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Cyclosporine A-induced apoptosis in renal tubular cells is related to oxidative damage and mitochondrial fission

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ABSTRACT

Cyclosporine A (CsA) nephrotoxicity has been linked to reactive oxygen species (ROS) production in renal cells. We have demonstrated that the antioxidant Vitamin E (Vit E) abolished renal toxicity in vivo and in vitro models. As one of the main sources of intracellular ROS are mitochondria, we studied the effects of CsA on several mitochondrial functions in LLC-PK1 cells. CsA induced ROS synthesis and decreased reduced glutathione (GSH). The drug decreased mitochondrial membrane potential (m) and induced physiological modifications in both the inner (IMM) and the outer mitochondrial membranes (OMM). In the IMM, CsA provoked mitochondrial permeability tran-sition pores (MPTP) and cytochrome c was liberated into the intermembrane space. CsA also induced pore formation in the OMM, allowing that intermembrane space contents can reach cytosol. Furthermore, CsA altered the mitochondrial dynamics, inducing an increase in mitochondrial fission; CsA increased the expression of dynamin related protein 1 (Drp1) that contributes to mitochondrial fission, and decreased the expression of mitofusin 2 (Mfn2) and optic atrophy protein 1 (Opa1), proteins involved in the fusion process. All these phenomena were related to apoptosis. These effects were inhibited when cells were treated with the antioxidant Vit E suggesting that they were mediated by the synthesis of ROS.

Keywords: Cyclosporine, Reactive oxygen species, Mitochondrial fission, Mitochondrial fusión, Apoptosis

Introduction

Cyclosporine A (CsA) is a drug used to treat many autoimmune diseases and in the prevention of transplant rejection. Nephrotoxicity constitutes its main adverse effect and can cause acute or chronic kidney damage (Naesens et al., 2009). Although they have been intensively studied, the intimal cellular mechanisms of CsA nephrotoxicity remain at present not completely elucidated (Parra Cid et al., 2003). Many studies have demonstrated a significant role for reactive oxygen species (ROS). In fact, we have shown that glomeruli of rats treated with CsA increased ROS and lipid peroxidation products (Parra et al., 1998). As the antioxidant Vitamin E (Vit E) avoided kidney failure and inhibited glomerular ROS synthesis, our data suggested that ROS have a pathogenic role in CsA nephrotoxicity (de Arriba et al., 2009; Parra Cid et al., 2003; Parra et al., 1998). These results were confirmed in cellular models, showing that CsA induced ROS synthesis in mesangial or tubular cells and the inhibition of this increase by Vit E (de Arriba et al., 2009; Parra Cid et al., 2003; Parra et al., 1998; Wang and Salahudeen, 1994).

The mitochondrial respiratory chain is responsible for most of the ROS synthesis in cells (Jackson et al., 2002; Orrenius, 2007), and we hypothesized that CsA could induce modifications in mitochondrial physiology which can trigger an increased ROS synthesis. Mitochondria are complex organelles that have an outer (OMM) and an inner membrane (IMM) that folds into a complicate network of tubules and lamellae called cristae (Jackson et al., 2002; Nunnari and Suomalainen, 2012). The IMM is the site of oxidative phosphorylation, and the electron flow through the protein complexes of the respiratory chain is coupled to the exit of protons to the intermembrane space generating a membrane potential (m). ATP synthesis is driven by the flux of protons into the mitochondrial matrix through the ATP synthase and a decrease in m could compromise cellular energy metabolism (Jackson et al., 2002).

Mitochondrial respiration accounts for about 90% of cellular oxygen uptake and 1–2% of the oxygen consumed is converted to ROS (Jackson et al., 2002; Jezek and Hlavata, 2005; Nunnari and Suomalainen, 2012). The main ROS produced by mitochondria is superoxide anion ($O_2^{\bullet -}$), a highly reactive compound. Several cellular antioxidant systems in mitochondria or cytosol inhibited its oxidant capacity (Jackson et al., 2002; Jezek and Hlavata, 2005; Nunnari and Suomalainen, 2012). Among these systems there are antioxidant enzymes like mitochondrial Mn-superoxide dismutase or cytosolic Cu–Zn-superoxide dismutase, glutathione peroxidase and catalase. Also, several non-protein antioxidants can inactivate ROS like glutathione (GSH) or vitamins C and E. The thiol-containing compound GSH acts as the primary cellular homeostatic redox

buffer in cells, and when ROS are produced, the proportion of oxidized glutathione (GSSG) increases, in a reaction mediated by glutathione peroxidase.

CsA increased mitochondrial ROS and this effect could be related to an increase in ROS production, a decrease of the antioxidant systems or both (de Arriba et al., 2009; Parra Cid et al., 2003; Parra et al., 1998). The increase of ROS can induce direct consequences on mitochondrial functions. Besides contributing to cellular energy metabolism, mitochondria participate in several cellular functions like fatty acid synthesis, gluconeogenesis, intracellular calcium control and regulation of cellular apoptosis (Nunnari and Suomalainen, 2012). We and others have demonstrated that CsA induced cellular apoptosis in tubular or endothelial cells (Justo et al., 2003; Perez de Hornedo et al., 2007) and it has been hypothesized that mitochondrial ROS synthesis and apoptosis are two related phenomena. Mitochondrial injury leads to the decrease of m, mitochondrial permeability transition pore (MPTP) formation and release of apoptotic mediators, such as cytochrome c and other proteins to cytosol, caspase activation and cellular apoptosis (de Arriba et al., 2009). As Vit E inhibited these processes, we confirmed that ROS were crucial in CsA mitochondrial effects (de Arriba et al., 2009).

Mitochondria are organelles that form elongated tubules that continuosly divide and fuse to form a dynamic interconnecting network (Berman et al., 2008; Mannella, 2008). The two opposing processes are called fission and fusion and their regulation is dependent on a complex interplay of several proteins (Hoppins et al., 2007; Soubannier and McBride, 2009; Westermann, 2008). Under physiological conditions, mitochondria are elongated. Upon stress stimulation, they become fragmented, and this process may contribute to mitochondrial permeabilization and the release of apoptotic factors (Hoppins et al., 2007; Soubannier and McBride, 2009; Westermann, 2008). However, it is not defined whether CsA induces mitochondrial dynamics modifications and their possible relationship with cellular apoptosis.

Our aim was to analyze the mitochondrial structural and functional alterations that CsA induced in LLC-PK1 cells. We evaluated the role of CsA in ROS synthesis and mitochondrial membrane potential (m), events mainly related to the IMM physiology. We also studied the effect of CsA on OMM, analyzing the influence of CsA on membrane permeabilization. Furthermore, we examined the role of CsA in mitochondrial dynamics and the expression of the main proteins involved in the mechanisms of mitochondrial fission and fusion, and its relationship with cellular apoptosis. Finally, to establish the possible role of ROS in these processes we tested the effect of the antioxidant Vit E.

Materials and methods

Materials

LLC-PK1 cells were obtained from ATCC (Rockville, MD, USA). CsA, ethanol, RPMI 1640 medium, Hank's Balanced salt sodium, bongkrekic acid, ionomycin calcium salt, cobalt (II) chloride hexahydrate ($CoCl_2$), cytochrome c (rabbit monoclonal IgG, C5723), FITC goat anti-rabbit IgG (F0382), anti-actin (A-2066), DMSO (D2650), propidium iodide (PI) (P417), NP-40 (174385), PBS (4417), Triton X100 and RNAase (R7884) were purchased from Sigma–Aldrich (St. Louis, MO, USA). d-l-Alpha-tocopheril acetate (Vit E) came from Roche (Ephynal®, Roche, Basel, Switzerland). Fetal calf serum (FCS) and trypsin EDTA were obtained from
Biochrom (Berlin, Germany). MitoSOX Red, NAO (10-N-nonyl acridine orange), JC-1
(5,5′,6,6-tetrachloro-1,1′,3,3′-tetraethylbenzimidazoly-carbocianine iodide), Mito Tracker Orange MT (MT, M7510), Alexa-fluor 488 antibody anti-cytochrome c mouse IgG, Vybrant apoptosis assay kit#2, AO (acridine orange), 2′,7′ - dichlorofluorescein diacetate (DCFH-DA), monobromobimane (mBBr, M1378) and rhodamine B, hexyl ester, perchlorate (R6) (R648MP) were obtained from Molecular Probes (Leiden, Netherlands). Calcein-AM was purchased from Invitrogen (Paisley, Scotland, United Kingdom).
Goat anti rabbit IgG H&L horseradish peroxidase conjugated and goat anti mouse IgG H&L peroxidase conjugated were a gift from Chemicon International (Tremecula, CA, USA). Acrylamide/bisacrylamide was obtained from Bio-Rad Laboratories (Hercules, CA, USA). Bax-inhibiting peptide, V5, was obtained from Calbiochem (Merck KGaA, Darmstadt, Germany). Antibodies against optic atrophy protein 1 (Opa1), dynamin related protein 1 (Drp1) and mitofusin 2 (Mfn2) were obtained from Santa Cruz Biotechnology Inc (Santa Cruz, CA, USA).
Flow cytometer used was a BD FACSCalibur™ (BD Biosciences, San Diego, CA, USA) and confocal microscope, a laser-scanning Olympus FluoView™ FV1000 (Olympus, Tokyo, Japan).

Cell culture and drug treatment procedures

LLC-PK1 cells were grown in RPMI 1640 10% (v/v) FCS supplemented (2 mM l-glutamine, 2 g/l sodium bicarbonate, 125 g/ml ampicillin, 40 g/ml gentamycin and 125 g/ml cloxacillin) at 37 ◦C and 5% CO_2 atmosphere. When cultures reached a confluence of 80–90%, the medium was replaced with a fresh medium containing 0.5% FCS for 24 h.

CsA was dissolved in ethanol (10^{-2} M stock solution) and in 0.5% RPMI. In previous experiments, we checked that ethanol does not affect the cultures. Vit E and Bax-inhibiting peptide (V5) were dissolved in RPMI (10^{-2} M stock solution) and added to the cultures at a 10^{-4} M final concentration

Preliminary experiments showed that CsA 10^{-6} M altered mitochondrial status without inducing cellular necrosis and this was the dose used in most of our experiments. When needed, cells were pretreated for 30 min with Vit E or V5 at a 10^{-4} M final concentration. CsA was added to achieve 10^{-7}, 10^{-6} or 10^{-5} M concentrations. Cells treated with ethanol (CsA vehicle), Vit E or V5 were used as controls. Cells in a plate were approximately 25,000 per cm^2 (600,000–700,000 in a 6-well and 100,000–150,000 in a 24-well plates). Unless otherwise specified, in all the experiments the cells were treated for 24 h with CsA.

ROS and GSH

DCFH-DA experiments were performed according to the procedure previously published (Bass et al., 1983) and the fluorescence was determined at 560 nm. Cells were incubated for 45 min at 37 ◦C with 5 M DCFH-DA and washed twice. The fluorescence intensity was visualized and measured by confocal microscopy, and results were expressed in arbitrary mean fluorescence intensity units per cell (MFI/cell).

mBBr is a nonfluorescent thiol-derivatization reagent that passively diffuses across the plasma membrane into the cytoplasm where it forms blue-fluorescent adducts with intracellular GSH, thiol-containing proteins and carboxylic acids. GSH depletion in CsA-treated cells was assayed with mBBr. Briefly, LLCPK1 were incubated with 2 M mBBr at 37 ◦C and 5% CO_2 for 10 min. After washing cells twice, staining was observed by confocal microscopy after lighting with a UV laser (405 nm) and detected using a band-pass filter centered at 490 nm.

Analysis of mitochondrial permeability by calcein-AM

Cells were incubated with 1 M calcein-AM that diffuses freely into cytosol, for 20 min. Cytosolic endogenous esterases cleave the ester group of calcein-AM, impeding its movement out of the cell, but allowing its free diffusion into mitochondria. The calcein fluorescence is quenched by $CoCl_2$ (0.1 M for 20 min), which is unable to penetrate into mitochondria unless mitochondrial pores are opened (de Arriba et al., 2009).

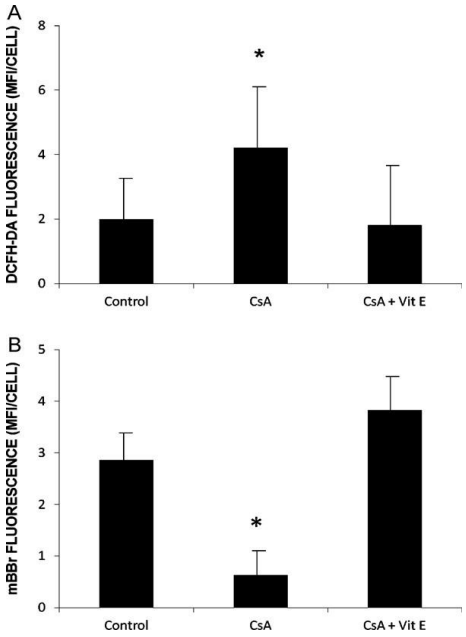

Fig. 1. Effects of CsA and Vit E on intracellular ROS and reduced glutathione (GSH). (A) DCFH-DA fluorescence increased in cells treated with CsA (10^{-6} M) for 24h with respect to control cells or cells pretreated with Vit E (10^{-4} M) before CsA (10^{-6} M) *p <0.05. (B) CsA (10^{-6} M) incubation of cells for 24h decreased GSH measured as monobromomimane (mBBr) fluorescence. Control or Vit E pretreated cells have similar fluorescence. *p < 0.05.

Measurement of the m by JC-1

LLC-PK1 cells were stained with 2 M JC-1 diluted in RPMI 0.5% FCS for 15 min at 37 ◦C and 5% CO_2, washed twice and analyzed by confocal microscopy as previously described (de Arriba et al., 2009).

FRET (fluorescence resonance energy transfer) NAO-MT

FRET occurs when the emission spectrum of one fluorophore (donor) overlaps with the excitation spectrum of a second fluorophore (acceptor), and the energy from excitation of the donor is transferred to the acceptor through quantum effects (Poot and Pierce, 1999). As a result, the emission by the donor decreases and consequently, the emission by the acceptor increases. The FRET efficiency depends on the inverse sixth-distance between the two fluorophores. We analyzed the interactions between CL and thiol-proteins in the IMM using a double label with NAO and MT green. The efficiency of FRET (FE) was calculated by dividing the MT red fluorescence by NAO green fluorescence.

Analysis of cellular cycle and apoptosis by PI

LLC-PK1 cells were incubated for 24 h with CsA. Supernatants were collected and cells were detached. After centrifugation (1200 rpm for 5 min at 4 ◦C), the cellular pellet was resuspended thoroughly in PBS (500 L) with 1% NP-40 and

0.5 mg/ml RNAase and incubated 30 min at room temperature. PI was added and cells were incubated for 15 min, washed twice with PBS and analyzed by flow cytometry.

Subcellular distribution of cytochrome c

Cells were grown in 6-well plates. After treatment with CsA (6 or 24 h) they were fixed in methanol 5 min at −20 ◦C. The supernatant was aspirated and 0.5% Triton X-100 in PBS was added. After 15 min, the supernatant was aspirated again and the cells were washed three times with a blocking buffer (2% FCS in PBS) following by an incubation with this buffer for 1 h. The cells were incubated overnight with the antibody anti-cytochrome c, washed three times, and incubated for 2 h with the secondary antibody FITC anti mouse IgG (1:200) at room temperature. R6 is an orange-red fluorescent dye (excitation/emission of ∼528/551 nm) that only is captured by active mitochondria and was added at 50 mM and cells were incubated for 20 min at 37 ◦C. The dual labeling was visualized using suitable lasers and detectors. The antibody was visualized by confocal microscopy (488 nm laser line/580 nm band-pass filter).

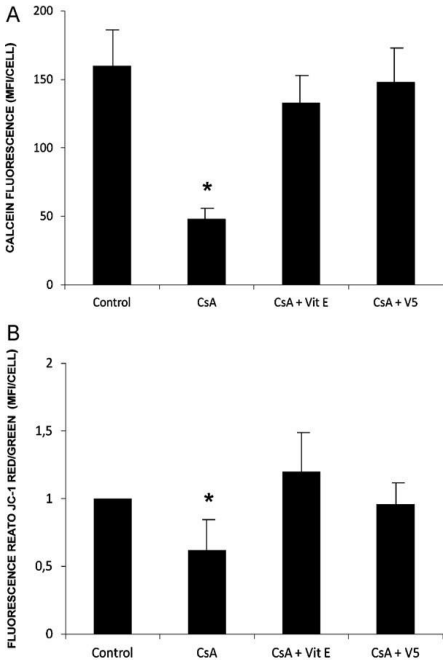

Fig. 2. Effects of CsA. (A) Calcein fluorescence in control cells, CsA-treated cells for 24h and cells supplemented with Vit E or V5 before CsA treatment (10^{-6} M). *$p < 0.05$. (B) Experiments with JC-1 showing the ratio between red and green fluorescences which is an index of m. After CsA treatment for 24h (10^{-6} M), JC-1 emitted predominantly green fluorescence because the dye was in monomeric form as a consequence of the diminished ΔΨm.

Analysis of Drp1, Opa1 and Mfn2 by Western blot

Proteins were processed by SDS–PAGE and transferred to nitrocellulose membranes overnight at 4 ◦C. After blocking, membranes were incubated with the primary antibody (anti-Opa1, anti Drp1 or anti Mfn2) and then with the secondary antibody.

Protein bands were visualized using chemiluminescence reagents (ImmunoStar HRP substrate kit, BioRad, CA, USA) and exposed to autoradiographic film (Kodak, New York, USA). Densitometric evaluation of the films was performed using the program Scion Image Ver. Alpha 4.0.3.2 (Frederice, MD, USA).

Statistical analysis

All experiments were performed at least five times, except FRET experiments with confocal microscopy (which were performed in triplicate). Results were expressed as mean ± standard deviation. They were studied by analysis of variance with Scheffé correction in group comparisons or Kruskal–Wallis test (SPSS 15.0 software, SPSS Inc., Chicago, IL, USA). The level of significance was set at $p \leq 0.05$.

Results

CsA increased cellular ROS and decreased reduced GSH, which is prevented by Vit E in LLC-PK1 cells

Our experiments with confocal microscopy showed that DCFH-DA fluorescence increased in cells treated with CsA respect to control cells. However, cells pretreated with Vit E emitted less fluorescence than control cells (Fig. 1A). The amount of GSH measured as mBBr fluorescence, was higher in control cells than in CsA-treated cells. Vit E preincubation inhibited the fluorescence intensity decrease induced by CsA (Fig. 1B).

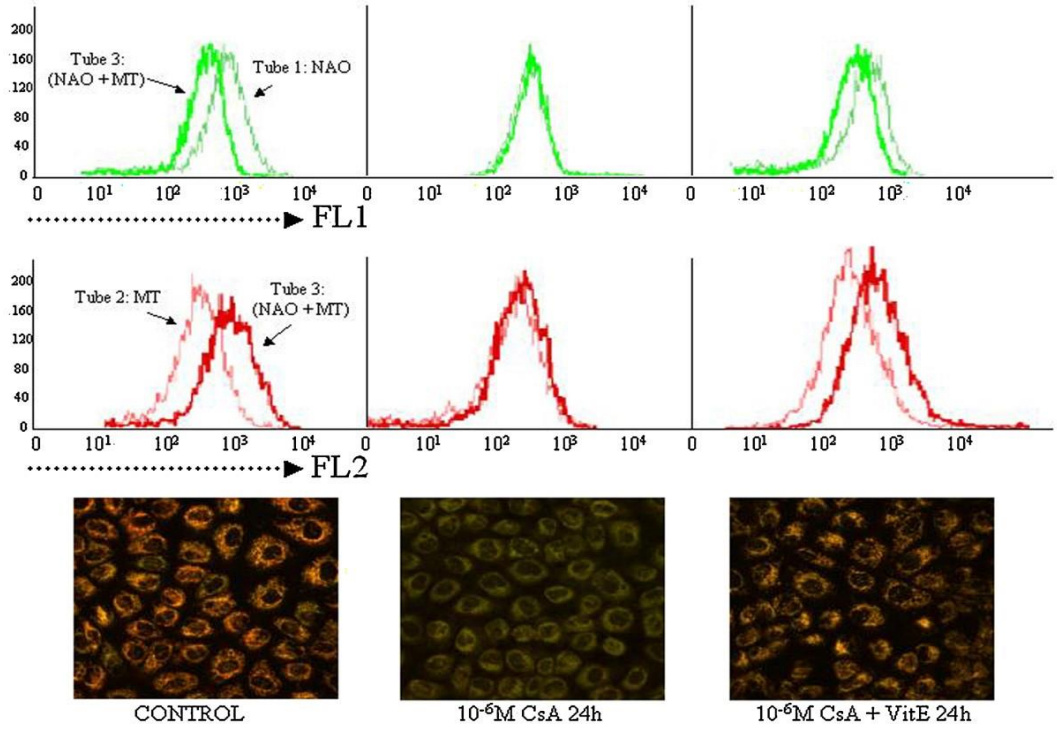

Fig. 3. FRET in control cells, cells treated with CsA 10^{-6} M for 24h, and cells treated with Vit E and CsA. Upper panel: representative flow cytometry experiment showed that NAO green fluorescence decreased in presence of MT due to a quenching phenomenon. MT red fluorescence increased in control cells suggesting that FRET was taking place. Vit E pretreated cells showed similar results. However, CsA treated cells had similar green and red fluorescence intensity in tubes with individual or double labeling, suggesting that FRET did not occur. Lower panel: confocal microscope images of a typical experiment showed that CsA treated cells have lost MT red fluorescence respect to control cells because in these cells FRET did not occur. Vit E pretreated cells have intermediate red fluorescence values.

CsA allowed the opening of mitochondrial pores

The experiments with confocal microscope showed that CsA decreased mitochondrial calcein fluorescence compared to control, Vit E or V5 pretreated cells (Fig. 2A). When we used ionomycin (500 M) as a positive control of pore opening, cells had similar fluorescence than cells treated with the highest concentrations of CsA (10^{-5} M) (data not shown). Bongkreic acid (50M) is a specific inhibitor of MPTP and cellular cultures simultaneously treated with CsA (10^{-6} and 10^{-5} M of CsA) and bongkreic acid had similar fluorescence values that control cells (data not shown).

CsA decreased ΔΨm.

Our experiments with confocal microscopy showed that CsA decreased the ratio between JC-1 red and green fluorescences respect to control cells (normalyzed ratio = 1). Vit E or the Baxinhibiting peptide V5 pretreatment of cells avoided this decrease indicating that both were able to preserve m (Fig. 2B).

CsA decreased FRET between NAO and MT and Vit E avoided this effect

Flow cytometry and confocal microscopy experiments showed that in control cells green fluorescence emitted by NAO decreased when cells were simultaneously incubated with NAO and MT. This event happened because MT is quenching NAO green emission. However, the red fluorescence emitted by MT increased (0.43 ± 0.27 MFI/cell) when cells were simultaneously incubated with the two fluorochromes. This finding indicated that FRET happened. However, our results obtained in CsA-treated cells showed that there was not transfer of energy between NAO and MT since the emissions in the tubes with individual dyes, were similar to the emission in tube with double dye (0.12 ± 0.04 MFI/cell, $p < 0.05$ versus control). This indicated that FRET was not taking place. The absence of energy transfer suggested that mitochondrial membranes have lost their normal structure. When cells were pretreated with Vit E before CsA, the results obtained were similar than results obtained in control cells (0.34 ± 0.22 MFI/cell). These data suggested that the antioxidant maintained both CL and proteins in close proximity in the mitochondrial membrane (Fig. 3).

CsA induced cytochrome c release from mitochondria to cytoplasm and Vit E inhibited this effect

Confocal microscope images showed that in control cells the mitochondria were filamentous with a tubular or thread-like appearance (stained with R6, red fluorescence) and cytochrome c was mainly located inside them (stained with antibody anticytochrome c and a secondary antibody-FITC, green fluorescence). CsA-treated cells for 6 h showed a decrease in planar cross-sectional area and in cellular confluence. Their mitochondrial network broke and mitochondria were fragmented into short rods and cytochrome c remained into mitochondria. However, when cells were treated with CsA for 24 h the cytochrome c fluorescence appeared scattered and mainly distributed in cytosolic space. The appearance of cells pretreated with Vit E was similar to control cells (Fig. 4).

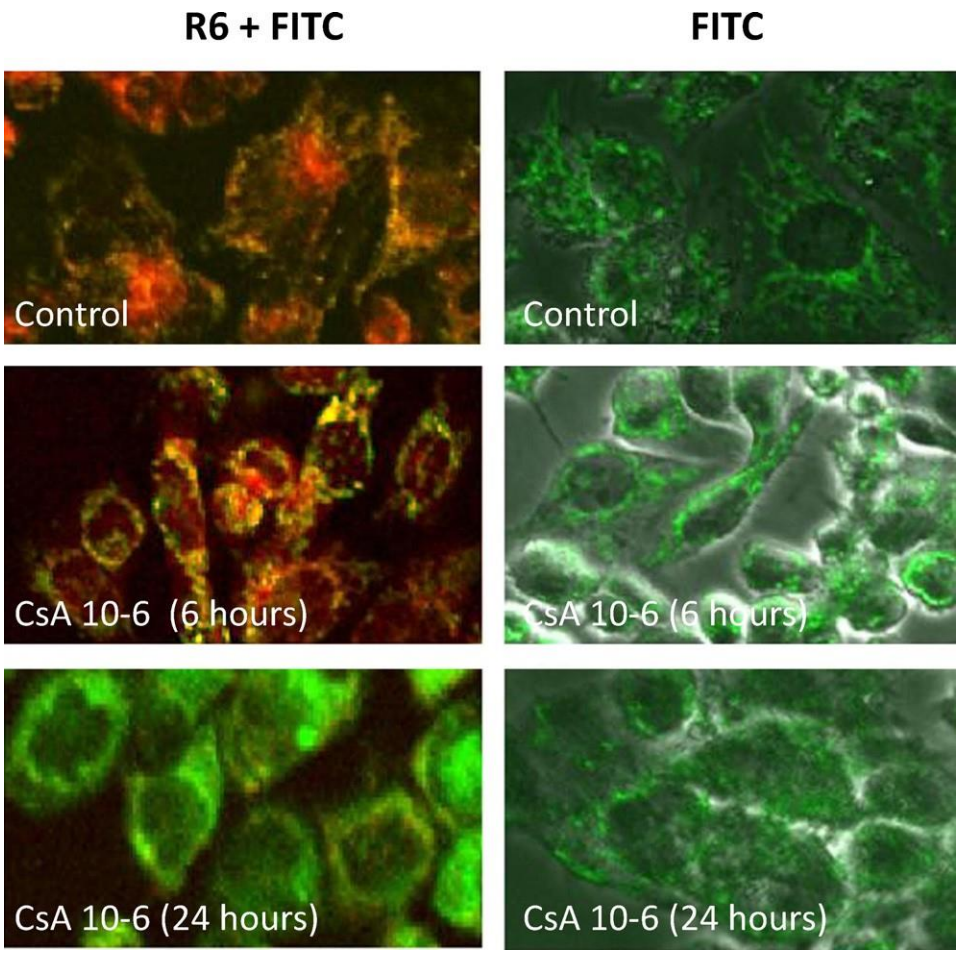

Fig. 4. Confocal mycroscopy images of an experiment with Rhodamine 6 – R6 (red fluorescence) to visualize mitochondria and antibody anti-cytochrome c and secondary antibody anti-IgG-FITC (green fluorescence). Left-hand images show double dye and right-hand images show FITC dye. Upper panel (control cells): the orange fluorescence is a consequence of the fluorescences colocalization and this indicated that citochrome c was inside mitochondria. The cells presented the mitochondrial tubular pattern typical of viable cells. Intermediate panel (CsA-treated cells for 6h 10^{-6} M): left-hand image shows that cytochrome c remained into mitochondria because both fluorochromes colocalize, but the linear mitochondrial pattern has changed and mitochondria were perinuclear and have a rounded appearance (right-hand image). Lower panel (CsA-treated cells for 24h 10^{-6} M): cytochrome c is distributed in cytosolic space and green diffuse staining was seen; the mitochondria were swollen suggesting cellular apoptosis.

Effects of CsA and Vit E on Drp1, Opa1 and Mfn2 protein expression

Western blot revealed that CsA increased Drp1 protein in LLC-PK1 cells. This effect was parallel to the decrease in the amount of Opa1 and Mfn2. These modifications were dose-dependent. Vit E pre-treatment avoided both the increase in Drp1 and the decrease in Opa1 and Mfn2 (Fig. 5A).

Vit E and V5 avoided cellular apoptosis induced by CsA

Cycle cellular analysis allowed the study of DNA content in LLC-PK1 cultures. Our results showed a dose-dependent increase in the number of apoptotic cells treated with CsA. Pretreatment of cells with Vit E or V5 before adding CsA decreased the percentage of apoptotic cells that was similar to control cells (Fig. 5B).

Discussion

CsA increases ROS production in several cellular models, modifying the oxidative balance, decreasing antioxidant levels and inducing lipid peroxidation (Parra Cid et al., 2003; RedondoHorcajo et al., 2010). These alterations were related to cellular apoptosis. Treatment of cells with antioxidants inhibited ROS production and apoptosis suggesting that both phenomena are associated (de Arriba et al., 2009; Parra Cid et al., 2003).

The immediate consequence of ROS overproduction is the decrease of antioxidant levels as we have demonstrated concerning GSH. This fact may pose cells in a situation more vulnerable to oxidative damage. Another consequence of mitochondrial ROS increase is the activation of MPTPs (Brady et al., 2004; Zoratti and Szabo, 1995). We have demonstrated that CsA induced MPTP in renal tubular cells (de Arriba et al., 2009). The opening of MPTP induced m collapse mediated by the loss of the electronic gradient in the respiratory mitochondrial chain (Brady et al., 2004; Zoratti and Szabo, 1995). There are data showing that CsA is an inhibitor of the MPTP (Bernardi, 1996; Crompton, 1999) and that CsA causes accumulation of Ca^{2+} into mitochondria (Fournier et al.,1987). However, accumulating evidence suggests that MPTP have two open conductance models, regulated and unregulated (He and Lemasters, 2002; Kim et al., 2003; Yamada et al., 2003). The opening of regulated pores is activated by Ca^{2+} and inhibited by Mg^{2+} and CsA. However, the conductance through unregulated pores occurs independently of Ca^{2+} activation and it is not inhibited by CsA. It has been suggested that oxidative stress may cause alterations of thiol groups of membrane proteins that originate misfolding and clustering of these proteins and opening of membrane pores (He and Lemasters, 2002). Furthermore, many experiments analyzing

mitochondrial effects of CsA have been performed in isolated mitochondria and results can be different in whole cell because of the possible influence of other cytosolic factors (Galindo et al., 2003). In summary, our data have confirmed that CsA altered the IMM physiology determining ROS increase, MPTP formation and decrease of ΔΨm.

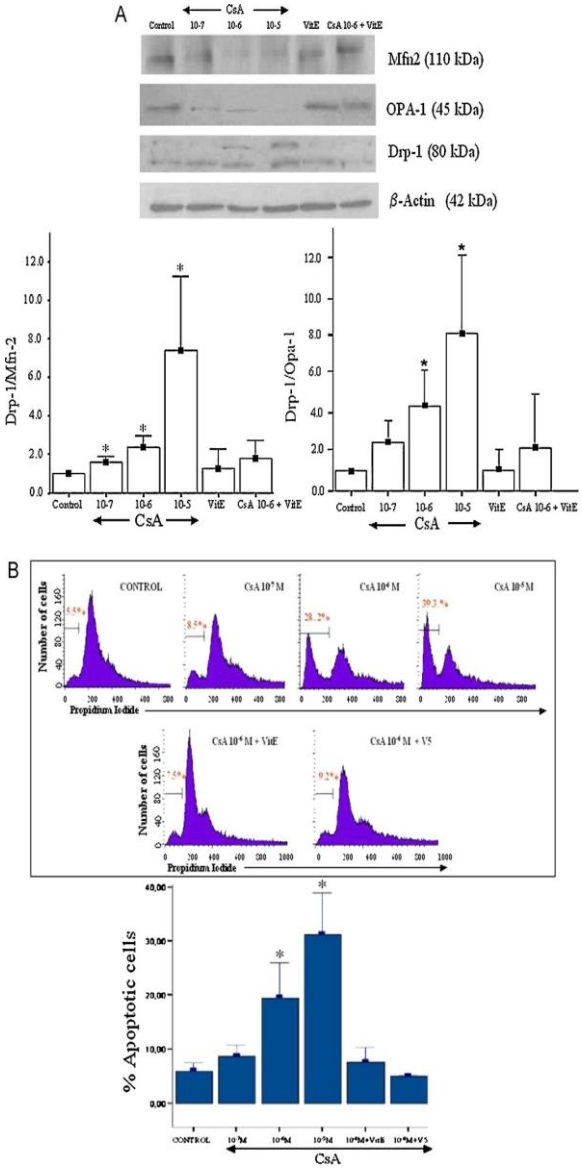

Fig. 5. (A) Western blot for Drp1, Opa1 and Mfn2. Cells were treated with CsA 10^{-6} M for 24h. Upper panel: Western blot of a representative experiment. Lower panel: mean values obtained in 5 experiments. Results are expressed as a ratio between the fission protein Drp1 and the fusion proteins Opa1 and Mfn2 respectively (*$p < 0.05$). (B) Flow cytometry experiments studying cellular cycle in LLC-PK1 cells treated with CsA 10^{-6} M for 24h. Upper panel: typical experiment that showed the cellular DNA content. Apoptotic cells were identified in the fraction sub-Go. Lower panel: mean of 5 experiments showed the percentage of apoptotic cells in control cells, CsA treated cells and cells pretreated with Vit E or V5 before adding CsA.

As the main mitochondrial ROS generators are located in the IMM, we studied the structural modifications induced by CsA on IMM. Our FRET experiments proved that CsA disarranged IMM, as it was shown by the decrease in energy transfer between NAO and MT. One of the main structural components of IMM is cardiolipin (CL). As we have previously shown, CsA induced CL oxidation in the IMM (de Arriba et al., 2009). This fact may have several consequences. First, the oxidation of CL can alter the IMM fluidity and modify its interaction with other membrane components. This phospholipid is also required to maintain an adequate function of the respiratory chain enzymes (McMillin and Dowhan, 2002). Furthermore, it is well known that CL has an intimate contact with cytochrome c which is mainly bound to CL in the IMM. When CL is oxidized, the binding of cytochrome c and CL disappears and cytochrome c is liberated into the intermembrane space (Iverson and Orrenius, 2004; McMillin and Dowhan, 2002; Perkins et al., 2009; Wiswedel et al., 2010). Also, it has been shown that ROS can oxidize other proteins like the complexes of the mitochondrial respiratory chain and ATP synthase (Jackson et al., 2002; Paradies et al., 2002), decreasing ATP synthesis and increasing mitochondrial ROS production.

Besides its effects on IMM, experimental data suggest that CsA also modified OMM contributing to cellular apoptosis mediated through the intrinsic pathway. A key element in the mitochondrialinduced apoptosis is the permeabilization of the OMM, which is regulated by several proteins belonging to bcl-2 family. The main proteins are Bax and Bak, which are counteregulated by other proteins of the same family (Lovell et al., 2008). In normal healthy cells, Bax is a cytosolic monomeric protein; however, once activated, Bax translocates to mitochondria, it is inserted into the OMM and oligomerizes to form large channels (Kinnally and Antonsson, 2007). These pores allow the liberation of the contents from the intermembrane space to cytosol, including several proapoptotic proteins like cytochrome c, Smac/Diablo, endonuclease G and the apoptosis inducing factor AIF (Brady et al., 2004; Zoratti and Szabo, 1995). We have demonstrated that after CsA treatment, the protein Bax increased in the mitochondrial and decreased in the cytosolic fraction (de Arriba et al., 2009). Furthermore, when cells were treated with the Bax inhibitor V5, the pore formation was inhibited, suggesting the direct involvement of Bax. Also V5 was able to inhibit de decrease of m induced by CsA, indicating that Bax also influences mitochondrial m. Finally, V5 inhibited CsA-mediated cellular apoptosis, supporting indirectly that pore formation in OMM is a relevant event in this process.

Preliminary data suggest the existence of a relationship between cellular apoptosis and intramitochondrial dynamics. Mitochondrial fission depends on Drp1, a cytosolic protein with GTPase activity that traslocate to OMM in response to several stimuli (Lee et al., 2004; Taguchi et al., 2007). Drp1 assembles in spirals around the fission sites in OMM, where it colocalizes with Mfn2 and Bax.

It is considered that these foci are constriction sites that separate elongated mitochondria into several shorter units during the process of mitochondrial fission (Karbowski et al., 2002, 2006). However, some authors argue that although mitochondrial fission and apoptosis can coexist, it remains unsolved whether mitochondrial fission has an impact on the rate of apoptosis or merely accompanies apoptosis as an epiphenomenon (Wu et al., 2011).

Mitochondrial fusion is a complex mechanism that involves several proteins and implies the joining of IMM and OMM of adjacent mitochondria. The mechanisms are not well defined, but Opa1 seems to participate in IMM fusion and Mfn1 and Mfn2 are involved in OMM fusion (Hoppins and Nunnari, 2009; Sheridan and Martin, 2010). Opa1 is located in the outer surface of the IMM throughout both inner boundary and cristae membranes and plays a role not only in mitochondrial fusion, but also in the maintenance of normal IMM architecture (Hoppins and Nunnari, 2009; Sheridan and Martin, 2010). Recent evidences show that Opa1 molecules are also placed in the base of cristae, locking the cristae and avoiding that their contents like cytochrome c could reach the intermembrane space (Brooks et al., 2009; Ulivieri, 2010; Yamaguchi and Perkins, 2009). Stimuli like intramitochondrial ROS production, can provoke the phenomenon of cristae remodeling characterized by the fusion of cristae and widening of the narrow cristae junctions, contributing to the liberation these contents to the intermembrane space (Brooks et al., 2009; Sheridan and Martin, 2010; Ulivieri, 2010; Yamaguchi and Perkins, 2009). Mfn1 and Mfn2 are inserted in OMM and their roles seem to be complementary: Mfn1 has a core role in mitochondrial docking and fusion, while Mfn2 could participate in the stabilization of the interaction between two adjacent mitochondria (Chan, 2006; Westermann, 2008). It has been suggested that fusion facilitate communication among different parts of the cell and transfer of contents along the mitochondrial chains.

Our experiments have shown that CsA inhibited fusion and induced mitochondrial fission simultaneously to ROS synthesis, mitochondrial pore formation and cellular apoptosis. When we analyzed the expression of several proteins involved in mitochondrial dynamics, we observed that CsA treated cells have a dose-dependent increased expression of Drp1 and decreased expression of the fusion proteins Mfn2 and Opa1. These modifications were inhibited when cells were treated with the antioxidant Vit E, suggesting a significant role of ROS in these processes.

Finally, CsA induced cellular apoptosis in LLC-PK1 cells in a dose and time-dependent manner. Treatment of cells with the Bax inhibitor V5 inhibited apoptosis, supporting that Bax, through pore formation in OMM may play a determinant role in cellular apoptosis induced by CsA. Furthermore, the inhibition of apoptosis by Vit E, suggest also a relevant role of mitochondrial ROS.

Conclusion

Our data support that CsA modifies mitochondrial structure and function through the synthesis of ROS (Fig. 6). CsA altered IMM provoking CL oxidation and decrease in the expression of Opa1, originating cristae remodeling and facilitating the liberation of cytochrome c into the intermembrane space. CsA also induces MPTP, with the consequent decrease of m. CsA also modified the OMM causing Bax-induced pore formation that mediates the liberation of several proteins involved in apoptosis like cytochrome c from the intermembrane space to cytosol. All these phenomena were parallel to mitochondrial fission and cellular apoptosis. As Vit E was able to inhibit all these morphological and functional modifications that CsA induced in LLC-PK1 cells, our data support a determinant role of ROS on mitochondrial induced apoptosisin this model.

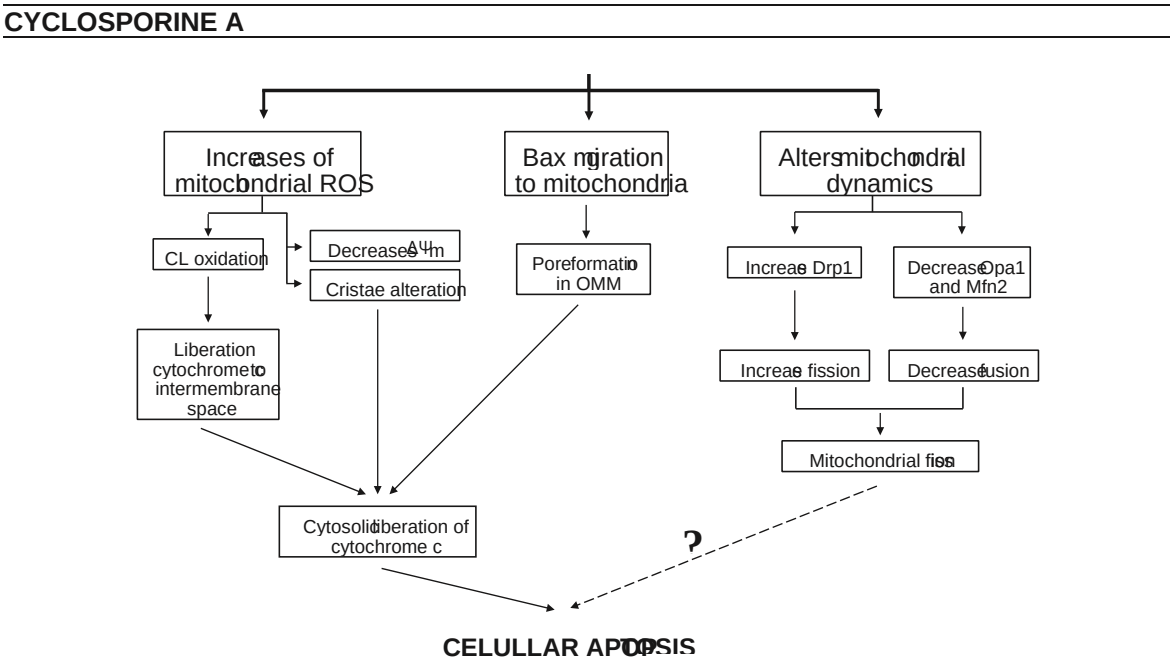

Fig. 6. Hypothesis theory explaining the possible mitochondrial effects of CsA. CsA modifies several mitochondrial functions in LLC-PK1 cells. The key points are ROS synthesis, pore formation and modifications in mitochondrial dynamics with increasing fission and decreased fusion. Some aspects of this diagram remain to be defined, particularly the possible relationship between mitochondrial fission and cellular apoptosis.

Conflict of interest statement

All authors declare that there are no conflicts of interest in this study.

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