Chapter 7

Anticalcineurinics: Role of Mitochondrial Transition Pore on Nephrotoxicity

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1. Introduction

Cyclosporin A (CyA) and tacrolimus (TAC) are calcineurin phosphatase inhibitors which are currently used in immunosuppression therapy for solid organ and hematopoietic stem cell transplantation to prevent and treat allograft rejection, and for autoimmune disease treatment [1]. Although drugs are not structurally related (Figure 1, Materials and Methods), they have identical cellular and molecular actions, including nephrotoxicity as a major side effect [2-4].

It is known that CyA and TAC may cause acute as well as chronic tubulointerstitial nephropathy characterized by tubular atrophy, loss of tubular cells and interstitial fibrosis [5], although the mechanisms of immunosuppression-associated renal damage are not fully understood [6, 7]. In any case, it is generally agreed that acute haemodynamic effects of CyA may be mediated by an imbalance of different vasoconstrictors and vasodilators, including renin-angiotensin system and nitric oxide among others [1,3]. Histologically, vacuolization, calcification and necrosis of tubular epithelial cells are characteristics, and endothelial cell swelling with variable degrees of hyalinosis. Abnormalities similar to those are found with TAC [4,8]. For chronic CyA nephrotoxicity, different studies have suggested a toxic effect of the drug on afferent arterioles and tubular epithelial cells, vasoconstriction and endothelial injury leading

to ischemia, as well as a direct toxic effect of CyA on tubular epithelium [5,9]. Moreover, apoptosis has been clearly evidenced in tubular and interstitial cells in patients, animals and cell culture models [9]. We have described that loss of mitochondrial potential usually precedes caspase activation and endonuclease mediated DNA laddering when proximal tubule cells are incubated in the presence of increasing doses of CyA [1,7]. Similar effects are found in TAC-induced nephrotoxicity [7, 10]. Mitochondrial depolarisation, caspase activation, cytosolic nucleosomal formation, and other morphological features of apoptosis induction are commonly found with both CyA and TAC in proximal tubule cells [1, 7]. Transforming growth factor-β (TGF-β), hypoxia and oxidative stress have also been considered to be important mediators of CyA and TAC-induced renal injury [6, 7]. Significantly, although both drugs have similar effects, TAC is recognized to induce fewer haemodynamic and fibrogenic alterations at equivalent doses of CyA [11], and studies have shown that the incidence of acute rejection is reduced by TAC use over CyA [12].

Although mitochondrial mediation in the damage is generally accepted to be common to both immunosuppressors, the exact mechanism has not been fully investigated. Several authors have found a decreased mitochondrial respiration with CyA, mediated by a reduction in complex II activity, with stage 3 and 4 activity reductions [13-15]. Others describe a preferential inhibition of respiration by CyA when substrates of complex I are used [16]. TAC also inhibits the succinate supported state 3 respiration [13]. ATP depletion is a key factor in cell death associated with renal failure; therefore phosphorylation has also been studied as a potential target for CyA induced nephrotoxicity. CyA induced inhibition of ATP synthesis ranges from 12% [17] to 40% [18], and may be partially prevented by calcium antagonists [19]. Like CyA, TAC inhibits net ATP uptake [13]. However, the most intriguing finding regarding mitochondrial mediation in CyA and TAC induced apoptosis is the relationship of both immunosuppressors with the permeability transition pore.

The mitochondrial permeability transition (MPT) pore is an unstable structure that mitochondria reversibly assemble in their inner membrane under several physiological and non-physiological conditions, mainly cell calcium overload. When assembled, inner and outer membranes physically touch each other at specific points; when assembled and opened, it allows non selective permeation of ions and solutes up to 1.5 kDa [20, 21], dragged by a water movement from cytosol into mitochondria resulting in mitochondria swelling that may be measured [22]. MPT pore assembly includes cyclophilin-dependent conformational changes in adenine nucleotide translocase (ANT), located in the inner membrane and in a voltage dependent anion channel (VDAC). Cyclophilin D, benzodiazepine receptor, creatine kinase, hexokinase, cytochrome c and Bax protein are also involved in MPT pore formation and regulation [23-25]. The exact molecular structure of the MPT pore is still controversial, as it is the MPT pore relation to energy transfer and apoptosis, and for these reasons the physiological function of the MPT pore is not well established [23, 26].

It is generally agreed that MPT pore opening is triggered by a sudden rise in mitochondrial matrix free calcium concentration, representing the third of three sequential phases of mitochondrial accumulation: respiratory chain acceleration, extramitochondrial calcium buffering and finally activation of the MPT pore [27]. Transitory openings are necessary to prevent mitochondrial calcium overload, by allowing calcium excesses release [26, 28, 29]. MPT

pore only closes again if intramitochondrial free calcium is reduced. However, no clear explanations have been offered about how mitochondria may release calcium without causing the death of the cell. Although there should be a physiological role for MPT pore transitory opening, many authors agree that a role of MPT pore in healthy cells has not been established, and it only plays a role in pathophysiological conditions [26, 28, 30], such as necrotic and apoptotic cell death, ischemia/reperfusion injury, heart failure and other cardiovascular or neurodegenerative diseases [26,31,32]. In opinion of Martin Crompton, a possible physiological function of MPT pore may be to establish contact between mitochondria in the formation of mitochondrial networks [28]. It is established that mitochondria can form tight intermitochondrial junctions, allowing the thus-conjugated mitochondria to operate as a bioenergetics continuum permitting efficient energy transfer between different parts of the cells [33]. Other authors have also hypothesized about other possible physiological roles of MPT pore, as the regulation of protein import, matrix volume and pH, cristae remodeling, redox equilibrium and control of metabolism [31, 34, 35].

In any case, it is accepted that MPT pore formation is considered a prelude of mitochondria triggered apoptosis; prolonged times of MPT pore activity are accompanied by loss of transmembrane mitochondrial potential [36, 37] and release of proapoptotic mitochondrial proteins as cytochrome c [38], SMAC/Diablo [39, 40], HtrA2/Omi [41], apoptosis inducing factor (AIF) [42] or endonuclease G [43]. Although there is no agreement as to whether these proapoptotic mediators leave the mitochondria through the MPT pore or by other pathways [44-46], or whether the leak takes place after or before pore opening [27], protein implicated in apoptotic balance Bcl-2, Bcl-XL, Bax and Bid [47-48] are implicated on MPT pore activity regulation. Agents capable to keep MPT pore timely opened as nitric oxide [49], free oxygen radicals [50, 51] or Bax [52] are proapoptotic. Those who block or interfere with MPT pore activity are antiapoptotic.

It is currently known that CyA is to date, the most specific inhibitor of the MPT pore by inhibiting the peptidylprolyl cistrans-isomerase activity of cyclophilin D [53, 54], and is considered as antiapoptotic on most cellular systems [26, 28]. On the other hand, no TAC binding proteins able to modify MPT pore activity have been reported to date. Therefore, MPT pore mediation on the nephrotoxicity of calcineurin inhibitors has always been considered a non-directly related phenomenon.

In this chapter we provide some insights into the proximal tubule mitochondria modifications that take place during CyA and TAC induced nephrotoxicity. We explore the potential implication of MPT modulation on cell protection, acute renal failure and mitochondrial ammoniogenesis.

2. Materials and methods

2.1. Drugs

CyA (Sandimmun Neoral®) was obtained from Novartis Farmacéutica S.A. (Spain) and TAC (Prograf®) from Fujisawa S.A. (Spain). Their chemical structures are represented in Figure 1.The

concentration used for both drugs were similar to the pharmacologically active recommended plasma level, and they were selected from our previous studies and experience [1, 7]. Cell cultures were exposed to different increasing concentrations of CyA (1-1000 ng/ml) and TAC (50-500 ng/ml), although most of the experiments were performed with 1000 ng/ml (CyA) and 50 ng/ml (TAC) based on the proven effectiveness of the drug to induce nephrotoxicity [1].

2.2. Experimental animals

All experiments were performed on miniature swine genetically selected to be isogenic for three loci of the major histocompatibility locus [55]. They were 3 months old and weighed 31.3 ± 0.7 kg (mean ± SEM). Isogenic minipigs were housed under controlled conditions and the study was approved by the Institutional Board for Animal Experiments. Animals were handled at all times according to the applicable legal regulations in RD 1201/2005, of 10 October, on the protection of animals used for experimentation and other scientific purposes.

2.3. Isolation and primary culture of renal proximal tubule epithelial cells

Proximal tubule suspensions were obtained as described [56]. The kidneys were aseptically removed from the animals and placed in a sterilized beaker containing Ham´s F-12 culture medium, pH 7.4. Briefly, renal cortex was sliced with a Staddie-Riggs microtome, and incubated with 0.6 mg/ml of collagenase A (Boehringer Mannheim, Germany) in Ham's F-12 medium for 30 minutes at 37 °C. Digested tissue was then filtered through a 250 μm metal mesh, washed three times in Ham's F-12 medium and centrifuged at 20,000 x g for 30 minutes in a 45% (v/v) Percoll gradient. The last band contains pure proximal tubules.

Proximal tubules were recovered from the deepest fraction, washed, and resuspended in culture medium (1:1 Ham´s F-12:DMEN supplemented with 25 mM hepes, 3.7 mg/mL sodium bicarbonate, 2.5 mM glutamine, 1% non-essential aminoacids, 100 U/mL penicillin, 100 μg/ml streptomycin, 5.10^{-8} M hydrocortisone, 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/mL sodium selenite and 2% fetal bovine serum), at a final concentration of 0.66 mg/mL, and plated on plastic culture dishes (60 mm) [57]. The culture was incubated at 37 °C in a 95% air/5% CO_2 atmosphere. The medium was renewed on the fourth day and every two days thereafter.

CyA or TAC were added to cell cultures as specified in every experiment.

2.4. Isolation of renal cortical mitochondria

The renal cortex was rapidly removed and placed into 100 mL of ice-cold isolation buffer (300 mM mannitol, 1 mM EGTA, 10 mM TRIS HCl, 1 mM PO_4H_2K, 1.74 mg/mL phenylmethylsulfonyl fluoride (PMSF), 0.2% BSA, pH 7.4 and gassed with N_2) and homogenized. The homogenate was centrifuged at 1075 x g for 10 minutes at 4 °C. The supernatant was centrifuged at 8635 x g for 10 minutes. The resulting pellet was washed twice at 8635 x g for 10 minutes and resuspended in isolation buffer to 32.5 ± 1.97 mg of protein/mL.

All experiments with isolated mitochondria are carried out in assay medium (300 mM mannitol, 10 mM TRIS HCl, 1mM PO_4H_2K, pH 7.4).

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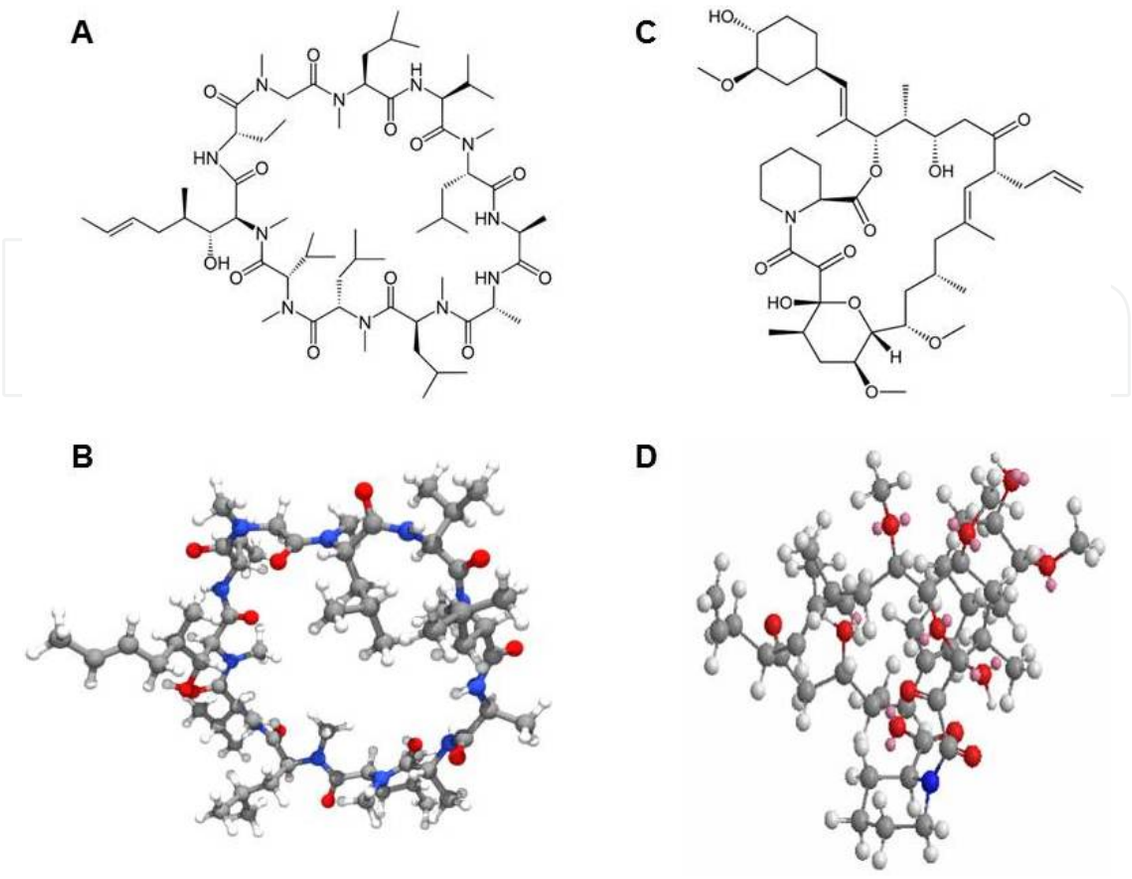

Figure 1. Formula of the nephrotoxic compounds. A and B represent the molecular structure of cyclosporin A; cyclic endecapeptide with chemical formula $C_{62}H_{111}N_{11}O_{12}$ and a molecular weight of 1202.16 g/mol. C and D represent the molecular structure of tacrolimus; macrolide whose empirical formula in its monohydrate form is $C_{44}H_{69}NO_{12}$, and with a molecular weight of 804.018 g/mol.

2.5. Mitochondrial oxygen consumption

State 3 and state 4 respiration was measured with a Clark-type electrode (YSI incorporated, Yellow Springs, Ohio, USA) in a metacrilate chamber at 37 °C equipped with magnetic stirring in the presence of 5 mM succinate before (state 4) and after (state 3) the addition of 2 mM ADP and 10 μM atractilosyde. Mitochondria were suspended in continuously stirred assay medium, maintained at 37°C at a final mitochondrial protein concentration of 0.40 ± 0.02 mg/ml. In parallel experiments, CyA 1μg/ml and TAC 50 ng/ml were added 30 minutes prior to addition of effectors or simultaneously with then (no preincubation). The content of oxygen was calculated using the following equation:

$$O_2(\text{mol}) = 0.21 \text{ x } 0.024 \text{ x } V_{\text{cuvette}} \text{x } (P_{\text{atm}} - P_{\text{H2O}})/22.4 \quad (1)$$

where 0.21 is the O_2% in the air, 0.024 is the O_2 solubility coefficient, 22.4 is the molar volume of an ideal gas (L/mol). The cuvette volume is 807 μl. Atmospheric pressure was 708.9 ± 1.96 mmHg and the vapour water pressure is 46.6 mmHg.

2.6. Calcium uptake

One mL of mitochondrial suspension was incubated with 5 μM fura-2, AM (Molecular Probes) for 30 minutes at 4 °C, washed three times at 8635 x g for 10 minutes and resuspended in isolation buffer at 4°C in a 1:1 proportion.

Fura-2 fluorescence was monitored with a SLM Aminco 8000 fluorometer at 37°C in stirred cuvettes, by exciting it at 340 nm (λ_{ex1}) and 380 nm (λ_{ex2}) and rationing the fluorescence intensities detected at 510 nm (λ_{em}). Mitochondrial matrix [Ca^{2+}] was determined using the following equation [58]:

$$\left[Ca^{2+}\right]=K_d Q\left(\left(R-R_{min}\right)/\left(R_{max}-R\right)\right) \quad (2)$$

where R represents the fluorescence intensity ratio F (340 nm)/ F (380 nm), R_{min} and R_{max} requires taking fluorescence measurements for the completely ion-free (EGTA 2 mM) and ion-saturated ($CaCl_2$ 5 mM) indicators. Q is the ratio of F_{min} to F_{max} at 380 nm. K_d is the dissociation constant of fura-2 for calcium (0.14 μM).

2.7. Mitochondrial swelling: MPT pore

Ca^{2+}-induced mitochondrial swelling was determined spectrophotometrically as described by Beavis and Col. [59], adapted to our experimental conditions, by using authors equation:

$$\text{Mitochondrial volume}\left(\mu l/mg\right)=\left[\left(1/Abs_{520\,nm}-0.1832\right)\text{ x }\left[\text{Protein}\right]\right]/0.06 \quad (3)$$

where 0.1832 is the theoretician inverse of absorbance for an infinite protein concentration, and 0.06 is a proportionality constant established by the authors [59]. Mitochondria were suspended in 3 mL of continuously stirred assay medium, maintained at 37°C at a final mitochondrial protein concentration of 0.54 ± 0.1 mg/ml. Swelling was initiated by the addition of succinate to the sample cuvette, and the absorbance changes at 520 nm were monitored with an Uvikon 930 spectrophotometer.

A mitochondrial basal volume of 3.56 ± 0.06 μl/mg protein was obtained.

2.8. Analysis of mitochondrial transmembrane potential by fluorescence microscopy

Mitochondrial transmembrane potential in intact renal proximal tubular epithelial cells (RPTECs) was followed by cellular distribution of Rhodamine. Cells were incubated at 37°C for 15 minutes in the presence of 10 mM Rhodamine 123 (Sigma) and washed twice in PBS. The cells were then observed with a fluorescence microscope Olympus IX70 (wide-band cube U-MWG; λ_{ex}: 510-550, λ_{em}: >590, λ_{dic}: 570).

2.9. Cellular glutamine metabolism

Glutamine uptake was determined on proximal tubules following spectrophotometrically the NADH production at 340 nm. Glutamine uptake was determined by difference with the

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glutamate. Determinations are based on following reactions of glutaminase and glutamate dehydrogenase, respectively [60]:

Glutamine → Glutamate + NH_4^+

Glutamate +NAD → Ketoglutarate + NH_4^++ NADH

2.10. Cytochrome c distribution

Release of cythochrome c from mitochondria into cytosol was measured using Western blot analysis. RPTECs treated for 48 h with 1 μg/ml CyA or 50 ng/ml TAC were harvested, washed once with ice-cold PBS, and gently lysed for 10 minutes in ice with 90 μl of lysis buffer (250 mM sucrose, 80 mM KCl, 500 μg/ml digitonin, 1 mM dithiothreitol, 0.1 mM PMSF, and protease inhibitors in PBS). Cell lysates were centrifuged at 12,000 x g at 4°C for 5 minutes to obtain the supernatants (cytosolic extract free of mitochondria) and the pellets (fractions containing the mitochondria), which were resuspended in 90 μl of lysis buffer. Equal amounts of protein were loaded (30 μg in each lane) and electrophoresed on 15% polyacrylamide gels as previously described [56].

Rabbit polyclonal antibody anti-cytochrome c (Santa Cruz Biotechnology, Inc, CA, USA) was used at 1:500.

The membranes were also probed with goat polyclonal antibody against a peptide of voltage-dependent anion-selective channel 1 of human origin (VDAC-1, 1:500; Santa Cruz Biotechnology, Inc.) for mitochondrial fractions and monoclonal anti-tubulin Clone B-5-1-2 Mouse Ascites Fluid (mouse IgG1 isotype) antibody (1:10,000; Sigma-Aldrich) for cytosolic fractions as internal controls for the technique. Proteins were visualized with the enhanced chemiluminescence detection system (ECL, GE Healthcare, Little Chalfont, Buckinghamshire, UK).

2.11. Apoptosis studies: Flow cytometry, microscopy and caspase activity assay

After CyA or TAC treatment, cell number was determined with a computer coupled video-microscopy system. Every count was assessed on 21 sectors images of 0.0775 mm^2 chosen at random from three different culture dishes.

Flow cytometry was performed on supernatant and monolayer harvested cells after dilution in $2x10^6$ cells/mL in 2% paraformaldehyde/PBS (30 minutes, 4°C, pH 7.4), permeabilization with PBS-Tween 0.5% (15 minutes at room temperature) and incubation with 40 μg/mL RNAse and 250 μg/mL propidium iodide (Sigma, St Louis, USA) at room temperature for 45 minutes. A minimum of 10,000 cells per sample were acquired in a FACScan equipped with a single argon-ion laser (Becton Dickinson, San Jose, CA), using standard Lysis II software. A gate was set on the basis of forward and scatter characteristics before fluorescence (FL2) was analysed.

Caspase 3 and caspase 9 activities were determined in RPTECs using the caspase 3 inhibitor, DEVD-FMK conjugated to FITC (FITC-DEVD-FMK) and the caspase 9 inhibitor LEHD-FMK conjugated to FITC (FITC-LEHD-FMK) as a fluorescent markers respectively, following the protocols of the CaspGLOW™ Fluorescein Active Caspase-3 and Caspase-9 Staining Kit from

BioVision, Inc., (Milpitas, CA, USA). Activated caspases detection was examined with the 20X PL-APO 0.7-numerical aperture objective of a Leica-SP2 confocal microscope (Leica Microsystems, Heidelberg, Germany). Different intensity measurements were assessed with the Leica Confocal Software LCS-1537 (Leica Microsystems).

2.12. Statistics

Pooled data are presented as mean ± SEM. Comparison between treatment groups were done by one or two ways factorial ANOVA. Least significant differences were computed for between-level comparisons. A p value <0.05 was considered to be statistically significant.

3. Results

3.1. Effect of CyA and TAC on primary growth of the renal proximal tubule cells

When primary cultures of pig RPTECs are exposed to increasing CyA or TAC concentrations, a dose dependent growth inhibition is produced, even with concentrations as low as CyA 1 μg/mL or 50 ng/mL. Figure 2 shows the growth dynamics of cultures in the presence of CyA 1 μg/mL and TAC 50 ng/mL, concentrations near to the peak plasma levels reached with oral administration of these two drugs. Both CyA and TAC inhibit growth from day 6, which impedes the culture confluence from day 8 (Figure 2).

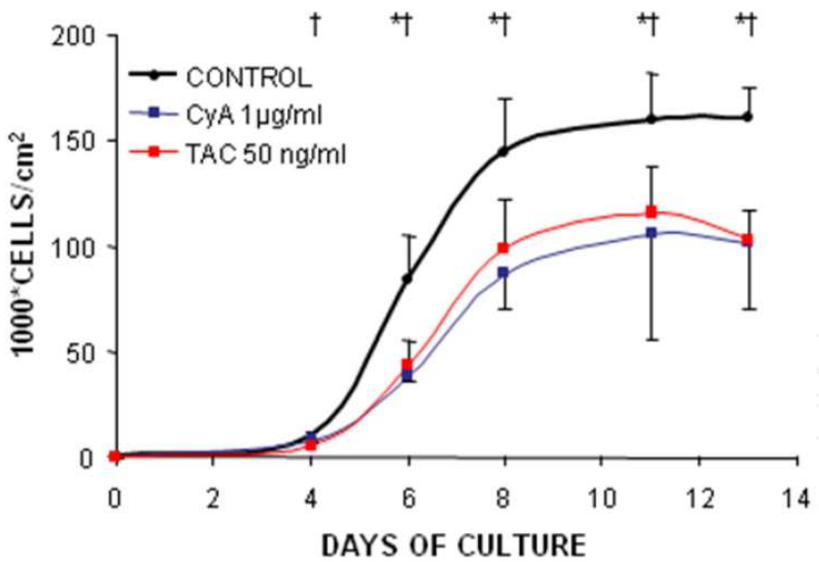

Figure 2. Cyclosporin A (CyA) and Tacrolimus (TAC)-induced cytotoxicity in pig kidney proximal tubule epithelial cells cultures. Culture cells were incubated with 1 μg/mL CyA or 50 ng/mL TAC. Viable cell number was determined by cell counting in a phase-contrast inverted microscope. Values shown are the mean ± SEM of cell counts per cm^2. *p<0.05 CyA vs. control; †p<0.05 TAC vs. control.

Figure 3 gives a 3-D histogram of the cell flow cytometry in cultures treated with CyA or TAC for 8 days. A distinct hypodiploid population can be observed, indicative of apoptosis

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induction with both treatments. Table 1 shows the dose dependence exhibited by the apoptosis induced by the two drugs.

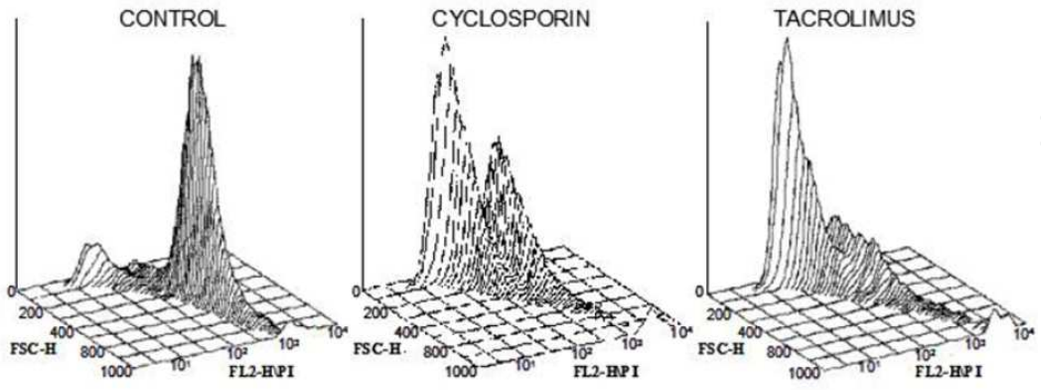

Figure 3. Effect of Cyclosporin A (CyA) and Tacrolimus (TAC) on apoptosis of pig kidney proximal tubule epithelial cells as assessed by flow cytometry analysis (FACScan). 3-D histograms of the cell flow cytometry in either control cultures or treated with 1 µg/mL CyA or 50 ng/mL TAC for 8 days. The hypodiploid population is greater in the CyA and TAC treated cells.

DOSE (ng/mL)	% hypodiploid cells (TAC)	% hypodiploid cells (CyA)
0	28.55 ± 2.9	28.55 ± 2.9
50	35.87 ± 5.8	
100		33.08 ± 3.8
500	44.42 ± 5.9*	
1000		42.32 ± 5.4*

Values are means ± SEM. *p<0.05 vs.dose 0 ng/ml. TAC, tacrolimus; CyA, cyclosporin A.

Table 1. Apoptosis assessed by flow cytometry analysis.

3.2. Effect of CyA and TAC on intramitochondrial free calcium

When suspensions of fura-2 AM loaded mitochondria were incubated in the presence of 21% oxygen and 5 mM succinate, but in the absence of ADP, an increase in intramitochondrial calcium concentration is observed (Figure 4A), paralleling an 8-fold increase in oxygen consumption (Table 2). Subsequent addition of 2 mM of ADP significantly increases the rate of oxygen consumption (Table 2) and drastically decreases intramitochondrial calcium concentration, although not reaching baseline levels (Figure 4A). This reduction in mitochondrial calcium when adding ADP is accompanied by a net calcium extrusion from mitochondria, as can be seen in Figure 4B where the same maneuver was repeated with non fura-2 loaded mitochondria suspended in the presence of the non-permanent acid form of fura-2. ADP

addition is followed by an increase in extramitochondrial free calcium, but there is a net difference between the initial free calcium mitochondria uptake when succcinate was added, and free calcium release after ADP addition.

In contrast, if fura-2 loaded mitochondria are first incubated with ADP 2 mM, no changes in mitochondrial calcium occur (Figure 4A). Subsequent addition of 5 mM succinate caused a delayed and lower increase in mitochondrial calcium, reaching a final intramitochondrial concentration similar to the one observed in the previously described setting (Figure 4A). Atractilosyde addition blocks mitochondrial calcium rise (data not shown) and reduce O_2 consumption in every condition.

In the presence of 1 μg/mL of CyA in the assay medium, mitochondrial calcium increases further when 5 mM of succinate is added, always in the absence of ADP (Figure 5). Simultaneously oxygen consumption increase is almost a 50% higher than observed in non-treated mitochondria (Table 2).

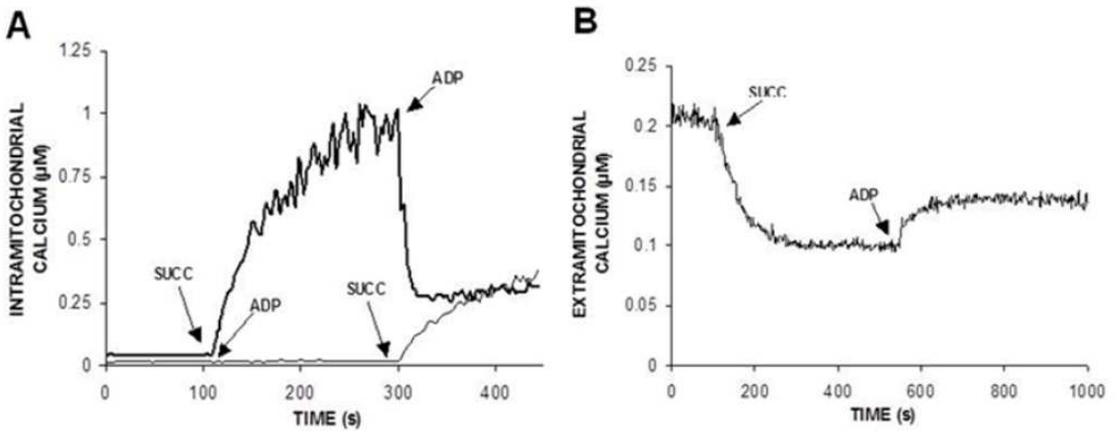

Figure 4. Succinate and ADP effects on mitochondrial calcium concentration. **A.)** The addition of 5 mM succinate (SUCC) to mitochondria in suspension induces an increase in the intramitochondrial calcium concentration. This calcium increase is inhibited by 2 mM ADP. **B.**) Decrease of the extramitochondrial calcium concentration induced by 5 mM SUCC. Mitochondrial suspension was incubated with 5 μM fura-2 and fluorescence was monitored with a fluorometer at 37°C.

The presence of TAC 50 mg/ml also causes a significantly greater increase in intramitochondrial calcium concentration and oxygen consumption when mitochondria begin succinate oxidation (Figure 5, Table 2).

In both cases, ADP addition significantly increases oxygen consumption (Table 2) and simultaneously the outflow of most of the calcium accumulated with or without CyA and TAC treatment (Figure 5). After ADP addition a stable intramitochondrial free calcium concentration is reached indicative of a new influx /efflux balance establishment. However, after 50-100 seconds a spontaneous increase in mitochondrial calcium concentration can be observed, although to a lesser extent. Again this new increase in mitochondrial calcium is higher and happens earlier with TAC than CyA.

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Groups	Control	CyA 1 µg/mL (NP)	CyA 1µg/mL (30 min)	TAC 50 ng/mL (NP)	TAC 50ng/mL (30 min)
Basal	341 ± 44	309 ± 39	541 ± 234	378 ± 36	469 ± 161
SUCC	2587 ± 126†	3290 ± 514*	2929 ± 286†	2918 ± 149*	2810 ± 240*
SUCC + ADP	3093 ± 187#	3675 ± 386*	3381 ± 163	3537 ± 335*	3440 ± 63#
SUCC+ ADP + Atractilosyde	2130 ± 151&	2218 ± 322&	2188 ± 177&	2114 ± 167&	2090 ± 135&

Mitochondria were preincubated with both drugs 30 minutes prior the conducting experiment or simultaneously (NP: No preincubation).Values are means ± SEM, n=22 *p<0.05 vs. "control" column; †p<0.05 vs. "basal line"; #p<0.05 vs. "succinate line"; &p<0.05 vs. "succinate + ADP line". SUCC, succinate.

Table 2. Mitochondrial oxygen consumption (µmol/g*h) induced by cyclosporin A (CyA) and tacrolimus (TAC).

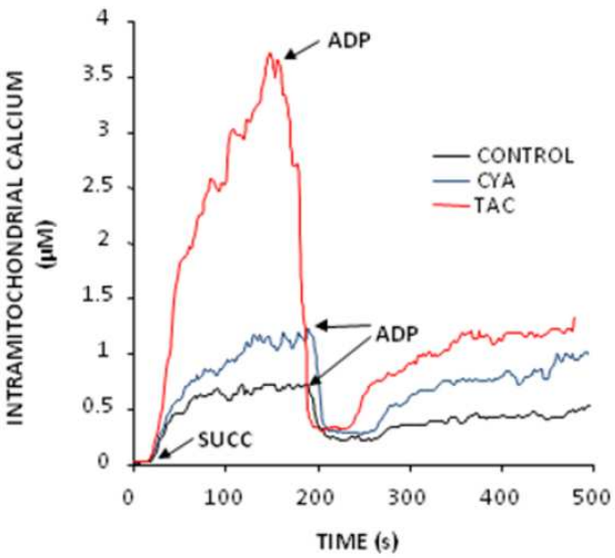

Figure 5. Cyclosporin A (CyA) and tacrolimus (TAC) increase intramitochondrial calcium concentration. In 5 mM succinate (SUCC) presence, 1 µg/mL CyA and 50 ng/mL TAC induce an increase in isolated mitochondria. 2 mM ADP causes the outflow of most of the calcium accumulated. Mitochondrial suspension was incubated with 5 µM fura-2 and fluorescence was monitored with a fluorometer at 37°C.

3.3. Effect of CyA and TAC on mitochondrial permeability transition pore

MPT pore opening was studied following mitochondrial electrochemical gradient restoration after respiratory chain activation (succinate addition), and subsequent mitochondrial calcium uptake. No extra calcium was added to the extramitochondrial medium, where free calcium was 0,2 µM (as determined in Figure 4B).

Figure 6 shows the temporary relationship between the changes in mitochondrial calcium influx, expressed as the first derivate of mitochondrial calcium concentration against time, and the change in mitochondrial volume expressed as the first derivate of the mitochondrial

volume versus time. Net calcium influx reaches a maximum of 7.3 ± 1.1 nM/s at 17.5 ± 0.9 seconds after addition of 5 mM succinate (Table 3).

Groups	Basal Calcium Influx (nM/s)	Maximal Calcium Influx (nM/s)	Time to Maximal Calcium Influx (seconds)
Control (n=11)	0.019 ± 0.011	7.3 ± 1.1	17.5 ± 0.9
CyA 1 µg/mL (n=4)	0.057 ± 0.015^{NS}	17.5 ± 2.7*	16.5 ± 0.9^{NS}
TAC 50 ng/mL (n=4)	0.006 ± 0.008^{NS}	88.8 ± 5.41*	17.5 ± 0.9^{NS}

Values are means ± SEM. *p<0.05 vs.control. Cya, cyclosporin A; TAC, tacrolimus. NS, non-significant.

Table 3. Characteristics of calcium influx induction after oxidation of succinate 5 mM.

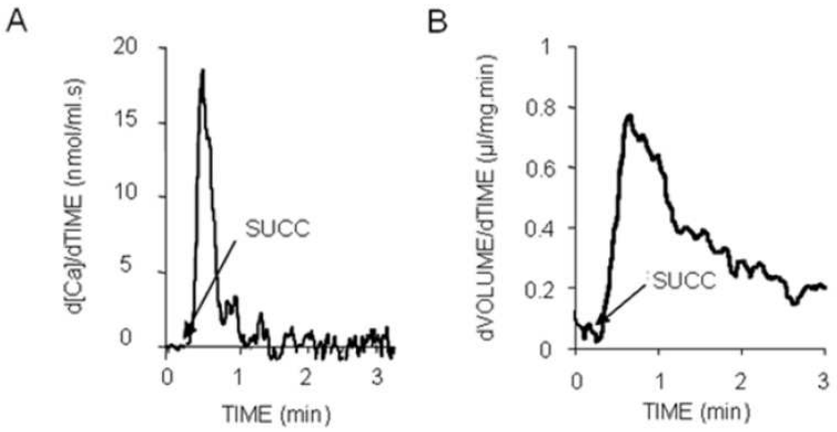

Figure 6. Temporary relationship between the changes in mitochondrial calcium influx and the change in mitochondrial volume. The addition of 5 mM succinate (SUCC) cause a net calcium influx (**A**) and at the same time a gradual change in mitochondrial volume occurs (**B**). Ca^{2+}-induced mitochondrial swelling was determined spectrophotometrically at 520 nm.

This influx subsequently decreases reaching a steady state when equilibrates with efflux (1st derivate = 0) (Figure 6A). A simultaneous gradual change in mitochondrial volume occurs, with a maximum value of 0.432 ± 0.055 µl/mg mitochondrial protein*min at 16.0 ± 1.5 seconds (Table 4). The influx volume then decreases to baseline values (Figure 6B).

The characteristics of this volume influx correspond to those of the MPT pore. As can be seen in Figure 7A, if the oxidation of the succinate is blocked by potassium cyanide (CNK), or if calcium inflow is impeded by ruthenium red, or if the mitochondria are pre-incubated with 2 mM of ADP, change in mitochondrial volume is impeded.

ADP immediately blocks pore formation and could induce pore closing at any time during the process. This can be seen in Figure 7B where the addition of ADP at different time points to a suspension of mitochondria with the pore previously formed and open, closes the pore at any time during the process, causing a volume efflux (1st derivate <0).

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Groups	Mitochondrial Basal Inflow (µL/mg prot.min)	Maximal Inflow (µL/mg prot.min)	Time to Maximal Inflow (seconds)
Control (n=21)	0.057 ± 0.009	0.432 ± 0.055	16.0 ± 1.5
CyA 1 µg/mL (n=17)	0.042 ± 0.007^{NS}	0.235 ± 0.018*	21.1 ± 1.9*
TAC 50 ng/mL (n=20)	0.062 ± 0.013^{NS}	0.405 ± 0.052^{NS}	16.1 ± 1.5^{NS}

Values are means ±SEM. *p<0.05 vs. control. NS: non-significant. CyA, cyclosporin A, TAC, tacrolimus.

Table 4. Characteristics of MPT pore induction after oxidation of succinate 5mM.

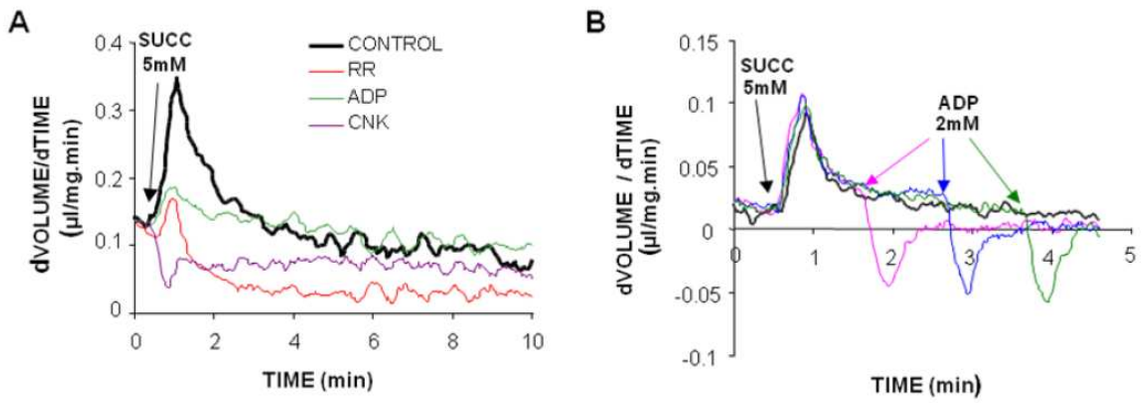

Figure 7. Inhibition of volume influx with well-known blocking of the mitochondrial permeability transition (MPT) pore. (**A**) Volume influx induced by 5 mM succinate (SUCC) is inhibited by 1 µM Ruthenium Red (RR), 1 mM potassium cyanide (CNK) and 2 mM ADP. (**B**) Close of the MPT pore and volume efflux induced by 2 mM ADP addition to a suspension of mitochondria, at different time point. Mitochondrial swelling was determined spectrophotometrically at 520 nm.

When CyA is present, 5 mM succinate addition to a mitochondria suspension causes a significantly reduced inflow through the MPT pore. The mitochondrial swelling observed in the presence of CyA is approximately half of that observed under control conditions (Table 4). It also takes significantly more time to reach its maximum (Table 4) and has an earlier closure (Figure 8A). Same results were obtained using the Met-Ile CyA, an analogue without anti-calcineurin activity (is devoid of immunosuppressive activity), but still capable of binding mitochondrial cyclophilin D (Figure 8A). However TAC did not modify overall kinetics of MPT pore opening (Table 4).

CyA-induced increase in mitochondrial calcium is not followed by a proportional opening of MPT pore, due to CyA dependent inhibition of MPT pore activation. TAC-induced increase in mitochondrial calcium is followed by the expected opening of MPT pore. To verify that volume inflow could be dragging calcium chelating anions into mitochondria, we studied again CyA and TAC effects on mitochondrial calcium in the presence of increasing doses of extramitochondrial phosphate, keeping constant the extramitochondrial calcium concentration. By increasing phosphate availability, the pore opening is followed by a much modest increase in intramitochondrial free calcium (Figure 9).

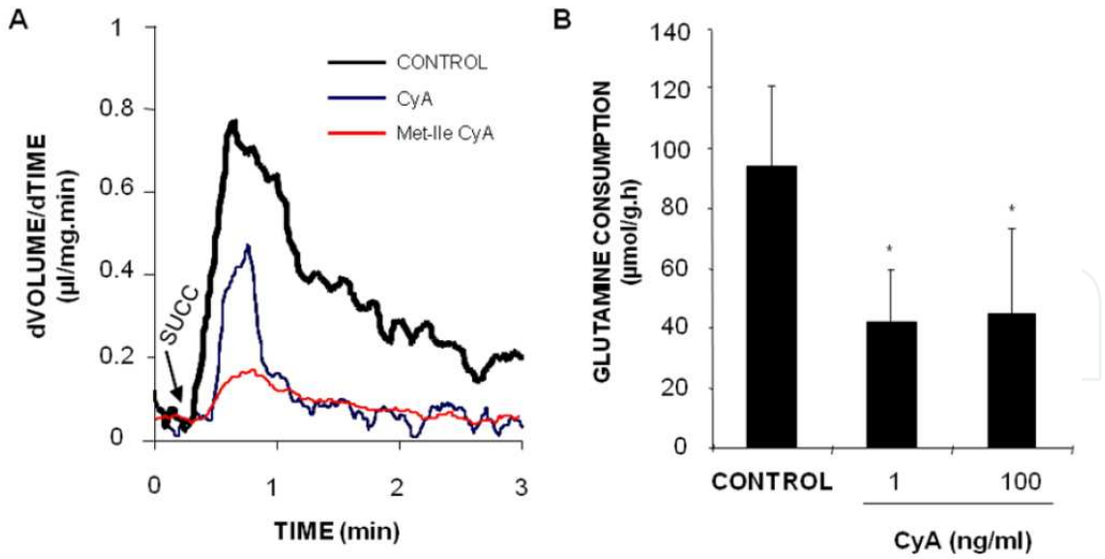

Figure 8. Effect of Cyclosporin A (CyA) on mitochondrial swelling and glutamine consumption. (**A**) In 1 µg/mL CyA presence as well as on Met-Ile CyA analogue, the mitochondrial swelling induced by 5 mM succinate (SUCC) is reduced. Mitochondrial swelling was determined spectrophotometrically at 520 nm. (**B**) CyA inhibited intramitochondrial phosphate-dependent glutaminase activity at 1 ng/mL and 100 ng/mL. Glutamine consumption was determined spectrophotometrically following the NADH production at 340 nm.*p<0.05 vs. control.

MPT pore opening inhibition by CyA will reduce the acute influx of phosphate into the mitochondria, causing free calcium to increase. This finding was indirectly supported when mitochondrial phosphate-dependent glutaminase (a selective enzyme of the proximal tubule mitochondria) activity was studied in intact proximal cells. CyA but not TAC (data not shown) inhibited mitochondrial phosphate-dependent glutaminase activity (Figure 8B).

3.4. Early and delayed MPT pore opening: role in CyA and TAC-induced apoptosis

Following succinate oxidation, opening and closure of MPT pore may be observed during the first minutes. However, if incubation with CyA or TAC is prolonged for 30 minutes more, a second increase in mitochondrial volume is produced (Figure 10) that is independent of any change on oxygen consumption rate. This second spontaneous swelling has some of the MPT characteristic: it can be inhibited by ruthenium red and by ADP. However, in contrast to that observed with the first opening of the pore, this time the change does not appear to be reversible, it is maintained over time and the presence of CyA does not impede or reduce it. This second opening is not observed in control mitochondria.

3.5. Mitochondrial potential and cytochrome c: apoptosis induction

Despite the fact that CyA is capable of a premature closing of the pore activated by succinate oxidation in the absence of ADP, and that TAC does not modify the usual response of the pore to such mechanism, both CyA and TAC cause the spontaneous, delayed opening of the transition pore, loss of cytochrome c to the cytosol (Figure 11B), and the gradual collapse of the mitochondrial potential gradient. In Figure 11A the mitochondrial potential dye, Rhoda-

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mine 123, which only stains the mitochondria when the mitochondrial potential is intact, diffuses out of the mitochondria and stains all the cytosol when the mitochondria is depolarised in the presence of CyA or TAC.

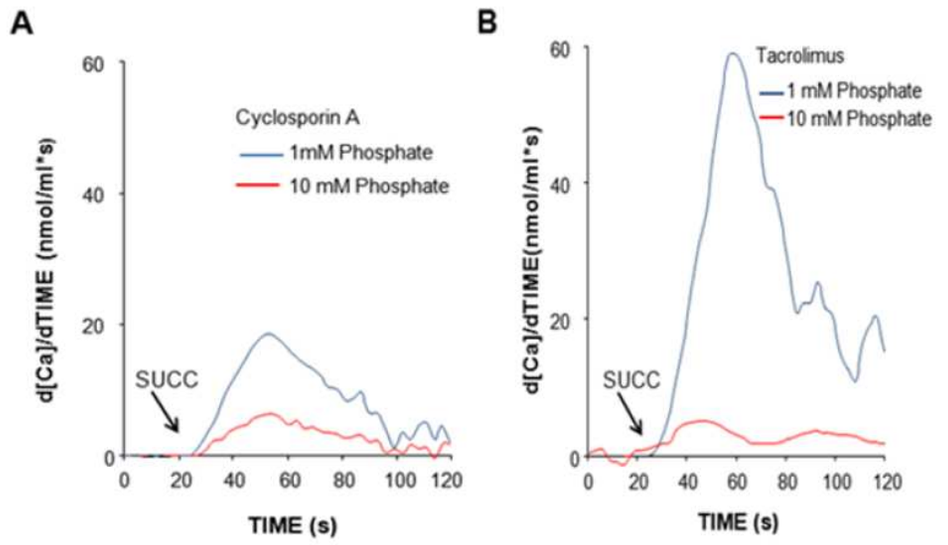

Figure 9. Effect of extramitochondrial phosphate on intramitochondrial calcium. When increasing extramitochondrial phosphate, the increase of intramitochondrial calcium induced by 5 mM succinate (SUCC) is much smaller so much in the presence of 1 µg/mL cyclosporin A (**A**) like of 50 ng/mL tacrolimus (**B**). Mitochondrial suspension was incubated with 5 µM fura-2 and fluorescence was monitored with a fluorometer at 37°C.

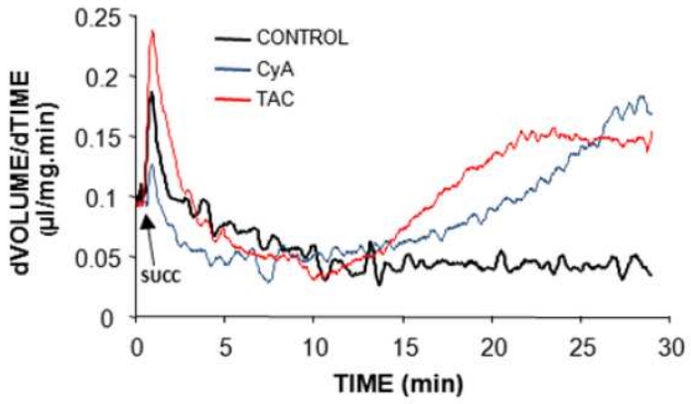

Figure 10. Early and delayed mitochondrial permeability transition pore opening by cyclosporin A (CyA) and tacrolimus (TAC). Incubation of a mitochondrial solution with 1 µg/mL CyA or 50 ng/mL TAC during at least 30 minutes produced a second increase in mitochondrial volume. Succinate-induced mitochondrial swelling was determined spectrophotometrically at 520 nm.

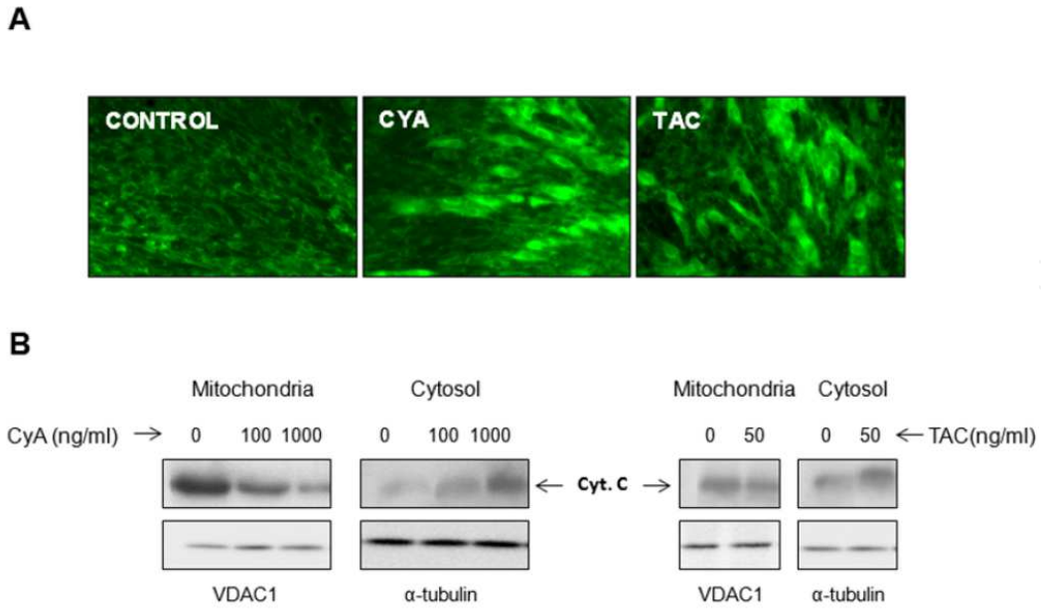

Figure 11. Mitochondrial depolarisation and cytochrome c release induced by Cyclosporin A (CyA) and Tacrolimus (TAC). (**A**) Renal proximal tubular epithelial cells (RPTECs) were treated during 48 hours with 1 µg/mL CyA or 50 ng/mL TAC and incubated with 10 mM Rhodamine 123. Cells were then observed with a fluorescence microscope. Rhodamine 123 diffuses out of the mitochondria and stains all the cytosol when the mitochondria are depolarised. (**B**) Western blot analysis of cytochrome c of cytosolic and mitochondrial fractions of RPTECs treated with CyA and TAC for 48 hours. Voltage-dependent anion-selective channel (VDAC1) and α-tubulin antibodies were used as internal loading controls for mitochondrial and cytosolic fractions respectively.

4. Discussion and conclusion

Anticalcineurinic based immunosupressors have dramatically changed the prognosis spectra of solid and bone marrow transplant. A decade ago, antiapoptotic properties of CyA and TAC on different tissues and animal models allowed to consider these drugs as the final solution in immunosuppression [61]. However, kidney injury showed to be an important drawback in their clinical applications, more dramatic when kidney function was normal prior to heart, liver or bone marrow transplantation. m-TOR inhibitors, the long-time awaited alternative to anticalcineurinics, have not fulfilled all the expectations because of the high incidence of tumors observed. A new look at the mechanisms and potential solutions to anticalcineurinics-induced nephrotoxicity deserves attention. We have discussed elsewhere the renal pathways involved in CyA or TAC apoptosis [1], but it may deserve to look at their effect on MPT pore activity to throw some light on largely described but not explained observations.

Both CyA and TAC cause increase in intramitochondrial calcium, even in the absence of rises in extramitochondrial calcium. But the mechanisms and consequences are different with both.

MPT pore will open as soon as the matrix free calcium reaches a given concentration able to drive the cyclophilin D dependent conformational change in ANT. Free calcium will be driven inside mitochondria when:

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a. The electrochemical gradient has been created,

b. There is extramitochondrial calcium in significant concentrations.

Both conditions take place during kidney trasnplantation when organ is removed from the cold storage and grafted in a donor. In our *in vitro* model, we induced the electrochemical gradient by adding O_2 and succinate to our mitochondria suspensions. Most of the papers previously published on isolated mitochondria, use also extramitochondrial additions of calcium to study the MPT pore. However, this maneuver mimics too closely a near to death cell condition, and makes it difficult to extrapolate conclusions to MPT pore activity under physiological conditions.

We observe the same inhibition of CyA permeates mitochondria and binds cyclophilin D, sparing it from calcium activation, and MPT pore assembly is barely activated. Imported free calcium rises inside the matrix and reaches values well over control values. We observed the same reduction in MPT pore activity with CyA analogous able to bind cyclophilin but unable to block calcineurin. Other authors reported an increased capacity of calcium buffering of CyA-treated mitochondria [24, 27, 62].

CyA is considered as an antiapoptotic factor because of this inhibition of MPT pore activity [26, 27, 63-65]. However, CyA does not inhibit calcium entry into mitrochondria. Crompton proposed MPT pore opening as the mechanism to prevent matrix free calcium accumulation and hypothesized that matrix calcium would be extruded through the pore, creating calcium currents until other cell calcium extrusion mechanisms would finally eject calcium out of the cell [24, 28]. Our results do not support such hypothesis. During pore opening, matrix calcium inflow stops, probably because VDAC is recruited to form the pore itself, but no calcium outflow was observed. Instead, based on our studies at different extramitochondrial phosphate concentrations, it is possible that water entry into mitochondria through the pore will have a "solvent drag" effect on phosphate or another anions able to reach the matrix, combine with free calcium, precipitate it as calcium phosphate granules, and release cyclophilin allowing the pore to close. If this mechanism is true, mitochondria will act as "calcium cleaner", trapping extramitochondrial calcium in order to keep cell calcium low, but with a limited capacity. As mitochondria gets older, more and more microcalcifications would be observed inside matrix and free calcium will progressively rise until opening again the pore, this time in an irreversible way as there is not a chance for free calcium to be precipitated any longer. Different indirect observations support this hypothesis from the early descriptions of calcium stores discovered in mitochondria of damaged tissues, to the mitochondria microcalcifications considered one of the hallmarks of CyA toxicity [66]. Interestingly, as CyA reduces influx into proximal tubule isolated mitochondria, we observe a dose-dependent inhibition of phosphate dependent glutaminase activity, an enzyme that is critical in proximal tubule ammoniagenesis extremely dependent on phosphate supply. It is possible that MPT pore inhibition by CyA is related to the reduced ammoniagenesis described with CyA in clinical and experimental settings.

When CyA or CyA analogues bind cyclophilin matrix free calcium rises faster than in control mitochondria. Although initially there is a transient reduced and delayed MPT pore opening, with time and calcium accumulation overwhelms CyA dependent cyclophilin inhibition, and

a second irreversible type of MPT pore opening takes place. Membrane potential begins to disappear and cytochrome c is released out of mitochondria.

We certainly show that MPT pore opening does not cause calcium entry into mitochondria as suggested by others [25].

Actually, there is another condition requested for MPT pore to open; there should be a short supply of ADP. Other way ANT will be recruited for ADP/ATP exchange and MPT pore will not be opened. We showed it clearly in Figure 7B. In other words, ADP delivery to mitochondria may prevent matrix calcium detoxification by disassembling the pore. Bruce Molitoris showed transient internalization and inactivation of tubule proximal Na^+ pumps during ischemia-reperfusion [67,68]. Our group identified the same phenomenon in porcine models of toxicity with CyA [69]. ATPase inhibition use to be considered as a deleterious process leading to cell death. But at the same time, by inhibiting Na^+K^+ATPase activity ADP delivery to mitochondria is drastically reduced, giving the MPT pore a chance to detoxify mitochondria trapped calcium.

TAC shares with CyA some protective characteristics against apoptosis, as well as the ability to cause proximal tubule cell damage mediated by mitochondria [1, 7]. TAC does not bind cyclophilin, and is not implicated in changes in the normal function of MPT pore. Dynamics of aperture, maximal inflow, and mitochondria swelling is similar to those observed under control conditions.

TAC is able to bind several members of the FK-506 binding protein (FKBP) family; immunophilin homologs located in the outer membrane of mitochondria with chaperone activities on several enzymes. FK-506 binding protein 8 (FKBP-38) interacts with Bcl-2 [70], anchoring it to mitochondria outer membrane where exerts antiapoptotic properties [70]. FKBP-38 acts as a docking molecule to localize Bcl-2 at the mitochondrial membrane [70, 71]. Similar effects have been described for Bcl-XL [71].

However, TAC does not prevent MPT pore from opening. The most intriguing finding we got about TAC interaction with MPT pore was the higher increase in the calcium influx into mitochondria (more than 12 times control influx) when electrochemical gradient was built up by O_2 and succinate addition to mitochondria. Ruthenium Red inhibited such inflow, showing that calcium was imported through the VDAC channel (data not shown). Correspondingly, matrix calcium increase was followed by a 12% increase in O_2 consumption. We could not observe any direct effect on the oxidative activity, phosphorylation or MPT pore activity.

So, only mitochondrial calcium influx seems to be targeted by TAC. Recently several authors described the ability of FKBP-12, another TAC receptor in the outer membrane of mitochondria to modify VDAC ion channel function. This effect is related *in vivo* to the endoplasmic reticulum (ER)-mitochondria calcium signal transmission axis, and it is essential to explain coupling between these two organelles. A subfraction of the ER is localized in isolated mitochondria associated to VDAC (Mitochondria Associated Macromolecular Complex, MAM). Increase in VDAC calcium permeability observed in our experiments with TAC may be a reflection of what is taking place *in vivo* in the ER to mitochondria calcium transfer process.

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Whatever mechanism lies under the relevant calcium influx into mitochondria under TAC treatment MPT pore will be opened, matrix calcium transiently reduced and MPT pore opened again as matrix calcium rises this time in an irreversible way. As with CyA, cytochrome c translocation and membrane potential lost were lately observed. Although both CyA and TAC actions seem to be able to converge into a final pathway of apoptosis and cell death, we have tested if time course activation on intermediate caspases is similar in CyA and TAC-treated proximal tubule cells. Not very surprisingly, time course was different (Figure 12). In CyA, caspases 3 and 9 activation followed a similar time course, but with TAC, caspase 9 activation was early and preceded caspase 3 activation.

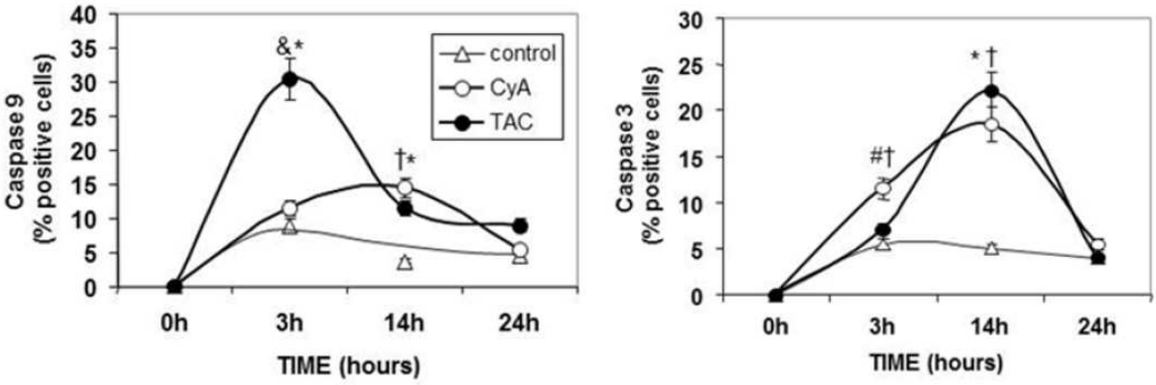

Figure 12. Effect of cyclosporin A (CyA) and tacrolimus (TAC) on time course activation of caspase 9 and caspase 3 in renal proximal tubular epithelial cells (RPTECs). RPTECs were treated along time with 1 µg/mL CyA or 50 ng/mL TAC. The fluorescence label of caspases allows for direct detection and quantification of activated caspases by fluorescence microscopy. Data are represented as the mean ± SEM of four experiments. *p<0.05 TAC vs. CyA treatment; &<0.05 TAC vs. control; †<0.05 CyA vs. control; #p<0.05 CyA vs. TAC treatment.

In summary, MPT pore has been classically considered as a mechanism aimed to cause cell death through mitochondria depolarisation. But MPT pore is only lethal when its activity becomes irreversible. Its physiological role is probably other, playing a role in intramitochondrial free Ca^{++} detoxification during periods of intense cytosolic Ca^{++} scavenging by mitochondria. Therefore, inhibition of the MPT pore opening may have unexpectedly bad results, as it may limit calcium detoxification. Phosphate availability to mitochondria may play a role in injury adaptation when mitochondrial calcium scavenging is necessary. So, these findings provide some insights about a potential physiological role of MPT pore activation in health and disease, a clear and distinct link between ADP production and delivery and mitochondria calcium detoxification capacity, the double-edged sword of CyA inhibition of MPT pore activity and the potential implication of TAC in the ER-mitochondria calcium transfer.

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