were permanent. This is still true to some degree, as discussed in Section 8 on sensitive periods. However, it is now apparent that the brain is also constantly revising some proportion of its synaptic connections, strengthening some and dismantling others. These changes can underlie learning and memory, sensitization or changes associated with stress and disease. Hormones too can exert profound effects on synaptic patterning in the adult brain (see Chapter 18). These effects are transient, and portions of the female brain may remodel with each successive estrous cycle (in rodents, and perhaps with each menstrual cycle in women, although currently unknown). It is now apparent that hormones sculpt the neuroarchitecture of the developing brain and establish permanent sex differences in synaptic patterning. For instance, the density of axodendritic spine synapses is twice as great in one hypothalamic region (the arcuate nucleus) in females as males whereas the opposite is true in another hypothalamic area (the preoptic area), with males having double the number of dendritic synapses as females. Surprisingly, it appears that a specialized type of glia, known as astrocytes, contribute to establishment of sexually dimorphic synaptic patterning. What makes it surprising is that this cell type is not generally thought of as a primary target for steroid action, in part because there is little evidence that they possess receptors for estradiol. Further examination indicates that this original supposition was correct, astrocytes are not the primary site of hormone action, but instead they are responsive to hormonally induced signals originating in the neurons. The chemical messengers used for neuron-to-astrocyte-to-neuron communication appear to be unique for each brain region, with two having been clearly identified so far (Fig. 3). For example, in the arcuate nucleus, estradiol induces neurons to synthesize and release the neurotransmitter GABA. The GABA in turn acts on the neighboring astrocytes initiating a signal transduction cascade that leads to growth and branching of astrocytic processes. The resultant change in astrocyte morphology appears to then feedback on neuronal morphology and alter synaptic patterning via an as yet undiscovered mechanism. In the nearby preoptic area, a different set of chemical messengers communicates between astrocytes and neurons. Here, estradiol induces the synthesis of a prostaglandin (PGE2) in neurons, which is secreted and acts upon the adjacent astrocytes initiating release of glutamate. The glutamate from the astrocytes then acts back on the neurons causing the formation of dendritic spines, and thereby altering synaptic patterning.

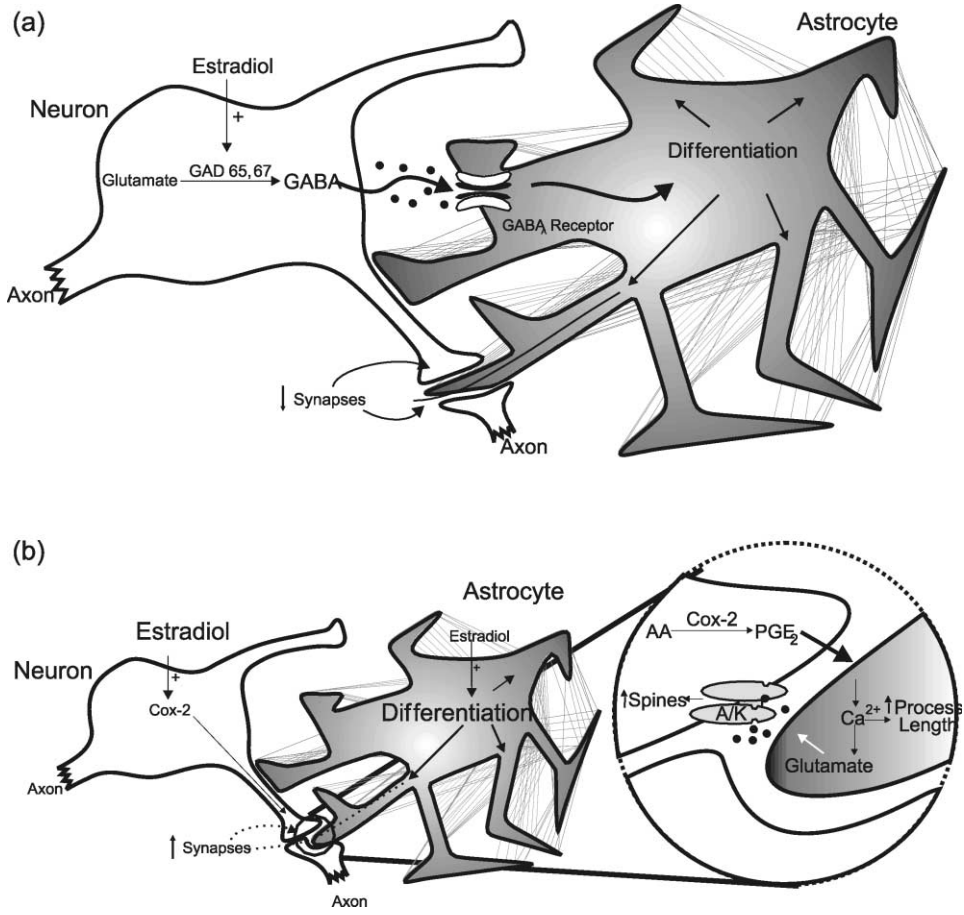

Fig. 3. *Cell-to-cell signaling helps to establish brain sex differences*. The brain consists largely of two cell types, neurons and glia. Astrocytes are a subset of glia and have recently been found to be highly responsive to modulation by gonadal steroids. The mechanisms of communication and relationship between astrocytes and neurons varies between brain regions and allows for heterogeneous regulation and establishment of local sex differences in synaptic patterning. (a) In the arcuate nucleus of the hypothalamus, estradiol increases synthesis of the neurotransmitter, gamma-aminobutyric acid, known as GABA, which is released and binds to GABA-A receptors on astrocytes, resulting in differentiation. Increased differentiation of astrocytes then decreases the density of synapses on dendritic spines via an unknown mechanism. (b) In the preoptic area, estradiol increases synthesis of prostaglandin E2 (PGE2) which is synthesized in neurons by cyclooxygenase 2 (COX-2). PGE2 is then released from neurons and acts on astrocytes, causing them to release glutamate. Glutamate then binds to receptors on the neurons and increases the density of synapses. Note that the ultimate effect of estradiol on synaptic patterning is the opposite in the two brain regions. These two examples demonstrate the general principle of region specific crosstalk between neurons and astrocytes to establish sex differences in the brain.

The use of experimental animals to elucidate mechanisms establishing sex differences in the brain is essential as there is no technical way to approach such a question directly in the developing human. An MRI can be used to obtain an indirect measure. Using this technique, it was determined that human brain development proceeds in the same way as

other mammals in that myelination is a relatively late process that occurs in tandem with the pruning of excessive synapses. That is, the general pattern for mammalian brain development is for exuberantly formed excessive synapses to be pared down by experience. Frequently used synapses are spared while those less active are allowed to degenerate, a process known as "activity dependent survival". Part and parcel of the survival selection process is myelination, which coats the neurons in a protective fatty sheath and increases the rate of neuronal transmission. MRI can distinguish between white matter, areas of myelination, and gray matter, regions consisting of cell bodies and dendrites, the principle site of synapses. Relative changes in white versus gray matter can be taken as an index of the processes of myelination and synaptic pruning, respectively. In a cross-sectional study, changes in white versus gray matter were examined in boys and girls ranging in age from 7 to 17. Both sexes showed an increase in white matter and decrease in gray matter with age, but the relative changes in males were significantly greater, having over a 3-fold greater loss in gray matter volume and more than 2-fold greater increase in white matter than females [7]. Its important to note that females also showed a significant age-related change in gray and white matter, but that the change appeared to be occurring at a different rate compared to males. This echoes a general principle of brain development in males and females; the same things happen, they just happen to different degrees and/or at different times.

10. Degree of cerebral lateralization

Distinct from but related to sex differences in volume and synaptic patterning, is the issue of lateralization. The brain is a bilateral structure and it has been known since the landmark studies of Rodger Sperry [17] in which the corpus callosum of severely epileptic patients was severed, that the left and right cerebral hemispheres are differentially involved in a variety of cognitive tasks and that they communicate with each other. Evidence suggests the degree of hemispheric specialization is especially strong in humans since it is functions such as language and mathematical ability, uniquely human traits, which illustrate the extremes of lateralization. However, most mammals also exhibit cerebral asymmetry. In humans, the left cerebral hemisphere is considered specialized for language and other communicative functions, while the right hemisphere is the predominate sight for spatial processing. Established sex differences in verbal and quantitative abilities led naturally into the hypothesis that the degree of cerebral lateralization also varies between males and females. There are two aspects of lateralization to be considered: (1) the size of the individual hemispheres and (2) the degree to which the hemispheres communicate. The corpus callosum is a large commissural fiber system that connects the left and right hemispheres and intense interest has been focused on this readily measurable structure. The history of reports on sex differences in the corpus callosum is a case study of the vagaries and reversals that can occur when the human brain is the endpoint. In the early 1980s several reports indicated that females had a larger over all corpus callosum, and that the splenium, the bulbous posterior end consisting of fibers connecting the main visual and auditory cortical areas, was the most dimorphic. This difference was detected in fetuses as young as 26 weeks and

lasted throughout gestation and into postnatal life. Numerous later studies found no sex difference in corpus callosum area in fetuses or adults up to 93 years of age. Some even found that males had a larger corpus callosum than females. The latter finding was consistent with the animal literature in which male rats have a larger corpus callosum than females and the difference is a function of perinatal testosterone exposure. Two variables likely accounted for the inconsistencies in findings in humans. Initial studies were conducted on post-mortem tissue and measured area as opposed to volume. The subsequent use of MRI, a particularly useful approach for this heavily myelinated structure, allowed for careful volumetric reconstruction as well as a correction for overall head size. Second is what has become an emerging principle in studies of the human brain, dynamic changes occur with aging. Although previous studies had looked at the brain at different ages, they had been viewed more as static snapshots in which males and females were compared as opposed to what is now understood to be a longitudinal process that can vary in rate and degree between males and females. Controlling for the degree of right handedness of subjects, especially males, further reduced variability in the data. Thus sex differences in a structure can wax and wane over the course of a lifetime as males and females diverge usually in late adolescence and early adulthood only to converge again in old age. A thorough understanding of changes in the brain across the lifespan thus becomes critical for the diverse fields of neonatology, psychiatry and gerontology. It can now be said with some confidence that there are sex differences in some regions of the corpus callosum, with females generally having a larger volume than males, which is presumably predictive of greater intrahemispheric transfer of information (reviewed in Ref. [20]). A consistent observation, however, is that circulating levels of testosterone in adult males correlates with increased corpus callosum volume [10].

Establishing that anatomical sex differences in cortical asymmetry relate to function is made possible with the use of electroencephalography (EEG), positron emission topography (PET) and more recently functional magnetic resonance imagery (fMRI). These techniques have allowed scientists to take a picture of the “working” brain and ask questions about how it responds during specific cognitive tasks in men and women. In general, all of these techniques have confirmed and expanded the concept of reduced lateralization in females compared to males. When asked to perform verbal processing tasks, brain activation is localized to the left inferior frontal gyrus in males whereas in females activity was found in both hemispheres and distributed over a wider area [15]. These observations are considered functional evidence for the clinical observation that men are more likely than women to suffer global aphasia after a left hemisphere stroke. However, as with purely anatomical studies, this area of research has not been without controversy. A recent study attempted to clarify previous contradictions by proposing that it is the time demanded to process a word task relative to the interhemispheric conduction delay that gives females an advantage by having a larger, and therefore, more efficient corpus callosum [9]. This hypothesis is difficult to prove definitively given the current temporal limitations of fMRI, which generates data in the order of 6–8 s while word processing occurs on average in 500 ms.

Central to this chapter is the question of how much early hormone exposure contributes, if at all, to the sex differences observed in cortical function. Indirect assessment of fetal exposure to steroids is gained by measurement of steroids in amniotic fluid. In one such

study, higher testosterone during gestation correlated with stronger measures of right handedness and greater right hemisphere dominance in verbal tasks. Naturally occurring experiments, such as the use of diethylstilbesterol (DES), a highly potent estrogen, given as treatment to women at risk of miscarriage, allows the potential to move beyond correlational analysis. Studies of adult men and women exposed to DES in utero report changes in verbal tasks associated with hemispheric lateralization. Variables such as the dose and duration of exposure across subjects and studies precludes overarching generalizations other than there are statistically significant effects of DES exposure on verbal processing within a particular study. It should be emphasized, however, that the effects that are found are subtle and restricted to a subset of verbal tasks (i.e. effects on dichotic listening of consonant–vowel syllables as opposed to word fluency tasks). More importantly, the effects noted on DES exposed subjects still placed them within the normal range of the population, meaning these individuals were not "impaired", simply their performance was shifted in the direction of one sex or the other. Studies of other populations experiencing unusual early hormone exposure, such as girls with congenital adrenal hyperplasia (CAH) or Turner's syndrome, and boys with Klinefelters reveal inconsistent results and neither add nor detract from the postulate of early hormonal influences on cortical asymmetry.

An alternative to the "natural experiment" approach in humans is direct comparison between humans and experimental animals. This is generally not possible when the animal, such as the rodent, has few cognitive behaviors in common with humans. However, non-human primates can be asked to perform similar cognitive tasks to those of young children and the primates can be manipulated hormonally prior to testing. Children as young as 15 months will perform an object reversal task in which they are rewarded with a cheerio by choosing the brightly colored object under which it is hidden. After several training trials, the object hiding the reward is switched and the child is asked to "learn" this reversal. Young boys perform better on this task than young girls. Conversely, if the task involves concurrent discrimination in which the same object hides the reward but the order presented is changed, girls outperform boys (this test is done on slightly older children, up to 35 months). In both cases, as the children get older, the sex difference disappears. Because of its simplicity, the same test, using the exact same brightly colored objects and the same reward, can be learned by young Rhesus monkeys. In this case an identical sex bias was observed, with males excelling at object reversal and females better at object discrimination. These tests are particularly useful in that it is hard to imagine a role for socialization since the objects and rewards are the same in both cases. But even more compelling is that in the monkeys, perinatal treatment with androgens reversed the performance of females such that they were equal to males and better than untreated females on the object reversal task [11].

Another domain in which parallels can be drawn between humans and animals is the collection of activities known as play. Play is not unique to humans as it is expressed by juveniles of probably every mammalian species. The purposes and adaptive advantages (if any) of play are a fascinating topic of inquiry, a discussion of which is beyond the current scope. However, a type of play categorized as "rough-and-tumble play" is directly relevant to the development of sex differences in the brain. This refers specifically to play that involves contact between individuals and can be quantified as the frequency, duration and

intensity (subjectively scored) of physical interactions. Across species, males are consistently found to exhibit higher rates, longer durations and greater intensity scores of rough-and-tumble play. What is particularly interesting about the behavior is that it occurs at a time when there is no sex difference in circulating gonadal steroid levels, in large part because there are no circulating gonadal steroids in either sex. Thus what is observed is either a function of developmental hormones, which is consistent with all of the experimental data in animals, or due to socialization, or a combination thereof. An obvious tool to address this question is girls with CAH an enzymatic deficiency that results in excessive androgen production by the fetal adrenals, resulting in increased androgen exposure of the fetal brain. These girls show a slight increase in rough-and-tumble play compared to their unaffected sisters, but do not reach the level of their brothers. When examined for sex differences in toy preference or preferred sex of play-mates, effects seen in CAH girls are more strongly in the direction of boys, but again do not reach the same level [3]. A similar pattern is observed in characteristics of children's (3–5 year olds) drawings in which CAH girls are intermediate between unaffected brothers and sisters. While a highly informative and useful approach, the extensive study of CAH girls is not without caveats. A recent initiative called The Tomboy Project, in which girls are identified as tomboys by their parents, aims to assess many of the same endpoints as those studied in CAH girls but without the concerns attendant to individuals that have been diagnosed with a disease and undergo lifelong treatment [2]. Many readers, the author included, who considered themselves "tomboys" as children might find themselves bristling at the notion that such girls should be "studied". But consider this. If men are better at math because they played with trucks as boys, then should not girls who played with trucks be better at math as women? In other words, many hypothesis about effects of early environment and socialization on adult cognitive functioning can be tested by clever and careful examination of naturally occurring variations in children's behavior combined with a prospective assessment in adulthood.

11. Why is it important to understand sex differences in the brain?

While the above arguments may or may not be convincing, it is still reasonable to step back and ask what is the goal of research on sex differences in the brain? There is unanimity regarding the utility of examining sex differences in say, cardiovascular function or susceptibility to diabetes, but discussions of determinants of behavior, particularly sex-based behavior, generate varied and sometimes emotional responses. The potential for outright abuse of scientific information as well as the more subtle, and perhaps more damaging effects of a diffusion into the collective mindset of scientific "facts" about boys and girls, warrants caution. So why study this minefield of a topic? One way of answering the question is to turn it around and ask, what are the consequences of *not* asking questions about biological differences in male and female brains? From a medical standpoint, it is apparent that males and females experience disproportionate risks for a constellation of neurological disorders and that their response to a neuronal insult can vary in part as a function of sex (discussed in detail below). From a societal standpoint, understanding that boys and girls are different, and in what ways they are different, is as important as

understanding in what ways they are the same. Styles of learning and coping strategies may vary in a predictable way that can be exploited to the greater advantage of both sexes. Finally, a topic not touched on in this chapter is the considerably higher rates of aggression, and hence violence, perpetrated by males, and which is a cost to society that goes beyond measure. It may be naïve to think that conducting studies in rats and mice of early hormonal effects on aggression will inform us about a behavior so ingrained, so complex and yet so simple that it cuts across every culture on the planet. But the alternative, concluding that a behavior with deeply engrained biological roots, including the hormonal environment of development, is solely a product of culture and society, is equally naïve. Complete understanding requires knowing all of the sources of variation and the magnitude of their contribution to diversity in behavior, both normal and aberrant. It is the goal of neuroendocrinologists to add to, not to dominate, the discussion of such variables.

12. Summary

(1) The brain begins as bipotential and its ultimate phenotype as male or female is determined in large part by exposure to steroid hormones during development.
(2) The effects of hormones on the developing brain are considered *organizational* and occur during a restricted sensitive period.
(3) Steroids then again act on the brain in adulthood to *activate* many sex-typic behaviors.
(4) The aromatization of testosterone to estradiol is a vital component of masculinization of the brain in rodents. In primates the majority of masculinization involves androgens, although a potential role for estradiol cannot be excluded.
(5) Neuroanatomical sex differences include the size (or volume) of particular regions or structures, the density of a projection or the synaptic patterning.
(6) Most, if not all, volumetric sex differences are a function of hormonally mediated differences in naturally occurring cell death.
(7) The mechanisms determining sexually dimorphic projections and/or synaptic patterning appear to be region specific and remain poorly understood.
(8) Some of the neuroanatomical sex differences observed in animal models are also present in the human brain.
(9) Several behavioral sex differences observed in animal models are also seen in very young children.
(10) The study of sex differences in the brain requires an appreciation for complex and often uncontrollable variables combined with rigorous scientific method.

13. Future directions

Compared with many subdisciplines in the field of neuroscience, the study of hormonal effects on the brain is relatively old, tracing its origins to the mid 1940s. By contrast, advances in this field have been comparatively slower than that of many others. The reasons for this slow rate of progress can be speculated on at length and are probably a combination of variables, not the least of which is the complexity of the problem.

Nonetheless, it is clear that there is a widening gap in knowledge of the mechanisms by which sex differences in the brain arise compared with the steady cataloging of anatomical, physiological and behavioral differences. The great challenge ahead is to begin to elucidate the cellular and molecular pathways of steroid mediated brain differentiation. Increasing understanding of the multiple ways in which steroids can alter cell functioning, much of what you have been learning in this text, overlays an additional level of complexity. Adding to this challenge is an emerging appreciation that the mechanisms for organizing a male versus female brain appear to be entirely unique for each brain region.

On a broader scale, establishing the mechanisms of steroid-induced differentiation offers insight into the normal mechanisms of brain development. All of the parameters altered by steroids; apoptosis, neurite extension and branching, synaptogenesis, are fundamental processes central to developmental neuroscience. How these occur and how they go awry, provide information about brain development in general. Steroid effects on these processes may also offer additional insight into the etiology of disorders or diseases in which there is a differential level of risk as a function of sex. Dyslexia, stuttering, autism, attention deficit hyperactivity disorder and Tourette's syndrome all occur at a higher frequency in boys than girls, whereas major depressive disorder, anorexia/bulimia, general anxiety disorder and obsessive compulsive disorder are more prevalent in women than in men. Many variables, both biological and cultural, likely contribute to the differences in both risk and rates of diagnosis. But the fact that disorders more prevalent in males form a constellation of developmental disorders, while those in females are largely of adult onset, raises the question of whether the differential hormonal milieu experienced between the sexes at these two life stages might be a contributing variable. Definitive answers to these questions are a long way off but progress begins with more research.

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Mechanisms of sex-based neuropathologies

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1. Introduction

Appreciation that sex influences normal behaviors dates back several millennia to Aristotle, who described the phenomenon that castration of immature male birds led to suppressed development of characteristic male singing and sexual behavior. About 150 years ago, Brown–Sequard claimed that the administration of aqueous extracts from animal gonads increased sexual virility and a variety of other human mood disorders, including depression. However, an appreciation that sex, gender, hormones and the different cultural environments of men compared to women also affect the incidence, the responsiveness to toxic agents, the risk, and the outcome of diseases that impact the central and peripheral nervous system has been recent.

Neuroscience is an area where it is critical to understand not only how basic physiological processes and anatomical structures of the nervous system in males and females are similar and different, but also the role of sex, gender, hormones and culture in brain development, behavior, and neuropathology. Classic studies established that sex differences may be evident as early as during fetal development (reviewed in Ref. [8]; see also Chapters 2 and 17). Exciting recent work establishes that the adult brain remains plastic. Therefore, sex-dependent changes that are modulated by hormonal environment during fetal life may be further modulated by hormonal milieu and environmental events during postnatal through adult life (reviewed in Ref. [10]).

The goal of this chapter is to describe differences in the incidence, risk, and outcome of diseases that affect the nervous system of men and women and how these differences may result from genetic, physiological, and environmental differences of both sexes.

2. Sex differences in the nervous system

Differences in normal function and pathological changes involving the adult nervous system may result from differences that occur as early as during fetal development since hormones and receptors that are present during this period influence later behaviors and strategies of learning and memory. Furthermore, these differences in fetal environment

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may suggest ways in which prenatal exposure to hormones may contribute to traits associated with disease (see Chapters 2 and 17). It is clear that no simple or single factor determines sex differences in any nervous system trait in health or disease. For example, in the past it has often been argued that differential occurrence of depression, which occurs in a higher proportion of women than men, reflects women's greater social orientation and/or stresses associated with women's multiple social roles. More recently, it has become apparent that, in addition to differential societal pressures, genetic and physiological differences between males and females greatly influence the incidence of depression.

Data from studies using experimental animals suggest that exposure to androgens during the pre- and neo-natal period determine male patterns of behavior and male patterns of hormone secretion. The organizing capacity of androgens appears to depend upon exposure during a critical developmental window in time and upon responsiveness of specific areas of the central and peripheral nervous system structures that extend beyond those that are traditionally associated with neural control of reproduction (see Chapter 2).

Neuroanatomical differences in male and female brains have been clearly established through several classic papers (reviewed in Refs. [2,3]). These differences are developmentally programmed and result in sex differences in the multiple characteristics of the central nervous system including differences in nuclear volume, neurogenesis and cell death resulting in differential neuronal number, differentiation and migration, cell size, neural cellular density, neuronal and glial morphometry, neuritic branching patterns, synapse formation and innervation. These differences in the morphological substrate may explain sex-related differences in gene expression, signal transduction and intra- and intercellular signaling, neuronal and physiological and behavioral responses to a variety of stimuli, and phenotypic differences in the amount and patterns of neurotransmitter release. In particular, differences in the volume of the sexually dimorphic region preoptic area have been studied extensively and are influenced by the perinatal hormone environment but not by hormonal conditions in the adult animal (reviewed in Refs. [2,12]). These early studies showed that castration of males and the administration of testosterone to females during the perinatal period of development could change the female-like pattern to a male-like neuroanatomical substrate and established the concept that differences in the wiring of the brain are programmed at birth. In birds, differences in singing behavior between males and females correlate with differences in the size of brain regions that control vocalization. However, it is important to point out that young male birds must hear the adult male song to initiate its own repertoire.

There are now many documented sex differences in a wide range of species including sub-human primates and humans. Sex differences in the human brain include the higher cognitive centers (reviewed in Ref. [14]). These differences have been observed in adults and the nature and origins of these differences are subjects of active investigation. Sex differences in human brain include

- sex-specific decreases in regional brain volume during development
- increased neuronal density in the temporal cortex in women
- greater inter-hemispheric coordinated activation of brain regions in women
- larger volume of hypothalamic nuclei in men

- differences in both resting blood flow and activation pattern accompanying self-induced mood change
- differences in whole brain serotonin synthesis and decreased serotonin receptor serotonin-2 binding in the frontal, parietal, temporal and cingulate cortex of women
- higher and more symmetric cerebral blood flow in women
- greater brain glucose metabolism in women.

Recent studies suggest that many of the sex differences in brain structure size become apparent as the brain develops in children. Many questions remain.

The concepts that have emerged from studies with laboratory animals and other non-human species have generally been confirmed in humans. However, important differences in details exist. For example, androgens act as masculinizing agents in all species, but different biologically active metabolites appear to be the critical mediators in different species. Taking together the large body of literature in this area, the important principle that emerges is that the central nervous system remains plastic through the life span. This was first observed in studies that used experimental animals, and appears to be shared in humans.

One of the most fascinating sex-determined differences in the nervous system involves instances of diametrically opposed responses to some stimuli in males compared with females. For example, aggression in male mice is considerably more intense than in female mice, and this difference is thought to be influenced by exposure to testosterone during the neonatal critical period and during adulthood [1]. Recent studies suggest that the story may be more complex and may involve different responses to key mediators of aggressive behavior. Sex-determined opposing responses to nitric oxide (NO), a compound that participates in cell-to-cell signaling, appear to be intriguing. The neural form of NO (*n*NOS) plays an important role in the expression of aggressive behavior in males. Changes in NO are measured by changes in NO synthase, the rate limiting enzyme in the synthesis of NO. The differential role of NO in aggressive behavior was discovered when *n*NOS knockout mice were created. Investigators observed that male knockouts were hyper-aggressive but inappropriate aggressiveness was never observed among the *n*NOS knockout female mice. Even when given an opportunity to defend their pups, *n*NOS knock out female mice were very docile, even more than their wildtype sibling females. These studies suggest that NO from neurons has important but opposite effects in the mediation of aggression in male and female mice (reviewed in Ref. [20]).

3. Effects of steroids on the central nervous system depend upon sex, gender, cellular, developmental, temporal, environmental context

A fundamental concept that has become clear from numerous studies to date is that the molecular and behavioral effects of steroids are highly context-dependent (reviewed in Ref. [14]). The genetic sex and gender background, and the cellular, metabolic, developmental, temporal, environmental context of steroid exposure are critically important in the differential response of males and females.

That the brain displays sex-related differences in structure and function has become a dogma. Early hypotheses have been repeatedly confirmed by demonstrations of sexual

dimorphisms in the rodent and avian brain morphology and of sex-related differences in synaptic density of regions that determine sexual behavior, hormone secretory patterns of rodents and areas that influence vocalization in birds. Sexual dimorphism at all levels of the central and peripheral nervous system have been reported and include differences in nuclear volume, neuron number, size, density, morphology; and gene expression, signal transduction, neuronal neuritic branching patterns, synapse formation and elimination, and physiological and behavioral responses. Since reproductive steroids regulate virtually all stages of brain development, from neurogenesis to neural migration, differentiation, synaptogenesis, survival and death, it should not be surprising that the brain exhibits such a wide range of sexual dimorphisms. However, not all differences between males and females are determined by exposure to steroids: some differences in the course of development of embryonic mesencephalic and diencephalic neurons appear under genetic control since they are evident well before the appearance of any differences in reproductive steroid levels. The sexual dimorphisms in animals and humans provide an anatomical and biochemical underpinning for sex-based differences in neuropathologies such as the incidence and/or risk of Alzheimer's disease, cerebrovascular stroke, cognitive dysfunction, and depression and other psychiatric disorders. These neuropathologies exhibit differences in prevalence, characteristic symptoms, age of onset, susceptibility to recurrence, responsiveness to stress, rate of progress, and/or response to treatment. Although other neuropathological phenomena exhibit sex-based differences, this chapter will focus on the ones listed above.

4. Alzheimer's disease

As our average lifespan increases, Alzheimer's disease has emerged as a major public health problem. In people over the age of 65, the prevalence of dementia and Alzheimer's disease doubles every 5 years; and 30–50% of women over 85 years suffer from dementia of some type (reviewed in Refs. [9,13]). Clearly the emotional, physical, social and financial costs to caregivers, families and the patients themselves are enormous. The financial cost of care alone is greater than for heart disease and cancer combined, in part, because the length of time that someone survives and needs care can be long.

Women have been reported to have a higher age-specific prevalence and incidence rates of Alzheimer's disease than do men (reviewed in Ref. [9]). In some reports, the disorder afflicts twice as many women than men. This sex-related difference in the incidence of Alzheimer's is further exacerbated since the average life expectancy of women is approximately 3 years greater than for men. Several studies that have shown that the incidence of dementia is greater in women than in men have established that this is due predominantly to increased incidence of Alzheimer's disease, rather than vascular dementia, in women. The predominance of the disease in women has raised the question of whether the lack of estrogen in postmenopausal women poses an increased risk for the disease. This question is relevant because women with Alzheimer's had significantly lower total estrogens and serum estradiol levels than did matched controls. Although several studies have approached the question of whether estrogen therapy decreases the risk, delays the onset, or stops the progression of neurodegeneration caused by Alzheimer's disease, the

findings to date are not clear. In some instances estrogen treatment has been shown to decrease the risk for Alzheimer's disease or induce a modest improvement in cognitive function in individuals who have already shown manifestations of the disease. However, other studies have reported no difference in cognitive function between estrogen- and placebo-treated individuals. A few recent studies failed to detect slowing of progression or improvement of cognitive and functional outcomes in women with mild to moderate Alzheimer's disease that were treated with conjugated equine estrogen. The results of these studies strongly suggest that estrogen may fail to reverse or even halt a disease process that has already been initiated. Therefore, while it is very probable that estrogen may be an effective primary preventative, acting to delay or attenuate the "initiation phase" of a disease, its efficacy as a hormone of repair that influences the "propagation phase" of an ongoing disease is less clear (reviewed in Refs. [5,19]). Thus, it appears that estrogens may influence certain aspects of cognitive function in healthy aging women, and decrease the risk for Alzheimer's disease. They are apparently less efficacious acting against an existing neurodegenerative condition to slow the progression of pathological decline once a disease process has been initiated and is manifest (Fig. 1).

Discrepancies among the results of the several studies that have been performed thus far may be due not only to the stage of the disease at which estrogen therapy was initiated but to several other differences in the design of these studies. First, the numbers of

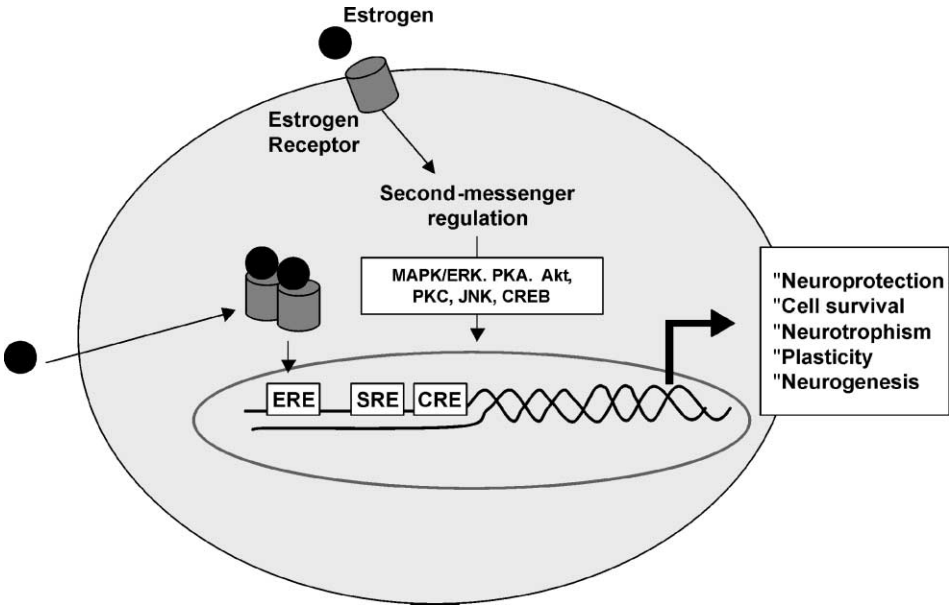

Fig. 1. Schematic representing some of the multiple mechanisms of estrogen action. Estrogens may act by binding to intracellular receptors that act as transcription factors by binding to promotor regions of responsive genes. Alternatively, estrogens may bind to receptors that are located in the membrane or to membrane domains, such as caveolae, to activate multiple second messenger signalling pathways, which in turn may have genomic actions.

individuals included in some studies are very small. Since the effects of estrogen may be subtle, failure to detect a statistically significant difference may result from inadequate statistical power. Second, some studies are prospective in design. In these cases, the specific estrogen preparation, whether it is combined with a specific progestin, the length of treatment and other variables can be controlled by the investigators. Other studies are epidemiological and retrospective in design. These studies are often able to include a larger population of subjects, but may be confounded by several factors. Frequently, individuals who have taken different hormone treatment regimes are included, the complete health background of subjects in the study is not known, the length of treatment may not be consistent, and the methods of reporting effects of treatment may not be ideal. Third, the criteria for establishing that a person truly has Alzheimer's disease, and is not suffering from some other forms of dementia are not always consistent. Since ultimate evaluation of the occurrence of Alzheimer's depends on neuropathological observations of the brain tissue, reporting of Alzheimer's disease based upon less stringent criteria must be considered in the interpretation of the results. Fourth, multiple different forms of estrogen therapy have been used in the various studies. Sometimes estrogen treatment is combined with progesterone or androgen. These factors may affect estrogen's ability to protect the brain against the effects of aging and Alzheimer's. In addition, the duration of the hormone therapy and whether patients were using hormone therapy at the time of testing may influence some measures of cognitive function, but not others. Fifth, the specific psychometric measures used to assess cognitive function vary widely. It is possible that estrogens influence only a subset of cognitive functions and the measures of these specific subsets may not have been used. It is important to consider that memory is not a unitary system but rather is composed of numerous component processes. When one tests only certain components of memory or when multiple endpoints are measured, but this is not considered in the statistical analyses, improper conclusions may be drawn. Sixth, one of the most common criticisms of observational studies is that they suffer from the "healthy user bias", that is that women receiving hormone therapy tend to be better educated and healthier than non-users. Therefore, many studies may be examining two very different groups of women in the non-users and the hormone treated women. This is particularly important when considering the incidence of Alzheimer's disease since the level of education appears to influence the incidence of the disease.

That estrogens influence cognition and memory in Alzheimer's disease should not be surprising since it has been associated with these parameters in healthy populations. A large number of clinical studies have investigated the effects of hormone replacement on these components of cognition (reviewed in Ref. [16]). The majority of data show that estrogen can enhance cognitive function in both young and older women. These studies complement those performed in Alzheimer's patients and show that estrogens do not exert actions on all aspects of memory, but instead may influence specific subtypes of memory. For example, some, but not all, studies show that estrogen treatment appears to specifically enhance immediate and delayed recall of verbal information. Other reports indicate that the beneficial actions of estrogen on cognition include improvement of visuospatial memory. Interestingly, the aspects of memory that appear to be affected by steroids are those that appear to be different in non-treated men and women. For example, men tend to outperform women on tasks that require mental and spatial

rotation, whereas women tend to outperform men when tested for spatial location in a static environment. Patterns of cognitive task-activated cerebral blood flow are different in women and men and this difference was eliminated by induced hypogonadism and restored by hormone replacement.

Studies in experimental animals suggest that estrogens are truly pleiotropic hormones that may influence the incidence and severity of Alzheimer's disease through multiple mechanisms. First, estrogens promote the growth of cholinergic neurons by reversing the decrease in neuronal choline uptake and enhancing the activity of choline acetyl transferase induced by ovariectomy. Since this neurotransmitter is critical to cognitive function and appears to be particularly vulnerable to cell death in Alzheimer's disease, it has been reasoned that factors that help to maintain the function of acetylcholinergic neurons will improve memory and cognition and decrease the incidence of Alzheimer's. Second, estrogens appear to influence the expression of apolipoprotein E. Certain isoforms of this lipoprotein appear to increase the risk of developing Alzheimer's disease and influence the rate of disease progression, therefore, factors that influence the expression of apolipoproteins may influence the etiology of the disease. In addition, estrogens stimulate the secretase metabolism of amyloid precursor protein. Finally, ovariectomy results in an increase in levels of Aβ deposition in the brain, whereas estradiol treatment reversed this increase. Since neuritic dystrophy, neurofibrillary tangle formation, gliosis and microglial reactivity, and other degenerative changes seen in the brains of people suffering from Alzheimer's disease are a result of altered metabolism of Aβ peptides, it is possible that modulation of Aβ metabolism may be one of the ways by which estrogen replacement therapy prevents or delays the onset of Alzheimer's disease in women.

5. Stroke

Stroke is a neurodegenerative condition that greatly impacts the health and quality of life of older people. It is the third leading cause of death for middle-aged and older women and a major health problem that affects half million Americans each year (reviewed in Ref. [11]). Interestingly, the risk of stroke is less in premenopausal women than in men of the same age. However, after the menopause the incidence of cerebrovascular disease rises rapidly. These clinical observations parallel epidemiological studies on the prevalence of stroke with regard to age and sex: at a younger age, women are protected compared to men against stroke, but women lose this protective advantage in their postmenopausal years. Together, these data suggest that secretion of endogenous estrogens and/or progestins play protective roles and decrease the risk of stroke. Since stroke imposes major morbidity and mortality in postmenopausal women, it is important to determine whether estrogen or hormone replacement therapy may decrease the risk and/or severity of cerebrovascular disease.

Growing evidence indicates that estradiol may influence the ability of the brain to withstand ischemic insults resulting from both cardiovascular disease and cerebrovascular stroke. However, as in the studies on the incidence and potential protective influence of estrogens in Alzheimer's disease cited above, the studies on steroids and the risk of cerebrovascular stroke are not without controversy and sometimes are difficult to evaluate. Over 5 years ago, Paganini-Hill [11] assessed the results of studies that were performed

prior to 1995 and concluded that the bulk of the evidence suggests that estrogen treatment decreased the risk of stroke. However, more recent studies tend to bring this conclusion into question. Most recently, the Women's Health Initiative [21] reported that hormone replacement therapy, consisting of a constant dose and simultaneous treatment with conjugated equine estrogens combined with medroxyprogesterone acetate increased the risk of stroke. It is critical to emphasize that the effects of estrogen alone on stroke risk will not be available for a few years since this arm of the study was not terminated and is ongoing. These results will be important contributions to the understanding of the relationship between stroke and the use of estrogen.

There are many reasons that may explain the seemingly contradictory findings. First, there are very different kinds of cerebrovascular stroke and the types of stroke examined in these studies are not always specified and may be different (reviewed in Ref. [5]). The two major types of cerebrovascular stroke are called ischemic stroke and hemorrhagic stroke. Clots in the cerebrovasculature produce ischemic infarcts (ischemic stroke); whereas, the bursting of cerebral vessels causes subarachnoid hemorrhage (hemorrhagic stroke). The overlapping and often mixed etiologies of these two very different types of stroke can result in confusion in the interpretation of seemingly contradictory data. If estrogen treatment decreases or increases the risk of specific stroke subtypes, effects of estrogen may be distorted and/or masked when strokes are grouped together and classified differently among the studies. Second, hormone treatment regimes are variable in each of the studies. In fact, in some studies it is not clear which hormone treatment was used, the length of time of the treatment, or whether the treatment was ongoing at the time of the study. It may be important to distinguish between ever and current users of hormone treatment, since these two categories may contain two distinct populations of women. We do not know whether ever users of hormone treatment may demonstrate long-lasting actions of estrogen that do not require current exposure to the hormone, while current users may exhibit primarily short-term actions of estrogen. Clearly, mixing the two populations can confound or obscure important effects of estrogen. Third, estrogen treatment may indirectly reduce the likelihood for stroke by modifying risk factors that underlie stroke. For example, estrogens may protect by exerting beneficial effects on diabetes, on the serum lipid profile, and/or the incidence of coronary heart disease. Since these factors increase the risk of cerebrovascular stroke, it follows that estrogen therapy may decrease the risk for stroke in parallel with its ability to improve lipid profiles, to influence the etiology of diabetes, or to exert protective actions against coronary heart disease. Fourth, hormone therapy may not always be beneficial since estrogens may, under some circumstances, impose an increased risk for stroke by influencing factors that mediate coagulation and fibrinolysis. Concerns of the thrombotic potential of estrogens arose from early reports that oral contraceptives appeared to increase the risk of venous thrombosis, pulmonary embolism, and stroke. Similarly, estrogen therapy in postmenopausal women appears to be associated with a higher risk of venous thrombosis during the initial period of usage. However, whether these effects of estrogen treatment increase the risk of ischemic stroke in postmenopausal women is unclear. The dose and route of estrogen delivery are the key in determining clotting potential. At higher doses, oral estrogens, which enter the body via the enterohepatic system, can stimulate the production of thrombogenic factors predominantly through its actions on the liver. Alternatively, lower doses of

estrogen, delivered orally or transdermally, may not significantly affect hemostasis. It is important to remember that transdermal delivery of estrogen bypasses enterohepatic circulation and thus may prevent estrogen-mediated stimulation of thrombogenic factors in the liver. Fifth, interpretation of data from the Women's Health Initiative [21] has been widely accepted as definitive evidence that hormone therapy increases the risk of stroke. However, it should be emphasized that this study was performed on asymptomatic women between the ages of 59 and 70, in which a single formulation of estrogenic and progestin compounds was used. Furthermore, the conclusion that this form of hormone treatment increases the risk of stroke depended upon the statistical analyses that were used by the authors. Use of a more conservative test (the adjusted confidence interval) would have forced the authors to conclude that hormone treatment has no significant effect on the risk of stroke. Finally, as with data from studies that focus on a potential role of hormones in Alzheimer's disease, most studies indicate that estrogen does not protect against the risk of either non-fatal stroke or death in postmenopausal women with a previous history of stroke. Thus, overall studies of diseases that influence the etiology of heart disease, Alzheimer's disease and cerebrovascular stroke suggest that hormone replacement and estrogen replacement do not effectively protect against or reverse a disease process that has already been initiated. Most findings, including preliminary unpublished data from the ongoing Study of Women Across the Nation (SWAN) study highlight the importance of low, physiological doses in estrogen treatment of postmenopausal women if one seeks to take advantage of the protective actions of this hormone, without deleterious effects.

Several investigators have used experimental animals to understand the potential neuroprotective actions of estrogen in brain injury and disease and have focused attention on whether estradiol protects the brain after an injury has occurred. This question is very different than the approach of most clinical studies, which focus on the role of estrogen or combined estrogen plus progesterone in decreasing the risk of stroke, but have not paid as much attention to the potential effects of estrogens in influencing the outcome of stroke injury. Methods that transiently or permanently block one or more cerebral arteries in laboratory animals have been used to induce a reproducible stroke-like infarct in the brain. These experimental manipulations provide convincing evidence that estradiol is a neuroprotective factor (reviewed in Ref. [19]). Females consistently sustain less brain injury than males and estrogen replacement protects against ischemic injury in males and females. It appears that use of physiological levels of estrogen treatment requires a period of treatment prior to the injury to observe protection, whereas use of pharmacological doses of estrogen allows for protection against cerebral ischemia even when it is administered as late as 3 h after injury. Pretreatment with low, physiological levels of estradiol exerts profound neuroprotective effects in permanent cerebral ischemia and that this protection requires the presence of estrogen receptor alpha and likely involve changes in gene transcription that favor cell survival (reviewed in Ref. [5]).

6. Pain perception and analgesic responses

The realization that sex differences in the area of pain exist has only recently been acknowledged. A growing body of evidence suggests that there are sex-related differences

in pain perception, response and the effectiveness of various analgesic agents (reviewed in Ref. [19]). More females than males report clinical pain conditions, including migraine, tension-type headache, fibromyalgia, rheumatoid arthritis, temporomandibular disorders and irritable bowel syndrome. Most studies of experimentally induced pain have consistently, though not unanimously, shown greater pain sensitivity among women than men and women experience more multiple or recurrent pains than men (reviewed in Refs. [4,6,16,18]).

Many mechanisms have been proposed to explain the sex-related differences. Studies have suggested that psychosocial factors, such as gender role expectancies, beliefs regarding one's ability to control and tolerate pain, and anxiety may explain greater pain sensitivity in females than in males. Other biological factors such as differences in resting blood pressure and genetic factors have also been considered. However, the most notable factor has been the influence of the gonadal hormones, especially estrogen. There are discrepancies in the literature, which are likely due to methodological differences across studies: the nociceptive assays used vary widely in both the painful stimulus and the measured responses. But the majority of studies conclude that hormones influence all aspects of pain.

Pain thresholds differ across the reproductive cycle. Clinical studies have observed that for most forms of painful stimulation, higher pain thresholds and tolerances are observed during the follicular compared to the periovulatory and luteal phases of the menstrual cycle. These results would suggest that when estrogens and possibly progestins are high, the perception of pain increases. The influence of exogenous hormones on pain response has received relatively little attention, although a few studies have examined the effects of oral contraceptives on pain perception. In general these studies suggest that oral contraceptives prevent the effects of cyclic hormone changes on pain perception and exposure to relatively constant doses of steroids decrease pain sensitivity.

Several recent clinical reports have shown that in addition to sex differences pain perception, sex-related differences exist in responsiveness to analgesic pharmacological agents. Opioids appear to produce better analgesia for women than men. Males may be more sensitive to agents that influence the gamma amino butyric acid (GABA) neurotransmission system; however, it is possible that the sex difference was not due to differential analgesic effects, but rather that males may experience less experimentally induced pain in the first place.

There is a large basic science literature that has studied hormonal effects on pain and analgesic responses. The use of experimental animals has deepened understanding of the influence of gonadal steroids on pain. In rodents, pain sensitivity peaks during late proestrus and early estrous, a period when estradiol levels peak. These results corroborate the clinical studies that show that sensitivity to pain is greatest during the periovulatory and luteal phase of the menstrual cycle in women. However, other studies report opposing findings in which pain thresholds are lower during estrous and metestrus compared to proestrus and diestrus. The effects of ovariectomy are also inconsistent: some report decreases in pain threshold, but others do not. As in clinical studies, studies using animals suggest that hormonal conditions influence the analgesic responses to pharmacological agents. Males appear to have greater sensitivity to opioid and non-opioid agents, such as cocaine, whereas females demonstrate enhanced analgesic response to nicotine, and cholinergic agonists.

Inconsistencies are apparent but a number of differences between the studies might account for the variable findings such as the type of stimulus used, time of day the test was performed and animal species tested. It is evident that further studies on differences in nociception across the estrous cycle need to be undertaken to improve our understanding of the underlying basis for these differences. Thus, future studies are likely to focus on whether differences in the perception of pain in females compared with males depend upon the steroidal milieu, other factors that vary across the menstrual cycle, environmental experiences and/or genetic differences. It will be important to assess to what extent we can actually manipulate nociception through steroid treatment.

Pain, either acute and transient or chronic, involves both peripheral and central processing arising from an unpleasant sensory and emotional experience associated with potential or actual tissue damage due to stimulation of nociceptors. Most nociceptive information arises from stimulation of simple nerve endings called nociceptors. Estrogens may influence the perception of pain through multiple neurotransmitters systems and thereby affect the function of nociceptors. In particular, its ability to modulate multiple aspects of excitatory amino acid and GABAergic neurotransmission may be the mechanisms through which this steroid modulates nociception. Estrogens increase *n*-methyl D-aspartate (NMDA)-induced currents and receptor densities and hence increase excitatory amino acid neurotransmission. In contrast they tend to decrease the expression of GABAergic receptor expression and currents and may thereby attenuate inhibitory tone. In parallel, excitatory amino acids increase and GABA suppress nociceptive behavioral responses in the test models of pain.

7. Depression and mood disorders

Approximately 20% of women experience an episode of major depression, a rate that is twice that of men (reviewed in Refs. [7,15]). Many different forms of depression occur more frequently in women than in men: seasonal affective disorder occurs three times as frequently in women than in men; eating disorders occur at a higher frequency in women than in men. In addition, some forms of depression are unique to women: premenstrual syndrome, post-partum depression, and depression in the perimenopausal period. Premenstrual syndrome is a condition that is characterized by cyclic changes in irritability, sadness, and anxiety that interferes with daily function and are confined to the luteal phase of the menstrual cycle, with remission within a few days of the onset of menses. Post-partum depression is defined as the onset of a depressive disorder within 4 weeks of delivery. Perimenopausal depression is defined by the onset of depression at middle age in association with the onset of menstrual cycle irregularity or amenorrhea and elevation in serum follicle stimulating hormone.

The reasons for these sex-related differences in the incidence of a variety of mental disorders are far from clear. Specifically, one cannot infer that the observed differences are a product or a reflection of sex-specific biology. Sex-related differences in the prevalence of depression could occur consequent to increased number and severity of stressors experienced by women or to social stigmatization of endorsement of depressive symptoms in men. Nonetheless, the potential for sex-dependent biology to play a significant role in

affective an cognitive disorders is suggested by the following: (1) sexual dimorphisms in brain structure and physiology have been identified in humans; (2) reproductive steroids regulate brain function; and (3) reproductive steroids play a role in the precipitation and treatment of mood disorders that are linked to period of reproductive endocrine change.

During the 19th century, several medical reports documented the fact that medical or surgical manipulations of a woman's reproductive system improved mood and behavior. More recently, several studies suggest that gonadal steroids regulate mood in some women and may be used in therapies in the management of depressive illness. Thus, there is a long held view that reproductive function in both men and women is intimately involved with mood regulation and depressive illness. Because mood disorders sometimes correlate with changes in reproductive function, investigators have hypothesized that these conditions may develop secondary to some abnormality in responsiveness to gonadal hormone secretion. The prevalent view at the present time is that these conditions result from a hypersensitivity to normal shifts in gonadal hormones, which affect neuroregulatory systems that play roles in affective disorders (reviewed in Ref. [15]). Differential responsiveness, and not differential hormone secretory patterns, has been thought to account for these sex-determined conditions since no differences in reproductive hormones have been consistently observed during these conditions in depressed women compared to asymptomatic women. There are several possible means by which otherwise normal steroid signals might elicit a change in behavioral state:

- altered set points for relevant neural systems;
- polymorphisms in gonadal steroid signaling pathway proteins or in systems regulated by gonadal steroids.

It follows from this reasoning that reproductive therapies may be able to correct both the endocrine anomaly and the mood disorder. Optimal antidepressant therapies are still a topic of active discussion (reviewed in Refs. [7,15]). The serotonergic antidepressants, particularly the selective serotonin reuptake inhibitors (SSRIs), appear to be the treatment of choice for severe premenstrual syndrome at this time. Modulating serontonergic function is consistent with the dominant theoretical view that the normal gonadal steroid fluctuations of the menstrual cycle trigger an abnormal serotonergic response in vulnerable women. Indications of abnormalities in markers of serotonergic transmission in women with severe premenstrual syndrome include evidence of a lowered platelet imipramine binding (a peripheral marker of serotonin function) in the luteal phase, decreased platelet serotonin content and serotonin uptake during the luteal phase, and decreased whole blood serotonin levels premenstrually. Interestingly, there are reports that women exhibit a superior response to SSRIs compared to men. The extent to which these differences reflect sex-related differences in pharmacokinetics remains to be determined.

With 22–33% of menopausal women reporting elevated levels of depression in epidemiological studies, the psychological benefit of hormone replacement may be of great significance. Estrogen treatment is widely believed to improve depressive symptoms in menopausal women, but study results are inconclusive because of large variations in study design and measures, hormonal status, and diagnosis of the subjects, the estrogen compound, dose, duration of use and failure, in some cases, to find an effect greater than

the placebo response. However, two recent well-designed studies found 17β-estradiol is an effective antidepressant treatment for depression in perimenopausal women (reviewed in Ref. [7]). Both studies clearly diagnosed depression, endocrinologically defined perimenopausal status and administered transdermal 17β-estradiol using randomized, placebo-controlled, double-blind designs and showed that estrogen may be an effective treatment for major or minor depression in perimenopausal women. One study reported remission of depression in 68% of the estradiol group compared to 20% of the placebo group after 12 weeks. The other reported a full or partial response for 80% of the estradiol group compared to 22% of the placebo treated group after 6 weeks of treatment. The strong effect of placebo treatment should be noted as it appears in many if not all studies involving pharmacological approaches to cognition and mood. Further studies are needed to confirm these positive findings and determine long-term effects of estradiol. In some cases the administration of estrogen enhances the therapeutic response to certain psychotropic agents including SSRIs.

Results from studies performed in experimental animals reveal that gonadal steroids influence several of the neuroregulatory systems thought to be involved in both the pathophysiology of affective disorders and the efficacy of antidepressant therapies. In some experimental paradigms, estradiol inhibits the serotonin reuptake transporter mRNA and influence the activity of specific serotonin receptor subtypes. In addition, several candidate neural signaling systems have been identified as potential mediators of the therapeutic actions of antidepressants. For example, antideptressants increase the expression and activity of the cyclic adenosine monophosphate (cAMP) response element binding protein and brain derived neurotrophic factor (BDNF). Genes for glial derived neurotrophic factor and its receptor have been proposed as potential targets of antidepressant related changes in cAMP responsive element binding (CREB) activity. Similarly, estradiol has been reported to influence many of the same neurotransmitter systems ovariectomy decreases and estradiol increases BDNF and increases CREB activity. Intriguingly, estradiol-induced changes in BDNF have been reported to mediate estradiol's effects on dendritic spine formation in hippocampal neurons. Thus, the therapeutic potential of gonadal steroids in depression is not only suggested by their widespread actions on neurotransmitter systems, but also by certain neuroregulatory actions shared by both estrogens and other therapies for depression.

8. Summary

Extensive literature documents that basic differences in genetic, physiological, and environmental experiences between males and females produce phenotypic differences throughout the lifespan in the incidence, risk, and outcome of neuropathological diseases. For a few of the neuropathologies, hormonal differences between men and women are a significant factor.

9. Future directions

Additional research is needed for us to fully appreciate how sex and gender influence normal behavior and the etiology of diseases that affect the nervous system. For example, it

will be important to assess whether differences in normal brain function and differences in the rates of normal aging in males and females predisposes them to differences in vulnerability to age-related pathologies. At the present time, it is not known whether differences in hormonal milieu, environmental differences or differences in social experiences influence the risk, incidence or manifestation of neuropathologies. Thus, future studies are likely to focus on the following questions. (1) To what extent do these differences determine sex-dependent differences in normal brain function and differential incidences or risk of neuropathologies? (2) Which of these sex-related differences are fixed and which remain plastic in the adult? (3) What influences change during the adult period of life? It is critical to keep in mind that these differences are not absolute and that it is not possible at the present time to look at a brain or brain image and know the sex of the person.

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Sex differences in autoimmunity

Thomas F. Fagan and Denise L. Faustman

1. Introduction

Autoimmunity occurs when the adaptive immune system generates antibodies directed to, or which cross-react with, an autoantigen, one that is present in an extract of normal tissue. Hence, there is no one autoimmune disease, but rather, a group of disparate diseases and conditions that share, mechanistically, a common etiology.

Symptoms of autoimmune diseases range in severity from mildly debilitating to life-threatening. Some, like pernicious anemia, affect specific organs or tissues, while others have profound systemic consequences, e.g. type I diabetes (see Table 1). In some cases the autoantigen is localized, as in Hashimoto's thyroiditis, but often the immune system is found to react with antigens throughout the body, as in systemic lupus erythematosus. In fact, as can be seen from Table 1, there is an autoimmune disease associated with almost every organ of the body.

In some autoimmune disorders the autoantigen has been identified. In multiple sclerosis (MS), for example, myelin basic protein, a component of the myelin sheath that surrounds nerve axons, is a known target, but other autoantigens are also thought to be involved in the etiology of this disease. In many autoimmune diseases, however, the autoantigen is unknown and the autoimmunity is difficult to prove conclusively. Hence, conservative estimates for the number of autoimmune diseases fall in the 15–20 range, whereas more liberal estimates suggest that over 80 autoimmune diseases exist. Given the latter estimate, autoimmunity becomes almost as common in the United States as cancer and heart disease.

The exact causes of autoimmunity are uncertain. What is clear is that selection mechanisms that normally ensure the immune system tolerates self-antigens have gone awry. Often this leads to the clonal expansion of immune cells that recognize a single autoantigen, but in many cases multiple self-antigens are targeted. What is important to remember about autoimmune diseases is that once the checks and balances that usually protect autoantigens have been circumvented, the immune system will begin to react to that self-antigen just as it would to any foreign one. Autoimmunity is, therefore, mediated by the same macrophages, B- and T-lymphocytes, and cytokines that normally mediate the targeting and removal of foreign antigens.

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Table 1
Female:male incidences of a subset of autoimmune diseases

Autoimmune disease	Tissue/organ affected	Female:male ratio
Hashimoto thyroiditis	Thryoid	10
Primary biliary cirrhosis	Liver	9
Chronic active hepatitis	Liver	8
Graves' hyperthyroidism	Thyroid	7
Systemic lupus erythematosus	Multiple	6
Scleroderma	Skin, systemic	3
Rheumatoid arthritis	Joints, synovium	2.5
Idiopathic thrombocytopenic purpura	Platelets	2
Multiple sclerosis	Nervous system	2
Autoimmune hemolytic anaemia	Blood cells	2
Pemphigus	Skin	1
Type I diabetes	Pancreas	1
Pernicious anemia	Blood cells	1
Ankylosing spondylitis	Spine, joints	0.3
Goodpasture nephritis/pneumonitis	Kidney/lungs	0.2

One of the mysteries of autoimmunity is that most of these diseases affect men and women disproportionately. Some, such as ankylosing spondylitis, a debilitating form of arthritis that affects the vertebrae, are more common in men. However, autoimmune disorders overwhelmingly have the greatest impact on the female population. In fact, of the over 8.5 million people in the United States that are thought to suffer from some type of autoimmune disease, the vast majority, 6.7 million, are women. Women, for example, are 10 times more likely to suffer from Hashimoto's thyroiditis, and are up to nine times more likely to develop systemic lupus erythematosus (SLE) [14]. So why are women so disproportionately affected by these diseases?

There are three main factors that are thought to increase a women's susceptibility to autoimmunity – hormones, genes and the environment. This Chapter will review the impact of these factors on development and potential treatments for autoimmune disease.

2. Hormones

While there is some clinical evidence for the importance of gonadal hormones in the etiology of autoimmune diseases, in most cases the relation between disease progression and these steroids is tenuous at best. Thus, while rheumatoid arthritis and MS typically go into remission during pregnancy, for example, there is no clear data to suggest that this is related to a shift in the balance of hormones. Indeed, in the case of rheumatoid arthritis, the remission has been proposed to be due to a change in the immune system provoked by incompatibility between mother and fetal human leukocyte antigen [19].

Estrogens and systemic lupus erythematosus. Recently, however, evidence has grown that estrogens may be a risk factor in SLE. This is hinted at by the predisposition of the sexes throughout a normal lifespan. Prior to puberty, the incidence of SLE in girls is about three times higher than in boys, but this ratio increases to about 10:1 after puberty, and

following menopause decreases again to about 8:1. Experimental animals too seem to support a role for estrogens. In some strains of inbred mice that spontaneously develop a form of SLE, estrogen receptors appear more numerous and at maturity have a higher affinity for the hormone than those from normal animals [4].

Studies have shown, however, that plasma estradiol levels in normal women and in those suffering from SLE are not statistically different. However, one interesting avenue of research suggests that it may not be estrogen but metabolites of estrogen that increase susceptibility to this disease.

These metabolites include 2-hydroxy- and 16-hydroxyestrone and their derivatives, produced by the enzymatic oxidation of estrogen at either position 2 or 16 of the steroid ring, respectively. Because the 16-hydroxy metabolites have been shown to be more "feminizing" and because the two oxidation reactions are irreversible and mutually exclusive, any perturbation that upsets the relative flux of these two metabolic steps may have a profound effect on target tissues. It is known, for example, that pregnancy can shift the balance toward the 16-hydroxy metabolites, while intense exercise, or a diet high in cruciferate vegetables, can have the opposite effect; the latter due to the intake of indole-3-carbanol, an inhibitor of the 16-hydroxylase. It is significant, therefore, that the levels of 16-hydroxy derivatives are elevated in both women and men that have SLE. It is also worth noting that some men with Klinefelter's, or XXY syndrome, meet the diagnostic criteria for SLE and that they have estrogen/androgen patterns that are typically female.

If estrogen metabolites may predispose one to SLE, then could androgen and its derivatives have the opposite effect and offer protection? In males, the incidence of SLE decreases after puberty, offering at least token support for this idea. But in addition, experimental evidence shows that in experimental animals with the disease, administration of androgens has beneficial effects including reduced mortality. In humans too, androgens affect development of the disease, as female patients with active lupus exhibit increased aromatization of testosterone to estrogen [13]. This finding has spurred the launch of clinical trials using the weak steroid dehydroepiandrosterone (DHEA) as a potential therapeutic for SLE.

Studies at the molecular level also point the finger at estrogen, at least in the case of lupus. It was recently found that human T-cells express both forms of the estrogen receptor, ERα and ERβ, and furthermore, in women with SLE, estrogen can act on these cells to upregulate the expression of calcineurin and the CD40 ligand [20]. Both of these molecules are necessary for the immune response. Calcineurin activates expression of the CD40 ligand, which in turn interacts with its cognate CD40 receptor on the surface of B-cells stimulating these cells into antibody production.

In fact, in mice that have been engineered to overproduce B-cells that recognize DNA autoantigens, estrogen facilitates the loss of immune tolerance, turning mice that would otherwise eliminate the autoreactive B-cells through clonal deletion, into animals with autoimmune disease [7]. Furthermore, in the peripheral B-cells of these animals, estrogen induces expression of the antiapoptotic factor, Bcl-2 [2], suggesting that the hormone may prevent the elimination of these cell lines by interfering with cellular pathway initiated programmed cell death (apoptosis).

While this type of evidence explains how estrogen may aggravate the immune system, perhaps turning what would otherwise be a low-grade autoimmunity into an aggressive

autoimmune disease, it does not explain why autoantigens become targeted in the first place. One possibility is that in the course of tissue remodeling, such as the cyclical growth and atrophy of the endometrium, for example, autoantigens, which are normally hidden from the immune system, are exposed. Here too, estrogen may impact the proliferation and reabsorbtion of non-immune cells by regulating Fas-mediated apoptosis; the Fas ligand (FasL) promoter contains an estrogen responsive element [16] and can be upregulated in response to the hormone in certain tissues, such as the ovaries [22]. Furthermore, estrogen may also regulate apoptosis in immune cells by modulating the Fas/FasL pathway, in addition to its effects on Bcl-2. In fact, Fas was originally identified in mice because its transcription is abolished by a mutation in the *lpr* gene, a mutation that, significantly, is associated with lymphoproliferation and autoimmunity in mice. In this regard it is worth noting that a mutation in the human ortholog of *lpr* is associated with autoimmune lymphoproliferative syndrome (ALPS), but in both the rare human and murine autoimmune disease, lymphoproliferation is a trademark that does not depict most autoimmune diseases. Additionally most human and murine forms of autoimmunity may have lymphoid cell populations with heightened apoptosis and no lymphoproliferation [3,5,11,12,15,17,23,24]. The lymphoid cells populations with heightened apoptosis have recently been associated with pathogenic characteristics and heightened ability to transfer disease [21].

3. Genes

In addition to the *lpr* gene there are several other candidate genes and loci that may predispose carriers to autoimmunity. However, as outlined below, there are several reasons why genetics is unlikely to provide complete answers to the questions why autoimmunity occurs and why it is found mostly in women.

A genetic basis for autoimmune disease is confounded by the problem of penetrance. Any genetic predisposition towards autoimmunity is not fully penetrant, as is apparent from familial studies, and more importantly, from epidemiological studies of identical twins. In the latter case, it is quite common to have one twin suffering from a debilitating autoimmune disease while the other is perfectly normal. Hence, environmental or behavioral factors, or stochastic processes perhaps beyond the genetic code must mediate any genetic predisposition to autoimmunity (see below). Indeed, it is now more appreciated, after the sequencing of the human and mouse genome with greater than 85% identity, that transcription, translation, phosphorylation, ubiquitination and many additional post-DNA influences create diversity.

Genetics also falls short in explaining why women are more susceptible than men to a given disease. For example, while there have been considerable advances made in recent years in identifying human leukocyte antigen (HLA) subtypes that may predispose carriers to specific autoimmune diseases, the simple presence of such HLA subtypes is not sufficient to explain the sexual dimorphism of most autoimmunities. HLA B27, for example, has been associated with spondyloarthropathy and uveitis, diseases for which the female:male incidence ratios are 0.3 and 1.0, respectively. More dramatically, HLA DR3 has been linked to diseases that affect males and females equally, such as myasthenia

gravis, but is also linked to Grave's disease, which has a female:male ratio of 7.0, and SLE, which depending on the data, can affect anywhere from 6 to 14 times more women than men.

Likewise, the sex chromosomes do not appear to provide the answer to the question of sexual dimorphism. Few disease markers have been found on either the X or Y chromosome, and there is no conclusive evidence that imprinting predisposes individuals to any autoimmunity.

In non-obese diabetic mice, however, the genetic defect that compromises macrophage proteasome function seems to affect female mice to a greater extent than their male littermates, and may explain the higher penetrance rates in female animals [25]. Similar proteasome misfunction may underlie the sexual dimorphism of some human autoimmune diseases but definitive data is still lacking.

4. Environment

The current expert thinking suggests that autoimmune diseases are most likely the product of genetic predisposition, stochastic immune responses, and environmental triggers. The latter could provide an adequate explanation for the greater predisposition of the sexes to particular autoimmunities, though for the most part any environmental explanation should be considered to be gender – rather than sex-based in the sense that male–female transsexuals are more likely to be exposed to "female" environmental triggers, and vice versa.

The two environmental factors most often studied are chemicals, such as toxins and drugs, and infectious agents. The link with chemicals may be somewhat easier to make. In mice, drug-induced lupus closely resembles the idiopathic disease and is more severe in female animals [26], while some years ago in Spain consumers exposed to contaminated cooking oil developed an illness closely akin to idiopathic scleroderma; in this incident most of the victims were women [1]. Some scleroderma type illnesses in men, on the other hand, are thought to result from exposure to industrial hazards, such as polyvinyl chloride, and industrial environments, such as mine shafts.

Links with infectious agents, particularly those that explain sexual dimorphisms, are harder to prove, though there are some very interesting correlations between specific infectious agents and specific diseases. Perhaps the most studied and debated correlation is between lupus and the Epstein–Barr virus. One of the many autoantigens that provokes the immune system in lupus is a nucleotide sequence found in the spliceosome. This sequence has strong similarity to an Epstein–Barr nuclear antigen (EBNA-1) that is commonly found in infected individuals. Could the immune system, which has been educated to attack the virus, mistake the spliceosome as foreign and set about to destroy it? This is the basis for the molecular mimicry theory, which has it share of proponents and detractors [9]. One argument against this explanation, particularly for lupus, is that the adult population has been almost universally exposed to this virus yet lupus is a relatively rare disease. Furthermore, Epstein–Barr is not known to discriminate on the grounds of sex. However, if mimicry does play a role in autoimmunity, it is most likely against a background of genetic susceptibility.

Other infectious agents have also been implicated in autoimmunity. *Borrelia burgdorferei* is thought to initiate a chronic form of Lyme disease, though the bacterium is not required for the prolongation of the disease, suggesting that antibodies directed against the organism may recognize self-antigens. There is no sex-bias in the incidence of chronic Lyme disease, but it closely resembles rheumatoid arthritis, which predominantly affects women.

Likewise, fogo selvagem, a type of Brazilian pemphigus foliaceus, an autoimmune disease of the skin, is transmitted by black flies, presumably by an infectious agent delivered when the fly bites. This insect borne disease affects women and men equally while the spontaneous form predominantly affects women.

One special case of environmental exposure that only affects females is the exposure to fetal antigens during pregnancy. Some of these antigens can be found in the mother's circulatory system 20 or 30 years post partum. A growing body of research has implicated these antigens in triggering immune responses that lead to autoimmunity. Again, this is not the obligatory or necessary trigger since many nulliparous women get autoimmunity and many multiparous women remain free of autoimmunity.

5. Immune education

Through-out life and especially during infancy, the immune system, composed of splenocytes, bone marrow cells and the thymus, produces vast numbers of new lymphocytes. These new lymphocytes have diverse new T-cell receptor rearrangements (T-cells) and diverse immunoglobulin chain rearrangements (B-cells). This vast diversity is then followed by extreme degrees of positive and negative selection so the immune system can both properly recognize and kill tumor cells and foreign invaders such as bacteria and viruses but not be too over reactive and kill self, i.e. autoimmunity. The positive and negative selection process is severe and it is estimated that over 90–99% of the lymphoid cells created will be killed prior to release.

Although the genetic basis of most autoimmune diseases is unknown some rare animal models of autoimmunity and rare forms of human autoimmunity have disclosed specific genetic errors or protein processing errors.

As Table 2 shows, some of the first genetic errors of autoimmunity were uncovered in those with simple inheritance. A single disease causing mutation was uncovered in the Fas and Fas ligand death receptors in both Lpr and Gld mice as well as a rare human disease called ALPS. These mutations prevent the normal death receptors on developing immune cells from being properly utilized and proper death of autoreactive cells does not follow. Not too surprising, these rare autoimmune diseases are symptomatically associated with tumor-like lymphoproliferative diseases in the periphery because of the excessive numbers of circulating cells that escaped negative selection.

These important discoveries were then followed by a hunt for similar mutant death receptors with "failure" to die lymphoid cells in additional multi-genetic murine and human spontaneous autoimmune diseases (Fig. 1). Surprisingly although some rare knockout animals appeared to have autoimmune tendencies with death receptor mutations, the majority of murine and human spontaneous autoimmune models did not have simple

Table 2
Death receptors and autoimmunity

	Treatment
Murine and human models with resistance to apoptosis	
Murine model	
(A) Lpr, gld mice	Genetic mutations in Fas and Fas ligand death receptors; apoptosis resistance
(B) Bcl2 −/− mice	Only some mice develop autoimmunity; others are resistant
(C) Diabetic model (NOD)	Reports apoptosis resistance of lymphocytes in NOD
Human model	
(A) Diabetes	Defective CD95 mediated apoptosis
(B) Autoimmune lymphoproliferative disease	Rare disease with Fas mutations
Murine and human models with heightened apoptosis sensitivity	
Murine model	
(A) Diabetic model (NOD)	Interruption in NF-κB signaling in NOD T-cells; T-cell apoptosis with TNF-α
	Abrogation or diminition of autoimmunity by TNF-α
	Induction of TNF-α in vitro induces T-cell apoptosis
	Induction of TNF-α in vivo eliminates a subpopulation of pathogenic T-cells
	NOD APCs have reciprocal elevations in NF-κB
(B) Lupus Model (NZB X NZW)F_1	NZB thymocytes have defective NF-κB signaling
	NZB mice have altered NF-κB; increased apoptosis Rx with fatty acids
	Treatment of lupus prone mice with TNF
Human model	
(A) Diabetes	Human diabetic blood has altered TNF-α sensitivity and apoptosis
(B) Lupus	Altered NF-κB signaling in lupus T-cells
(C) Crohn's	Mutation in Crohn's disease (NOD2) in NF-κB signaling pathway
	Mutation in Crohn's disease (NOD2) in NF-κB signaling pathway
	The Crohn's NOD gene mutation disrupts NF-κB processing

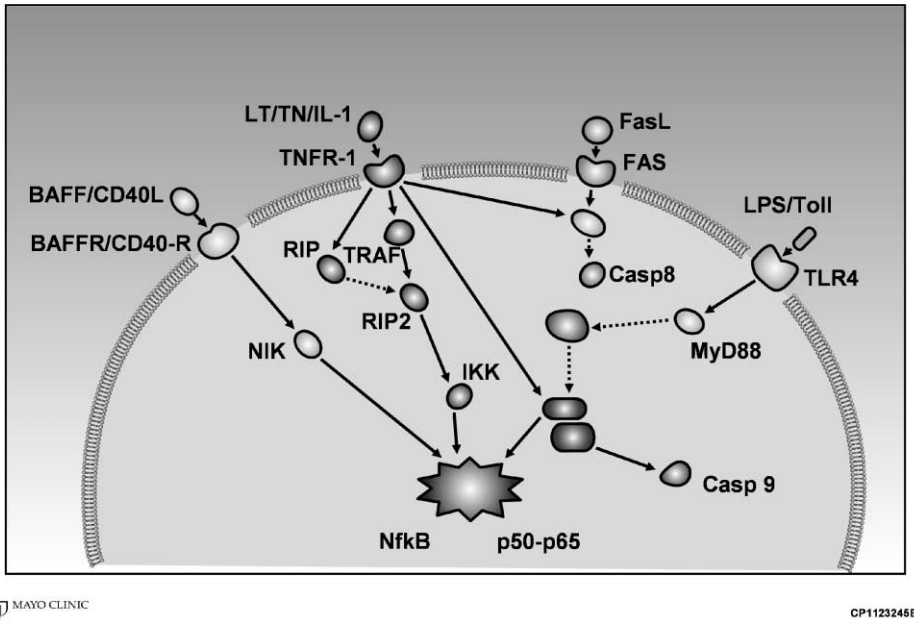

Fig. 1. Death receptors and relationship to autoimmunity. Multiple death receptor pathways from the lymphocyte surface now appear to be linked to autoimmune dysfunction. Although the first pathways identified were FasL and Fas mutations in murine models, these autoreactive diseases were associated with failure to die defects of all lymphoid cells and thus massive lymphoproliferative disorders. More recently, the NFKB pathway defects have become a commonly identified defect, first in the NOD mouse (Type I diabetes) and now in ZNBXNZW mice (lupus). Also, Crohn's patients and human lupus patients have subsets of lymphoid cells with interruptions of this pathway.

point mutations in death pathway receptors or post-receptor signaling pathways causing resistance to die. As Table 2 shows, the more common forms of human and murine autoimmunity have revealed just the opposite. Scientists are uncovering in both mice and humans with spontaneous autoimmunity, that "bad" T- and B-cells commonly have heightened apoptosis sensitivity (Table 2). What is clear is that normal lymphoid cells have a normal abundance of death receptors on their surface, i.e. CD40, TNF, Fas and Toll/LPS. Certainly, a mutant pathway that is now being found in murine lupus and diabetes as well as human lupus and Crohn's, is disruption of the TNF/NFkB pathway culminating is heightened apoptosis sensitivity to exposure to TNF.

Treating autoimmunity – controlling the immune system. As the etiology of most autoimmune diseases is poorly understood, historically the physician has at his or her disposal a limited number of options for therapy. There are two main approaches to treatment, attack the root of the problem, namely, the immune system itself, or manage the symptoms. Most treatments by far fall into the latter category, as in the management of blood sugar levels in diabetes or alleviation of the pain and inflammation associated with rheumatoid arthritis. More recently, however, progress has been made toward understanding the molecular basis for autoimmunity, and this has led to some promising experimental therapies and clinical trials.

Autoreactive T-helper cells, for example, have been implicated in the etiology of MS, psoriasis, rheumatoid arthritis, Crohn's disease and a host of other autoimmunity, but more important was the recognition that the balance between Th1 and Th2-cell activity may be the key. Th1-cells secrete a variety of mostly proinflammatory cytokines including interleukin-2, and interferon-γ, while Th2-cells secrete non-inflammatory cytokines, including IL-4 and IL-10, which moderate Th1-cell activity. Upsetting the delicate balance between the two cell populations may lead to the proliferation of auto-reactive T-cells and the propagation of an autoimmune condition. Increased production and activation of proinflammatory Th1-cells that recognize myelin basic protein antigens, for example, exacerbates MS, while psoriatic skin lesions can be ameliorated using non-inflammatory cytokines such as IL-10, a response grounded in the fact that these lesions are innervated by reactive Th1-cells.

Studies in small experimental animals show that skewing the balance of the T-helper cells in favor of a Th2 phenotype can alleviate autoimmune diseases that affect the internal organs. This type of strategy runs the risk of suppressing the whole immune system; nevertheless, some immunomodulatory agents have been used on MS patients with moderate success. Glatiramer acetate, an artificial analog of myelin protein, which is thought to activate myelin-reactive Th2-cells, reduces the rate of relapse by about 30% and seems to reduce the number of lesions occurring on the brain and spinal cord when used to treat those with the relapsing–remitting form of the disease [10]. Clinical trials are still underway for those with primary progressive MS. Glatiramer is better tolerated than interferon-β (IFN-β), currently approved for use in the United States, and which seems to delay progression of relapsing-remitting MS. IFN-β inhibits production of the proinflammatory interferon-α.

More recently, interleukin-4 (IL-4), which is secreted by Th2-cells, has been used in a small clinical trial for treatment of psoriasis. Recombinant human IL-4 reduced the number of skin lesions by almost 70% in 3/4 of the trial participants, was well tolerated, and shifted the cytokine profile of the lesions toward the Th2 phenotype [6]. This treatment, therefore, looks very promising, not only for psoriasis, but also for other Th1-associated diseases such as rheumatoid arthritis and Crohn's disease.

Other clinical trials have focused on the hormone-responsiveness of the immune system. Trials using DHEA as a potential treatment for SLE are currently underway as mentioned above. Indole-3-carbanol, an inhibitor of estradiol 16-hydroxylase, has also been used in a very brief 1 week metabolic study of lupus patients followed by a 3-month follow up. The results were encouraging with the 2-hydroxy-:16-hydroxy-estrone ratio almost doubling and the SLE disease activity index dropping. This trial was too short, however, to make any firm projections and there was no striking amelioration of disease activity during the trial.

Other potentially promising though as yet experimental approaches to tackling autoimmunity include re-teaching the immune system the difference between self and non-self. This strategy has been used to effectively cure rodents that are genetically predisposed to developing diabetes mellitus [21].

This re-education of the immune system is a two-step process involving the elimination of mature as well as naïve immune cells that recognize β-cells of the islets of Langerhans as self. Non-obese diabetic (NOD) mice have a mutation in the ability to produce the

LMP2 subunit of the proteasome that both interferes with normal antigen presentation and makes immune cells susceptible to the apoptotic effects of tumor necrosis factor-α (TNF-α). Thus, the very mutation that causes the diabetes also provides the means for achieving the first step in the process of purging the misbehaving immune cells from the circulation. The second step, establishing new immune cell lines that tolerate β-cells, is achieved by introducing into TNF-α-treated mice, the MHC class I molecule with self-peptide that is necessary for antigen presentation. This second step selectively kills the naïve cells that are the cell reservoir for continued direct beta cell death by autoreactive memory T cells. Although the naïve cells killed by MHC class I molecules with self-peptide are probably lineage related to the memory cells selectively killed with TNF-alpha, their different states of activation dictate how therapeutic and purposeful re-selection can be induced in the periphery. While this strategy has proven to work well for NOD mice – 75% of animals treated maintain normoglycemia after treatment – its applicability to human diabetes is still under investigation. If human diabetic patients do respond to TNF-α, for example, then many other autoimmune diseases, including SLE, and Crohn's disease, which are purported to carry similar genetic defects may also benefit from this type of therapy [8,18,23].

6. Future directions

For many years the autoimmune patient population was faced with a dilemma. For nearly 20 years no new drug for autoimmunity was approved by the FDA. This situation has changed dramatically over the past 10 years. The size of the autoimmune markets has driven technology and drug makers to think about this situation in detail. Most new drug approvals work on the concept that the immune system is activated and the immune system that is activated produces many active cytokines that cause damage to the host. Therefore, most new drugs on the market fall into two major classes, i.e. anti-cytokine therapy and immunosuppressive therapy. Indeed these drugs bring therapeutic benefits that out weight risks but none work on disease reversal. Obviously the future of autoimmune therapy is to get to the heart of the etiology of this disease by first identifying, even in advanced disease, the disease causing cells. Secondly, after cell specific identification, the next goal will be to develop drugs that only target and modify the disease causing cell populations. Such targeted drugs will obviously have a high impact for people worldwide with these devastating diseases.

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Influence of hormones and sex on platelet functions

Muthuvel Jayachandran and Virginia M. Miller

1. Introduction

Circulating platelets were first described anatomically as discs participating in hemostasis and thrombosis by Bizzozero in the 1880s. Although he also identified megakaryocytes in bone marrow, it was not until almost 20 years later that Wright, using his new polychrome staining solution, discovered similarities in shape and color of the red-to-violet granules in platelets and bone marrow megakaryocytes. Since Bizzozero's description of platelets over a century ago, much has been learned about how platelets contribute to hemostasis and thrombosis. However, much remains to be learned about mechanisms that regulate platelet functions in men and women. This chapter will provide a basic review of platelet formation, platelet–vessel wall interactions contributing to hemostasis or thrombosis, and how these processes are influenced by sex hormones. In this chapter, hemostasis defines a well-regulated process of rapid formation of a platelet plug, activation of coagulation cascade, deposition, stabilization and containment of fibrin at sites of vascular injury. Thrombosis defines the inappropriate activation of physiological hemostatic processes within non-injured arteries or veins, which blocks the blood flow to vital organs.

2. Platelet formation

Megakaryocytes are developed from hematopoietic stem cells and reside primarily in the bone marrow. Although it is accepted that platelets are produced from megakaryocytes, mechanisms by which platelets are formed and released into the circulation from megakaryocytes remain controversial. There are several models of platelet formation including platelet budding, cytoplasmic fragmentation via the demarcation of the membrane system and proplatelet formation (Fig. 1). Newly formed platelets contain a small amount of endoplasmic reticulum and mRNA and thus retain the ability to synthesize small amounts of protein. However, lacking nuclei, platelets are unable to synthesize new mRNA, and the mRNA in the so-called reticulated (young) platelets

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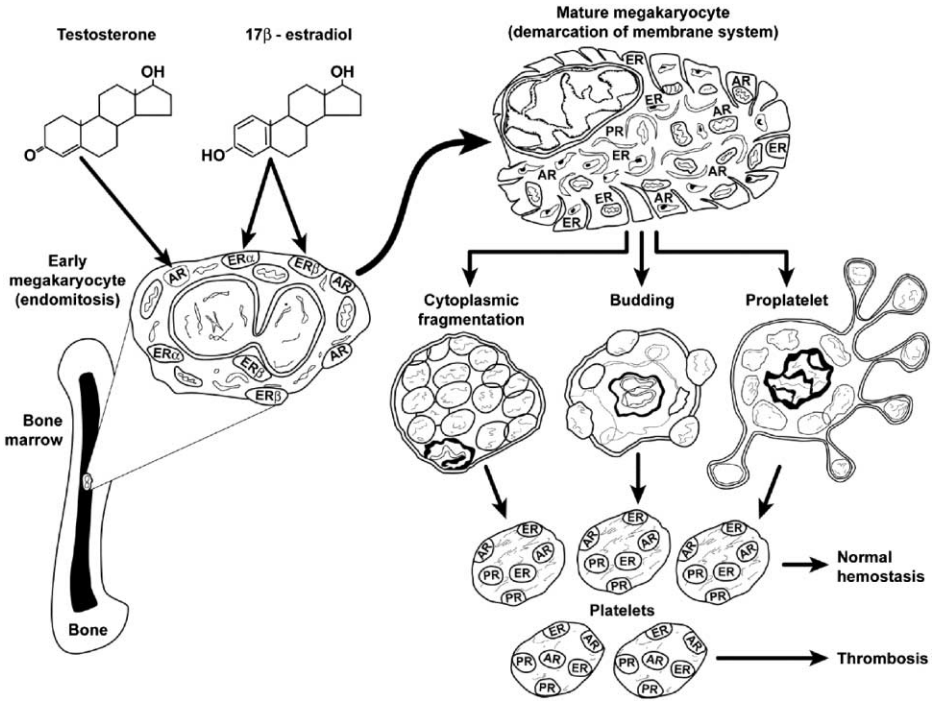

Fig. 1. Schematic of proposed mechanisms by which platelets are formed from bone marrow megakaryocytes. Sex steroid hormones by binding to hormone receptors can regulate gene transcription and translation in megakaryocytes. However, hormones binding to their receptors could initiate changes in platelet functions through non-genomic mechanisms once platelets are in the circulation. Abbreviations: AR, androgen receptor; ER, estrogen receptor; ERα, estrogen receptor alpha; ERβ, estrogen receptor beta; PR, progesterone receptor.

decays in about 24 h. Therefore, synthesis of protein in the platelet once in the circulation is limited.

There are between 150,000 and 400,000 platelets/μL of blood in humans. About 10–15% of circulating platelets are reticulated. Since the life of a platelet in the circulation is about 10–12 days, the circulating pool of platelets at a given moment in time reflects the genomic influence of hormones on the megakaryocytes when each platelet was produced. This concept is important when considering effects of hormones on platelet function because sex steroid hormones (testosterone, estrogen, and progesterone) affect physiological functions through binding to their specific receptors (please see Chapter 3). Both megakaryocytes and circulating platelets contain estrogen receptors (ERα and ERβ), androgen receptors (AR) and progesterone receptors (PR) [13,16]. These receptors act as transcription factors and mediate genomic (receptor mediated changes in gene expression, see Chapter 3) and/or rapid (seconds to minutes) non-genomic (see Chapter 4) effects in targeted cells and/or tissues. Genomic effects of sex steroids in platelets would occur only in megakaryocytes as these precursors of platelets contain nuclei whereas circulating platelets do not.

Evidence that sex steroids affect platelet production and content. In experimental animals, platelet turnover (indicated by the percentage of reticulated platelets) is increased by testosterone treatment of castrated male mice and ovariectomy of adult female pigs [27]. In preliminary experiments from our group, the number of reticulated platelets increased with sexual development in male pigs. Although these data are consistent with the idea that testosterone and/or the absence of female hormones stimulate platelet production, actual mechanisms by which testosterone and estrogen affect production of platelets are unknown.

In addition to changes in production of platelets, sex steroids affect the number of steroid receptors on platelets as well as platelet content of biologically active molecules. For example, concentrations of estrogen receptors as well as platelet-derived growth factor, nitric oxide synthase and matrix metalloproteinases (MMPs) increased in platelets following ovariectomy of adult female pigs [5,12]. Proteins and cytokines released from platelets stimulate migration and proliferation of smooth muscle and production of extracellular matrix of the blood vessel wall. Therefore, platelets produced under conditions of estrogen depletion stimulate a greater "response to injury" than platelets produced under estrogen-replete conditions [5]. These results have important implications for platelet-mediated vascular repair or development of occlusive vascular disease as would occur in post-menopausal women, which will be discussed in detail in following sections.

Sex steroid hormones, in particular estrogen, also regulate steroid receptor-associated proteins or molecular chaperone proteins including heat shock proteins. In an inactive form (absence of hormone binding), androgen and estrogen receptors are associated with a number of molecular chaperones including heat shock proteins (hsp 27, 32, 70, and 90) and large immunophilins. These receptor-associated proteins keep receptors in a conformation that increase affinity for the hormone and decrease affinity for DNA binding as well as transport of hormone–receptor complex from the cytosol of the nucleus. The hormone-receptor complex interacts with a wide variety of co-regulator proteins that either enhance (co-activators) or reduce (co-suppressors) activation of targeted genes. Therefore, hormonal regulation of chaperone binding proteins indirectly influences protein synthesis in megakaryocytes through regulation of binding of sex steroids to transcriptional sights. Gene transcription also is influenced by phosphorylation mediated signal transduction cascades, presumably by changing the interaction of steroid receptors with other cellular proteins necessary for transcription. Although sex steroid-mediated transcription would occur in bone marrow megakaryocytes containing nuclei but not in platelets, non-genomic effects of hormone receptor phosphorylation or signal transduction could occur in circulating platelets.

Polymorphisms of AR (CAG repeat polymorphism and GGC polymorphism), estrogen receptor α (*Pva* II, *Xba* I and B-variant polymorphism) and estrogen receptor β (ERβ2 and ERβcx) are associated with several diseases including breast cancer, prostate cancer, spontaneous abortion and changes in bone mineral density. However, little is know regarding how these polymorphisms affect platelet functions. In addition, much remains to be learned about how glycosylation and phosphorylation of these receptors translate into differences in platelet activity [18,21].

Since platelets from males and females contain androgen and estrogen receptors, understanding how platelet functions are regulated by sex steroids will be important to

understand development of occlusive cardiovascular disease in men and women. However, mechanisms of how androgen and estrogen receptors mediate effects of androgens, estrogens, and estrogen receptor modulators (tamoxifen and raloxifene) on nucleated bone marrow megakaryocytes and anucleated platelets are not defined. The following sections review experiments which define platelet functions from animals of varying hormonal status.

3. Platelet aggregation

Platelet aggregation is a process of cell-to-cell adhesion initiated by activation of specific membrane receptors for various types of agonists (e.g. adenosine diphosphate, (ADP) 5-hydroxytryptamine or serotonin, collagen, epinephrine, norepinephrine, thromboxane A_2 and thrombin). Activation of specific membrane receptors for these agonists initiates a chain of events such that the shape of platelets changes from discoid to spherical and platelet α- and dense granules within the platelet are activated to secrete their contents. Secretion of, e.g. P-selectin (cell adhesion protein) and glycoprotein IIb/IIIa (GPIIb/IIIa) receptors from α-granules and ADP and 5-hyrdoxytryptamine (5-HT) from dense granules may bind to receptors on adjacent platelets thereby recruiting additional platelets that form clumps (Fig. 2). Other substances released from platelets, such as prostacyclin and nitric oxide, may reduce aggregation.

Interaction of agonists with platelet membrane receptors initiates activation of several intracellular messenger molecules, including Ca^{2+} ions and the family of guanosine nucleotide regulatory (G) proteins. These proteins regulate hydrolysis of phosphoinositids by phospholipase C (PLC; human platelets contain seven isoform (PLCγ1,2, PLCβ1,2,3,4, and PLCδ1)) and A_2 (PLA_2) into diacylglycerol (DG) and inositol 1,4,5-triphosphate (IP3), thromboxane A_2 and cyclic adenosine monophosphate (cAMP; Fig. 2).

PLC and PLA_2 synthesized IP3, involved as messengers to mobilize intracellular $Ca^{2+}$and DG, activate protein kinase C (PKC) and myosin light chain kinase (MLCK). This mediates phosphorylation of specific proteins (e.g. 47 kDa pleckstrin and myosin light chain, respectively), platelet secretion and activation of fibrinogen receptor GPIIb/IIIa. Mitogen activated protein kinases (MAP kinases) and phosphorylation of proteins by non-receptor mediated tyrosine kinases also affect platelet–platelet interactions. Sex steroid hormones could modulate these complex cascades through transcriptional and translational regulation of enzymes in the megakaryocytes. Therefore, it might be expected for differences to be apparent in the aggregation of platelets derived from males and females.

Spontaneous aggregation of platelets (i.e. unstimulated platelets in the absence of an agonist) is not different in platelets derived from males and females. However, with stimulation, the size of platelet aggregates vary between males and females. Using two techniques to measure size of platelet aggregates (light transmission which cannot detect small aggregates (i.e. less than 100 platelets) and light scattering which can detect aggregates as small as two or three platelets), platelets from females had greater numbers of medium and fewer numbers of small aggregates than those from males when stimulated with ADP [9].

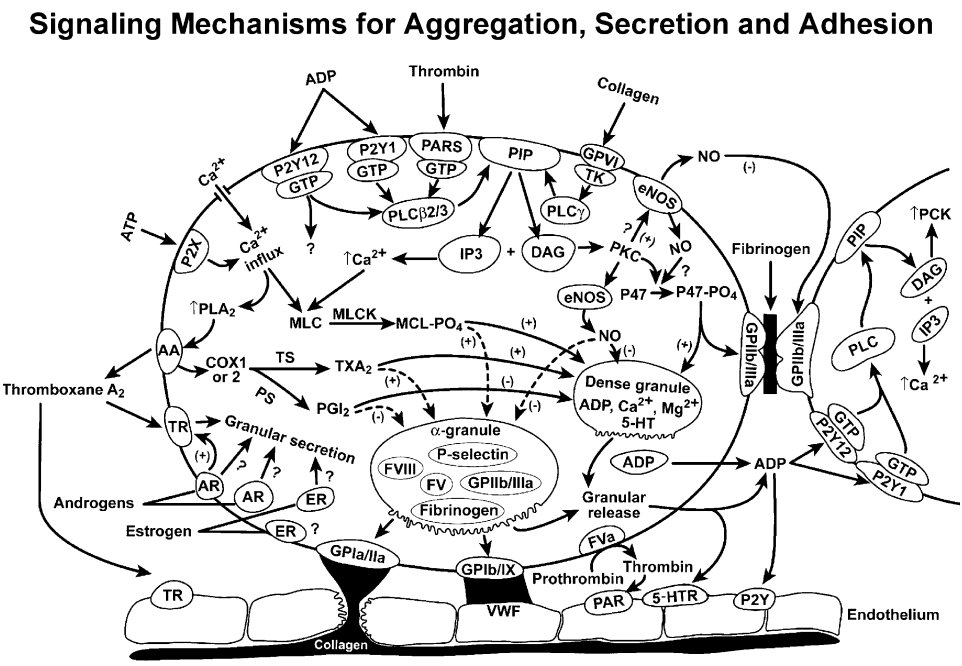

Fig. 2. Summary schematic of pathway activation resulting in platelet aggregation, secretion and adhesion. Agonists binding to surface receptors activate intracellular second messenger systems, which stimulate release of contents of α- and dense granules. These substances, like ADP and 5-hydroxytryptamine, stimulate other platelets as well as activate receptors on the vascular wall. Sex hormones binding to their receptors could influence these pathways genomically in the megakaryocyte and non-genomically by yet undefined mechanisms. Abbreviations: AA, arachidonic acid; ADP, adenosine diphosphate; AR, androgen receptors; ATP, adenosine triphosphate; COX-1 and -2, cyclooxygenase-1 and -2; DAG, diacylglycerol; IP3, inositol triphosphate; eNOS, endothelial nitric oxide synthase; FV, factor V, FVIII, factor VIII; fibrinogen receptor (GPIIb/IIIa); GPIa, glycoprotein Ia; GPIIb, glycoprotein IIb; GPIIIa, glycoprotein IIIa; GPVI, glycoprotein VI; GTP, guanosine triphosphate binding protein; 5-HTR, 5-hydroxytryptamine receptor; 5-HT, 5-hydroxytryptamine; MLC, myosin light chain; MLCK, myosin light chain kinase; NO, nitric oxide; P47, 47 kDa pleckstrin; PARs, protease activated receptors, PIP, phosphatidyl inositol bisphosphate; PGI_2, prostaglandin I_2; PKC, protein kinase C; PLA_2, phospholipase A_2; PLC, phospholipase C; PS, prostacyclin synthase; P2Y, purinergic receptor 2Y; TK, tyrosine kinase; TR, thromboxane receptor; TS, thromboxane synthase; TXA_2, thromboxane A_2; vWF, von Willebrand factor; (+) activation; (−) inhibition; ? indicates unknown interaction.

Aggregation in response to ADP was greater in platelets from male compared with female rats and castration reduced platelet aggregation in males but increased aggregation in females. Platelet aggregation induced by both ADP and collagen also was significantly greater four weeks after ovariectomy in pigs. Collectively, these data indicate that testosterone or loss of ovarian hormones increases mechanisms associated with platelet aggregation. These studies are consistent with recent observations of platelet aggregation in sexually immature (juvenile) and sexually mature male and female pigs. Aggregation in response to ADP decreased in female platelets and increased in male platelets concomitant

with increases in sex specific hormones (Fig. 3). Thus, there appears to be an influence of sex steroid hormones and/or hormones of the pituitary–gonadal axis on platelet functions during sexual development. There are few studies that directly examine effects of treatment of individuals with sex hormones on platelet aggregation. However, both high dose oral testosterone and estrogen increases thrombosis in both men and women [23,28]. This increase in thrombosis involves changes in production of proteins of the coagulation cascade in the liver in addition to platelet activation.

One mechanism by which testosterone affects platelet aggregation is through increases in receptors for thromoxane A_2. Thromboxane A_2, a metabolite of arachidonic acid, acts through membrane surface receptors to aggregate platelets. Testosterone treatment of humans and experimental animals (rat, guinea pig, mouse), up-regulates platelet thromboxane A_2 receptors and enhances aggregation in response to the thromboxane mimetic U46619 [1,29]. Testosterone-treatment also enhances aggregation of platelets from female rats suggesting that this action of testosterone is independent of the genetic sex. Alternatively, a metabolite of testosterone, dehydroepiandrosterone (DHEA) prevents human platelet aggregation in vivo. The mechanism by which this effect is mediated is not known.

Ovarian hormones also affect platelet aggregation. For example, fibrinogen binding to the platelet surface is greater during luteal compared to the follicular phase of the menstrual cycle suggesting that progesterone and estrogen may both affect platelet aggregation. Treatment of post-menopausal women with 17β-estradiol and medroxyprogesterone reduces in vitro platelet aggregation compared to platelets from women not receiving hormone treatment [3].

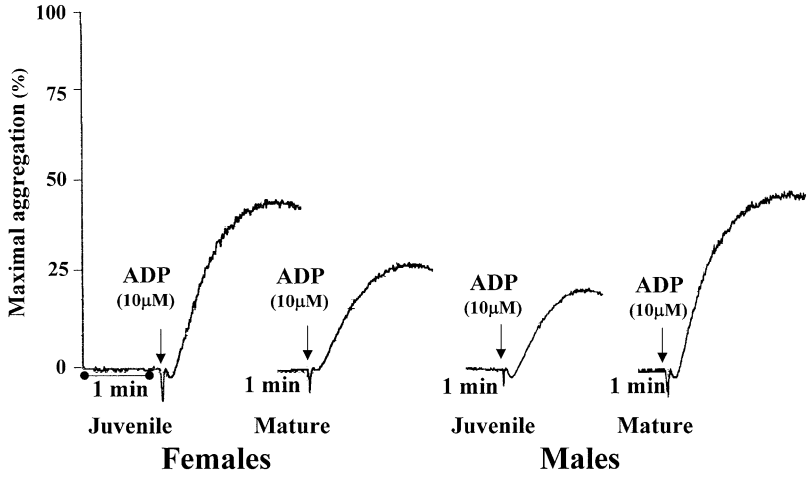

Fig. 3. Changes in platelet aggregation with sexual maturity in pigs. Platelets (250,000 platelets/mm^3) from immature (3-month-old) and sexually mature (6-month-old) male and female pigs were stimulated with 10 μM adenosine diphosphate (ADP) in platelet rich plasma. Aggregation decreased in females and increased in males following development of gonads and secretion of sex specific hormones.

One of the difficulties in assessing effects of hormones on platelet functions is the differentiation of acute, non-genomic actions compared to those mediated through genetic transcription in megakaryocytes. While changes in platelet aggregation with hormone treatment of whole animals or people reflect genomic effects on platelet content, acute exposure to hormones of isolated platelets in vitro are mediated by non-genomic actions as circulating platelets do not contain nuclei. For example, in vitro treatment of platelets with testosterone and estradiol for 30 min reduced ADP-induced aggregation in platelets from both male and female human volunteers. Exogenous estrogen modulates isolated platelet functions through changes in platelet intracellular calcium and release of nitric oxide. Nitric oxide either from within the platelets or from an exogenous source, like the endothelium, increases cyclic guanosine monophosphate (cGMP) in platelets [8]. This increase in cGMP is sensitive enough that it was used as a bioassay for nitric oxide production from endothelial cells early in the study of endothelium-derived relaxing factor (EDRF) before EDRF was known to be nitric oxide [11]. How increases in cGMP inhibit platelet aggregation remains to be defined and it is not known whether activation of ARs on platelets are associated with regulation of nitric oxide.

Expression of estrogen receptors (ERα and ERβ) were similar in platelets from human males and females over the age range of 24–65 years [14]. Also, expression of estrogen receptors (ERα and ERβ), PR-BB, and AR did not differ between male and female sexually immature or mature pigs (unpublished observation from our group). These observations suggest that platelet hormone receptors do not vary by genetic sex. However, expression of estrogen receptors increases in platelets of adult female pigs following ovariectomy. Much remains to be learned about how sex hormone receptors are regulated by their ligands in platelets.

4. Platelet secretion

As mentioned above, ligand–receptor binding initiates aggregation of platelets and release of contents from platelet granules in a process called secretion. However, under experimental conditions using solutions of diluted platelets, granule secretion can be induced under conditions of diminished platelet–platelet contact (aggregation) [26]. There are three major types of granules present in platelets: alpha granules, dense granules, and lysosomes. Alpha granules are the largest and most abundant granules (~ 80 granules/platelet) in human platelets and contain growth factors, coagulation proteins, adhesion proteins, cytokines, integrins, angiogenic factors and cell activating factors. Dense granules are about 10 times less abundant than alpha granules but have a higher concentration of small cellular activation molecules such as ADP and 5-HT. These molecules, once released, induce a secondary wave of platelet activation and aggregation by binding to their respective receptors on membranes of adjacent platelets (Fig. 2). Only a few lysomes are present in platelets.

Synthesis of secretory proteins in megakaryocytes would be under genomic influence of hormones as in other cell types. Content of platelet derived growth factors (PDGF-AA, PDGF-AB, and PDGF-BB) and transforming growth factors ($TGF\beta_1$ and $TGF\beta_2$) were similar in platelets of male and female blood donors from age 24–65 years. However,

ovariectomy of adult female pigs increased expression $PDGF_{BB}$ in platelet lysate. Since $PDGF_{BB}$ increases proliferation of vascular smooth muscle and reorganization of intracellular matrix, it might be predicted that changes in secretion of these platelet products at sites of vascular injury or endothelial dysfunction would result in an increased response to injury or exacerbate the repair process in hormone-deplete females. Indeed, proliferation of smooth muscle cells in culture was greater in response to platelet lysate from ovariectomized compared to gonadally intact pigs [5]. Similar changes in contents released from platelets in women after menopause could contribute in part to increased occlusive arterial vascular disease in this population of women. Although treatment of ovariectomized pigs with either 17β-estradiol, conjugated equine estrogen or raloxifene decreased content and secretion of some platelet-derived components (unpublished observations from our group), effects of lysates from hormone-treated animals on proliferation of smooth muscle remains to be determined.

P-selectin is a granular membrane protein and a cellular adhesion molecule that mediates the interaction of activated endothelial cells or platelets with leukocytes. It is released from α-granules of activated platelets and from Weibel–Palade bodies in endothelial cells. Expression of P-selectin is necessary for rolling and tethering of platelets and adhesion and rolling of leukocytes on the vascular endothelium. Cell membrane bound P-selectin interacts with P-selectin glycoprotein ligand-1 or glycoprotein Ib (GPIb) or sialyl Lewis X carbohydrate-expressed leukocytes, platelets, macrophages, and endothelial cells. Platelet content of P-selectin does not vary by sex in human platelets or with ovariectomy in sexually mature pigs [12,13]. Polymorphisms in the gene for P-selectin (C-2123G, A-1969G, and Thr715 Pro) are associated with coronary artery disease. A soluble form of P-selectin which circulates in the blood originates from an alternatively spliced form of P-selectin from endothelial cells and platelets. Soluble serum P-selectin is greater in patients younger than 55 years with coronary artery disease than those without disease but decreases in patients older than 65 years compared to persons without disease [4]. Circulating plasma P-selectin is lower in premenopausal women compared to men and estradiol (10 mg E_2 valerate) injection brings down plasma P-selectin levels in men [15]. Therefore, while P-selectin may represent a potential marker for individuals at risk for coronary artery disease, there are no studies that have determined differential expression of membrane P-selectin on platelets and endothelial cells by sex or hormonal status.

Some substances, like 5-HT, are taken up from the plasma by platelets or other cells by vesicle processes. Regulation of these uptake processes by hormones has not been studied. However, it has been proposed that uptake and subsequent release of 5-HT by platelets could contribute to vasospasm especially in areas of damaged or dysfunctional vascular endothelium (see below [2,6,25]).

Platelets also are important in generation of thrombin necessary for the conversion of fibrinogen to fibrin. In a cell-based model of coagulation, coagulation occurs in three phases: initiation, priming and propagation (for details see Ref. [20]). These steps result in the generation of thrombin from prothrombin on the platelet surface when Factor Xa binds to Factor Va. Little is known regarding how sex steroids regulate these various steps in thrombin formation. However, platelet thrombin generation differs from individual to individual but platelets from the same individual show reproducible thrombin generation.

Platelet aggregation, granular secretions (dense granules and α-granules) and signaling mechanisms are important determinants of both hemostasis and thrombosis. Alteration in either platelet aggregation or secretion results in a variety of disorders and syndromes in humans [22]. These syndromes include: Gray platelet syndrome (storage defects in granules e.g. α-granules), Glanzmann thrombasthenia (defective aggregation with all agonists due to the deficiency of GPIIb/IIIa), giant platelet syndrome (deficiency of platelet glycoproteins GPIb, V and IX), Scott syndrome (defective platelet procoagulant activity) and Bernard–Soulier syndrome (defective platelet adhesion to subendothelium due to a defect in GPIb), defective binding to vascular collagen (deficiency of GPIIa and GPIa), platelet type von Willebrand disease (spontaneous binding of vWF to GPIbα) and receptor defects for soluble agonists such as ADP, TXA_2, 5-HT, and epinephrine (Fig. 4). It is not known whether or not sex steroids affect these platelet disorders and there does not seem to be a propensity of a disorder by sex.

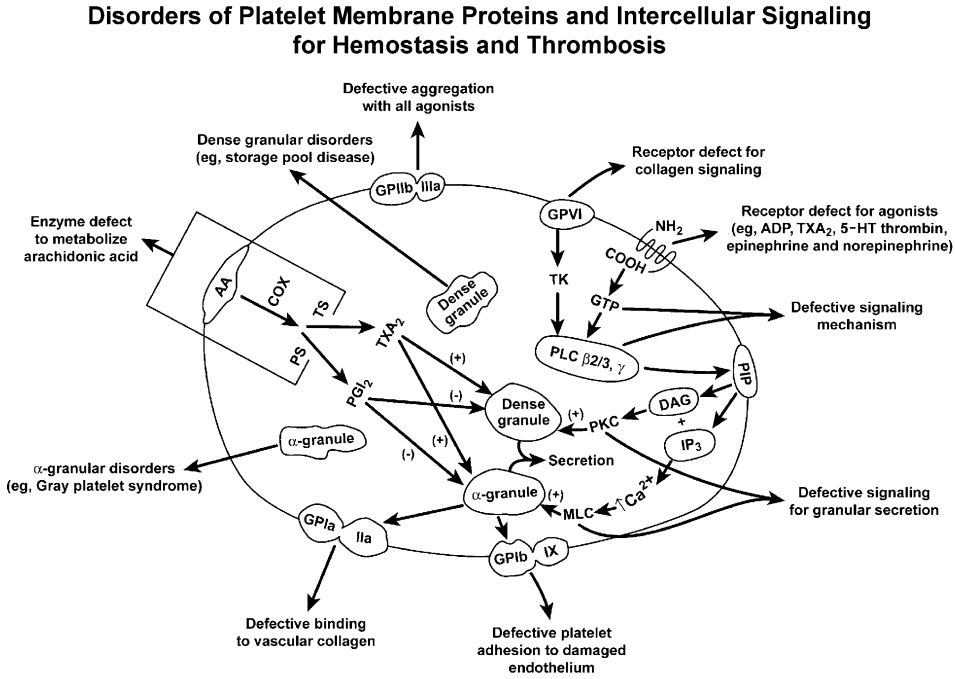

Fig. 4. Schematic identifying disorders of platelet membrane proteins and intracellular signaling pathways associated with hemostasis and thrombosis. Abbreviations: AA, arachidonic acid; COX-1 and -2, cyclooxygenase-1 and -2; DAG, diacylglycerol; IP3, inositol triphosphate; Fibrinogen receptor (GPIIb/IIIa); GP, glycoprotein; MLC, myosin light chain; PIP, phosphatidyl inositol bisphosphate; PGI_2, prostaglandin I_2; PKC, protein kinase C; PLC, phospholipase C; PS, prostaglandin synthase; TS, thromboxane synthase; TXA_2, thromboxane A_2; (+) activation; (−) inhibition (for details, see Ref. [22]).

5. Platelet–Vessel wall interaction: adhesion and wound healing

Interactions between platelets and the vessel wall are critical for hemostasis. Following mechanical or biochemical damage (oxidative stress, lipid peroxidation) to the endothelium, circulating platelets adhere to the damaged endothelium or exposed subendothelium through von Willebrand factor (vWF) and specific platelet membrane GPIb (Fig. 2). Adhesion follows the binding of plasma fibrinogen on platelet surface GPIIb/IIIa and adhered platelets release or secrete their granular (α- and dense granules) contents which results in recruitment of other circulating platelets to injured area.

Under normal physiological conditions, release of nitric oxide and prostacyclin from arterial and venous endothelial cells inhibit platelet aggregation. Receptors on the endothelial surface for products released from platelet granules including ADP and 5-HT release endothelium-derived relaxing factors including nitric oxide and prostaglandins. These EDRFs not only act to inhibit further platelet activation, but as they also are released toward the vascular smooth muscle, cause dilation of the blood vessel so that the platelet aggregate tends to be washed away by the flowing blood [7,10]. With damage to the endothelium as might occur with atherosclerosis, platelet-derived vasoactive products bind to receptors on the vascular smooth muscle and cause contraction. The platelet aggregate then is retained [24]. Depending on the anatomy of the vessel wall, platelet-associated thrombus formation may occur in various clinical disorders such as myocardial infarction, ischemic heart disease, peripheral arterial disease, and stroke. Few studies have examined effects of gender/sex and hormonal status on the platelet–vessel wall interaction in vivo. However, addition of autologous platelets to coronary arteries in vitro causes relaxation of arteries with endothelium and contraction of arteries without endothelium. Although no statistically significant difference was observed in the magnitude of these responses among coronary arteries from male, female, or ovariectomized female animals, there was a significant difference in the content of vasoactive substances released from the platelets [19]. A functional difference in vascular response between tissues derived from males and females was observed, however, when the platelets were added to isolated segments of femoral veins. Under these conditions, contractions of veins without endothelium from males were significantly greater than those from ovariectomized females [17]. A difficulty with interpreting these experiments is that the vascular responses will reflect the sum of the response to multiple factors released from the platelets in addition to hormonally modulated expression of receptors and transduction pathways in the endothelium and smooth muscle. Therefore, while the net mechanical effect, dilation in tissue with endothelium and contraction in tissue without endothelium, is the same among animals of different hormonal status, the mediators of those responses may not be the same. Therefore, in order to target specific pharmaceutical interventions directed at platelet–vessel wall interactions, it is important to understand how the various target molecules are influenced by both/either genetic sex and/or hormones.

Platelet-derived products are essential for healing of wounds, whether the wounds are internal to the blood vessel wall (as would result from intravascular interventions) or to

the surrounding tissue. Detailed discussion of the influence of hormones and sex on wound healing is found in Chapter 22. However, it is important to reiterate here that sex steroids regulate genomic transcription of platelet derived-factors that affect proliferation of cells, composition of extracellular matrix, activation of coagulation proteins (thrombin and fibrinogen) and migration of inflammatory cells into the wound and thus the healing process in general. An important family of enzymes associated with wound healing as well as instability of atherosclerotic plaques are MMPs.

MMPs are the family of structurally related zinc and calcium-dependent enzymes that contribute to the degradation of the extracellular matrix protein needed for vascular remodeling in wound healing and angiogenesis. MMPs (MMP-2, -9, and -14) and their inhibitors (TIMP-1, and -2) are present in human platelets and megakaryocytes. Expression of MMP-2, MMP-9, TIMP-1, and TIMP-2 were similar in platelets of male and female blood donors ages ranging from 24 to 65 years [13]. In pigs, loss of ovarian hormones increased platelet aggregation and secretion of MMP-2, decreased secretion of MMP-14 and did not change MMP-9. While MMP-2 stimulates platelet aggregation, MMP-9 prevents thrombin- and collagen-induced aggregation in humans. Therefore, regulation of MMP's represents a potential mechanism by which thrombosis could be modulated.

6. Future directions

Cardiovascular morbidity and mortality is higher in men than women and in post-menopausal women with a history of cardiovascular disease, treatment with exogenous hormones increases risk for future myocardial infarction, stroke and venous thrombosis. Some of this risk may be related to effects of sex hormones on platelet function. Because the risk of thrombotic events is present in men and women using exogenous hormones, effects on platelet functions is likely to be independent of genetic sex, however, this hypothesis has not been tested directly. Much remains to be learned about how sex hormonal status affects bone marrow megakaryocytes and platelet–platelet and platelet–endothelial cell interactions. To clarify how platelets participate in gender/sex difference in etiology and progression of cardiovascular disease, the following objectives need to be addressed:

1. Determine genomic and non-genomic actions of testosterone and estrogen on bone marrow megakaryocytes and platelet production from male and female animals.
2. Determine the location of sex steroid receptors in platelets (e.g. membrane or cytosolic) and how their activation affects platelet aggregation and secretion in the circulation.
3. Determine whether or not and how sex hormones affect phosphorylation-induced platelet aggregation and vessel wall interactions.
4. Determine how genetic polymorphisms in sex steroid receptors affect platelet functions including relationship to thrombotic risk.
5. Determine differences in expression and activity of MMPs in platelets in men and women and the influence of sex hormones on that expression.

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Sex differences in wound healing

Gillian S. Ashcroft

1. Introduction

Cutaneous wound healing is an innate response which involves a number of overlapping phases namely leukocyte recruitment, matrix deposition, epithelialization, angiogenesis and the formation of a mature scar. In the developed world, a significant and rapid increase in the elderly population has resulted in a substantial increase in age-impaired wound healing states. Costs for the treatment of such wounds amount to over $9 billion per year (in the USA), in addition to an incalculable degree of morbidity including reduced mobility, wound odor, exudate and pain. Impaired age-related wound healing states, both acute wounds that fail to heal and chronic ulcers, are characterized by excessive leukocytosis and subsequently enhanced proteolytic degradation of matrix constituents [1]. Intriguingly, reports have shown that acute wounds heal more slowly in males than females, and that the response is different in the two sexes. The impact of hormones on cellular and tissue responses has major downstream effects on the rate of healing, resulting from enhanced inflammation, impaired cytokine signal transduction, and an altered balance of protein production and degradation. Understanding of the complex interactions between the cell and the hormonal microenvironment, and the mechanisms underlying sex differences in repair, is essential in order to develop focused strategies to accelerate healing in the elderly. This chapter will review the basics of the wound healing response, identify age and sex differences in that response, and then review what is known about how estrogen and androgens contribute to mechanisms underlying such sexually dimorphic characteristics of wound healing.

2. The wound healing response

Immediately following injury a host of protective responses are initiated, which include coagulation and an acute local inflammatory response followed by fibroblast recruitment, proliferation, matrix synthesis and remodeling [6]. At 2 days post wounding, the number of neutrophils within the wound reaches its peak, followed by an increasing representation of monocytes. Inflammatory cells engage in essential functions including phagocytosis of debris, microbial agents and degraded matrix components, in addition to the secretion of

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proteases. Simultaneously, epithelial cells begin to proliferate, migrate and re-form the barrier to the external environment. A fibronectin-rich scaffold forms below the new epidermis, containing fibroblasts and new blood vessels. The synthesis of extracellular matrix (ECM) ultimately replaces the provisional clot with collagen types I and III, forming a connective tissue scar. The final phase is that of remodeling in which collagen is synthesized, degraded and reorganized. Elastase and matrix metalloproteinases (MMPs) produced by keratinocytes, fibroblasts and inflammatory cells degrade the matrix components of the wound during this phase. This proteolysis is regulated by tissue inhibitors of matrix metalloproteinases (TIMPs) and naturally occurring inhibitors of elastase such as secretory leukocyte protease inhibitor (SLPI). Under certain circumstances, such as with intrinsic aging, these processes are dysregulated leading to enhanced leukocyte recruitment and proteolytic activity, enhanced tissue breakdown, and ultimately delayed acute and chronic wound healing states.

3. Age/sex differences in wound healing

Age influences all phases of tissue repair and specific changes in the healing response may be modulated by host sex.

3.1. Inflammation

Age-related impaired cutaneous wound healing is associated with excessive inflammation, predominantly neutrophil recruitment, activation, and protease production. Aging modulates cell adhesion, migration, and functional responses, with pervading consequences for the wound repair process. In addition to an age-related enhanced capacity for inflammatory cells to adhere in both humans and experimental animals, such cells also express increased levels of pro-inflammatory cytokines such as TNF-α. One downstream effect of the increased cytokine expression is to drive leukocyte recruitment and the local inflammatory response. In vivo age-associated cutaneous wound healing is characterized by increased numbers of neutrophils locally resulting in enhanced and sustained elastase activity [7]. Both inflammatory cell adhesion in vitro, and numbers of wound tissue inflammatory cells in vivo, are increased in females compared to males [2].

3.2. Epithelialization

Epithelial cells must proliferate and migrate in order to restore a cutaneous barrier and to contribute to contraction of the wound. This process involves the interaction between numerous cytokines, cytokine receptors, integrins and proteases. In vivo cutaneous epithelialization is delayed with intrinsic age, both in healthy humans and in experimental animals. A number of factors contribute to such a delay: decreased keratinocyte proliferation, reduced expression of epithelial growth factor (EGF), and increased responsiveness to inhibitory cytokines. These age-related changes result in persistent exposure of the wound to microbial pathogens which further exacerbate inflammation and delay dermal healing.

3.3. Angiogenesis

The matrix derived from the endothelium changes with age, and the production of growth inhibitors, and the sensitivity to such agents, increases as cells are aged in vitro. In vivo, aging has been associated with reduced capillary growth, delayed angiogenesis, and impaired production of angiogenic growth factor. Despite a delay in neo-vascularization with aging, the ultimate quantity of new blood vessels may be affected by other factors as well. For example, following experimental acute excisional wounding of the upper inner arm, elderly healthy females without co-morbidity deposit greater quantities of new vessels than elderly men. The pathophysiological consequences and mechanisms of such events are still unknown.

3.4. Matrix/basement membrane deposition

An early and important component of the wound provisional matrix is fibronectin, produced by fibroblasts. Fibronectin and collagen protein decrease in wounds from aged subjects, moreover elevated neutrophil-derived elastase and MMP-2/-9 production have been associated with the excessive matrix breakdown in wounds of aged humans. Both matrix and basement membrane deposition is significantly delayed with age, with elderly females healing more quickly than elderly males. For example, using an acute wound healing model in healthy subjects, the deposition of collagen VII, a basement membrane molecule, is markedly delayed in the >60 year age-group, particularly in elderly males [4].

4. Role of hormones in wound healing

4.1. Estrogen

In the developed world the majority of women live one third of their lives in a state of systemic estrogenic deprivation [5]. Despite the extensive use of estrogens in hormone therapy (HT), mechanisms of action of estrogen in the wound healing process are largely unknown. The potential impact of estrogen on skin homeostasis and wound repair is underscored by the presence of estrogen receptors in fibroblasts, macrophages and epidermal cells. Topical and systemic estrogen treatments increase the rate of healing in male and female aged humans (particularly females). Estrogen affects cutaneous wound healing response by modulating all phases of the wound healing process: the inflammatory response, cytokine expression and matrix deposition, accelerating re-epithelialization, stimulating angiogenesis and wound contraction, and regulating proteolysis [1,3] (Table 1). However, the mechanisms underlying such effects on cutaneous wound repair have not been fully determined, emphasising the need for further investigation.

4.1.1. Effect of estrogen on the inflammatory phase

Excessive and prolonged inflammation associated with age-impaired cutaneous healing can be related to altered cell adhesion, migration, and functional responses such as

Table 1
Effects of estrogen on wound healing

Stimulates epithelialization
Dampens inflammatory response
Reduces proteolysis
Increases collagen deposition
Alters local cytokine profiles (↓ MIF; ↑ TGF-β)

phagocytosis. Leukocyte recruitment and proteolytic destruction of tissue is further exacerbated by the age-related increase in pro-inflammatory cytokines (such as TNF-α) released by such cells. The oxidative metabolism of activated neutrophils during phagocytosis is increased following estrogen administration, possibly by an increase in myeloperoxidase activity. Moreover, estrogen enhances the phagocytic capacity of such cells thereby reducing the risk of infection and the transition to a non-healing wound [8]. Estrogen also has direct effects on neutrophil transmigration from the vascular space into the site of injury and has been reported to inhibit neutrophil chemotaxis and alters the expression of neutrophil adhesion molecules (specifically L-selectin). One consequence of this estrogen-mediated inhibition of chemotaxis is an indirect increase in wound collagen and fibronectin levels secondary to reduced local levels of inflammatory cell-derived proteases [1,3].

The demonstration of estrogen receptors in monocytes and macrophages suggests that estrogen may have a direct effect on these cell types. One such factor regulated directly by estrogen is macrophage migration inhibitory factor (MIF), a pro-inflammatory cytokine produced by monocytes, T-lymphocytes, endothelial cells and keratinocytes. Estrogen modulates the local inflammatory response by down-regulating MIF expression in human and murine activated monocytes, leading to reduced inflammation, enhanced matrix deposition and accelerated wound repair (Ashcroft, unpublished data). Such effects are dependent upon estrogen receptor alpha (ERα) suggesting a focused approach to accelerating healing in vivo, for example by employing ERα agonists locally or by neutralizing MIF itself.

4.1.2. Effect of estrogen on the tissue proliferation/remodeling phase

Estrogen acts to accelerate healing and to protect against microbial colonization by stimulating reformation of the epidermal barrier. Estrogen has a mitogenic effect on keratinocytes, increasing the rate of re-epithelialization after wounding in both animals and humans. In a recent study, post-menopausal women who have never taken HT showed retarded re-epithelialization at 7 days post-wounding, whereas post-menopausal women who had taken HT for at least 3 months had levels of re-epithelialization similar to those females in the pre-menopausal group.

Estrogen treatment in human volunteers, both topically and systemically, enhances collagen and fibronectin deposition in vivo. A major contributor to matrix production is the cytokine transforming growth factor-β (TGF-β1) which induces the synthesis and inhibits the degradation of ECM, and stimulates the formation of granulation tissue.

Estrogen increases the secretion of TGF-β1 by dermal fibroblasts in vitro, regardless of donor age, and increases expression levels of TGF-β in wound tissue in vivo. Recent reports have documented a decrease in wound collagen deposition and MMP-mediated collagenolysis following ovariectomy in rats. These effects were reversed by estrogen replacement, implicating estrogen as a pivotal mediator involved in shifting the balance from matrix degradation to matrix synthesis. Thus estrogen may accelerate the healing response via the modulation of cytokines which influence matrix production, and by a direct or indirect effect on protease activity.

A major part of the tissue remodeling phase involves angiogenesis. It has been postulated that estrogen may influence angiogenesis by a direct effect on endothelial cells since estradiol increases the in vitro attachment of human endothelial cells to various types of matrix. Estrogen also stimulates the migration of endothelial cells in vitro following "wounding" of the monolayer, and these effects can be blocked by the estrogen receptor antagonist ICI 182, 780 suggesting a receptor-mediated effect. In vivo animal studies have produced contradictory results, reporting no effect or an ultimate decrease in vascularity dependent upon the experimental animal and the dose/route of drug administration. The consequences of specific effects of estrogen on angiogenesis during the in vivo cutaneous wound healing response are currently unknown and further studies are in order to delineate the role of estrogen on neo-vascularization in tissue repair.

Taken together, the literature suggests estrogen stimulates re-epithelialization, wound contraction and matrix deposition during the tissue proliferation phase of wound healing.

4.2. Androgens

The age and sex differences in wound repair in vivo may be influenced by a number of hormonal factors (Table 2), including decreased gonadal and local production of estrogens, altered levels of adrenal sex steroid precursors, and increased pituitary-derived leutinizing hormone and follicule stimulating hormone (LH/FSH) particularly in elderly females. Elderly males have altered local levels of bioactive estrogen secondary to reduced secretion of adrenal sex steroid precursors, and changes in the local conversion of precursors to estrogens by aromatisation, however other factors above and beyond

Table 2
Age/gender effects on hormonal profiles

Reduced systemic estrogen in males and females
Decline in adrenal sex steroid precursors (DHEA/S)
Increased LH/FSH (females)
Altered regulation of local estrogen/testosterone production (aromatase)
Role of androgens (?)

Estrogen levels locally are altered with age secondary to reduced adrenal and gonadal production, and dysregulated aromatase conversion of sex steroid precursors to local estrogens. Increased levels of LH and FSH, particularly in post-menopausal females, may influence wound repair since, for example, LH induces angiogenesis in other organs such as the ovary. The specific role of androgens has been largely neglected until recently.

Table 3
Effects of androgens on wound healing

Enhanced inflammation
Increased local production of TNF-α
Increased NFκB activity
Reduced matrix deposition
Delayed healing

estrogen may play a role in the male response to injury. One factor that has received little attention is the potential role of androgens in modulating wound healing responses. Elderly males generally maintain testosterone levels, albeit with a gradual reduction with increasing age, and androgens have been reported to be pivotal mediators of local and humoral immune responses in other patho-physiological processes. In this context, several reports indicate that androgens play a critical role in the immune response and account for sex differences in outcome, including susceptibility to sepsis, parasitic infection, and atherosclerosis related to enhanced monocyte adhesion to endothelium.

Castration of male mice results in accelerated cutaneous wound healing and is associated with a dampened inflammatory response and increased matrix deposition [4] (Table 3, Fig. 1). In hairless mice, castration enhances proliferation of hair follicles and increases skin healing by two separate mechanisms. The underlying mechanisms involve a direct effect of testosterone on murine macrophage TNF-α production via the androgen receptor, in parallel to down-regulation of TNF-α following castration or androgen

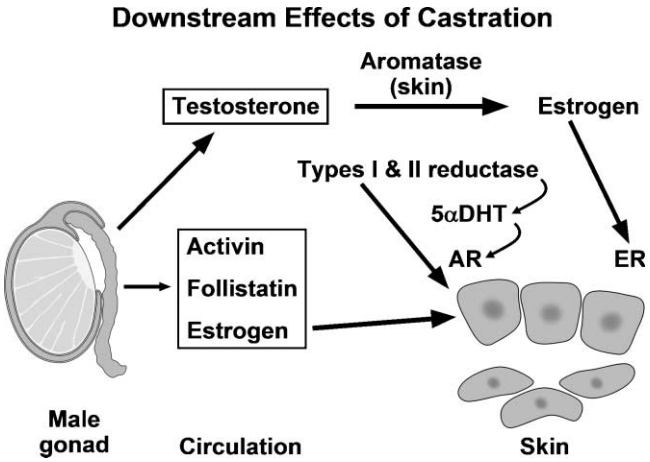

Fig. 1. *Downstream effects of castration*. Male gonads secrete a number of factors, which may influence wound repair. In addition to the predominant hormone testosterone and small quantities of estrogen, other factors are produced by the testes such as activin and follistatin to modulate wound repair in experimental animals. One experimental method used to study mechanisms underlying the effects of castration, is to treat male animals with an oral flutamide androgen receptor antagonist prior to, and during, the wound healing process. Abbreviations: AR, androgen receptor; ER, estrogen receptor; 5α-DHT, 5-alphadihydrotestosterone.

receptor antagonism. Such findings concur with models of burn injury in which systemic levels of TNF-α are markedly reduced in castrated compared to intact animals. Intriguingly, androgen receptor blockade accelerates healing in a similar fashion to castration suggesting a future target for therapeutic intervention to accelerate healing in elderly males.

5. Future directions

The current literature strongly implicates a crucial role for both androgens and estrogens in the wound healing response. Further understanding of the specific downstream gene targets of estrogen/androgen actions, the receptor/signaling pathways through which these hormones act, and the potential sex differences in such pathways, will enable more focused treatment approaches to be established. Such an approach would potentially minimize adverse and well-documented systemic effects attributable to estrogen. Moreover, the mechanism of androgen action can be further delineated using type I and II reductase inhibitors in order to determine the role of 5α-dihydroxytestosterone in the wound response. In addition, the synergistic effects of specific therapies, tailored to the individual and dependent upon sex, may be a feasible strategy. Such treatments may involve the combined use of estrogen agonists (possibly specific to a particular receptor isoform) and anti-androgenic compounds (Fig. 2). A further exciting possibility may involve the use of sex steroid precursors (such as dehydroepiandrosterone [DHEA]) which act differentially through the estrogen/androgen pathways, in addition to non-estrogen receptor/androgen receptor pathways that mediate specific functions but are, as yet, undefined.

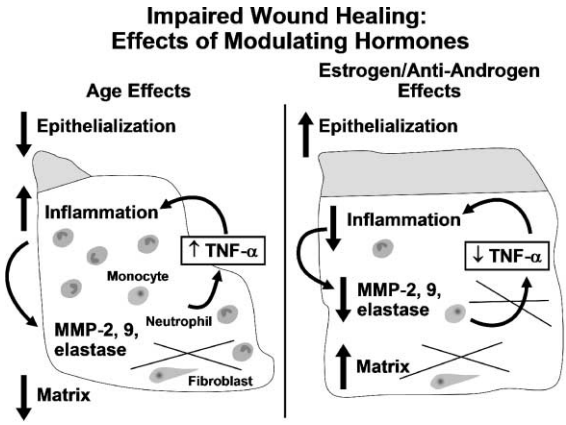

Fig. 2. *Impaired wound healing: effects of modulating hormones*. Aging is associated with delayed epithelialization resulting from impaired migration and proliferation, excessive inflammation leading to increased levels of proteases (MMPs, elastase) and matrix degradation. Reduced fibroblast production of, and responses to, specific cytokines (eg. TGF-1, EGF) results in reduced matrix production, compounding the excessive degradation at the wound site. Based on in vitro and in vivo studies, estrogen and anti-androgen treatment may accelerate healing by stimulating epithelialization, reducing inflammation, and enhancing matrix deposition. Abbreviations: MMP, matrix metalloproteinases; TNF-α, tissue necrosis factor.

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