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Vitamin E protects against the mitochondrial damage caused by cyclosporin A in LLC-PK1 cells

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Cyclosporin A (CsA) has nephrotoxic effects known to involve reactive oxygen species (ROS), since antioxidants prevent the kidney damage induced by this drug. Given that mitochondria are among the main sources of intracellular ROS, the aims of our study were to examine the mitochondrial effects of CsA in the porcine renal endothelial cell line LLC-PK1 and the influence of the antioxidant Vitamin E (Vit E). Following the treatment of LLC-PK1 cells with CsA, we assessed the mitochondrial synthesis of superoxideanion, permeability transition pore opening, mitochondrial membrane potential, cardiolipin peroxidation, cytochrome c release and cellular apoptosis, using flow cytometry and confocal microscopy procedures. Similar experiments were done after Vit E preincubation of cells.

CsA treatment increased superoxide anion in a dose-dependent way. CsA opened the permeability transition pores, caused Bax migration to mitochondria, and decreased mitochondrial membrane potential and cardiolipin content. Also CsA released cytochrome c into cytosol and provoked cellular apoptosis. Vit E pretreatment inhibited the effects that CsA induced on mitochondrial structure and function in LLC-PK1 cells and avoided apoptosis.

CsA modifies mitochondrial LLC-PK1 cell physiology with loss of negative electrochemical gradient across the inner mitochondrial membrane and increased lipid peroxidation. These features are related to apoptosis and can explain the cellular damage that CsA induces. As Vit E inhibited these effects, our results suggest that they were mediated by an increase in ROS production by mitochondria.

Introduction

Cyclosporin A (CsA) is a widely used drug that has led to improved patient and graft survival in transplant patients and has also provided significant clinical benefits in the management of many diseases (Calne et al., 1978; Langford et al., 1998a, 1998b). The clinical use of CsA is, however, limited by acute and chronic nephrotoxicity (Mason, 1990; de Mattos et al., 2000). As antioxidant therapy attenuates CsA nephrotoxicity, it has been speculated that toxicity could be mediated by reactive oxygen species (ROS) (Parra et al.,1998a,1998b; Jenkins et al., 2001; Padi and Chopra, 2002; Parra Cid et al., 2003).

In previous studies, we demonstrated that CsA induced ROS synthesis in isolated rat kidney glomeruli and mesangial cells (Perez de Lema et al., 1997; Parra et al., 1998b; Parra Cid et al., 2003) and we found that this effect was dose and time-dependent and was abolished by exogenous antioxidants such as Vit E (Parra et al., 1998b). However, although glomerular changes can be seen after CsA treatment, tubulointerstitial damage in kidney biopsies of patients with CsA nephrotoxicity usually determines long-term prognosis (Laine and Holmberg, 1996).

We also demonstrated that CsA increased renal lipid peroxidation and the urinary excretion and glomerular synthesis of Thromboxane (Parra Cid et al., 2003). It was hypothesized that CsA-induced ROS can attack cellular membrane lipids that lead to the increased synthesis of vasoconstrictors that mediate nephrotoxicity (Coffman et al., 1987; Parra et al., 1998a).

Several investigations have shown that CsA increased the synthesis of mitochondrial ROS (Galindo et al., 2003; Montero et al., 2004). However, the mechanisms involved are not well defined (Parra Cid et al., 2003). ROS are produced continuously by the mitochondrial respiratory chain and can attack nearby molecules such as proteins and lipids (Justo et al., 2003; Orrenius et al., 2007). When ROS generation overcomes the neutralizing capacity of antioxidant systems, the oxidation of thiol groups of proteins and other molecules can lead to mitochondrial dysfunction (Kowaltowski et al., 1998). In particular, cardiolipin oxidation may have important mitochondrial structural and functional consequences, because cardiolipin is the main lipid of mitochondrial inner membrane and it is related to proteins involved in energy metabolism and electron transfer complexes (McMillin and Dowhan, 2002).

It is known that mitochondria play a central role in cellular apoptosis (Desagher and Martinou, 2000; Green and Kroemer, 2004). Two main pathways for apoptosis have been defined: the extrinsic pathway that results from activation of death receptors, such as Fas, and the intrinsic pathway that may result from mitochondrial stress (Desagher and Martinou, 2000; Green and Kroemer, 2004).

A crucial role for mitochondrial-mediated apoptosis has been attributed to permeability transition pore (PTP) opening (Crompton, 1999; Kim et al., 2003). In healthy cells, the inner mitochondrial membrane is responsible for the maintenance of electrochemical gradient, essential for respiration and energy production. In this process, calcium is taken up and a low conductance permeability transition pore appears to flicker between open and close states. In situations of calcium overload or increased levels of ROS a continuous opening of PTP alters the permeability of mitochondrial membrane, allowing the release of proapoptotic factors to cytosol that can initiate apoptosis (Crompton, 1999; Kim et al., 2003).

Another mechanism that allows the release of factors from intermembrane space is independent of calcium and involves the permeabilization of the outer mitochondrial membrane by proapoptotic Bcl-2 family proteins, such as Bax and Bak (Jurgensmeier et al., 1998; Wei et al., 2001; Iverson and Orrenius, 2004).

A further feature associated with the mitochondrial alterations caused by PTP opening is the loss of negative electrochemidal gradient across the inner mitochondrial membrane that gives rise to the mitochondrial transmembrane potential ($\Delta\Psi m$). Dissipation of the $\Delta\Psi m$ renders a depolarized membrane and modifies mitochondrial function, increasing the synthesis of ROS and decreasing ATP synthesis, events often observed in the early stages of apoptosis (Kakkar and Singh, 2007; Kroemer et al., 2007; Rasola and Bernardi, 2007).

The aim of the present study was to establish the effects of CsA on several mitochondrial functions and their relationships with the apoptotic status in LLC-PK1 cells. As Vit E can reverse CsA nephrotoxicity in animal models, we also tested the effect of Vit E pretreatment on the mitochondrial changes induced by CsA in these cells.

Materials and methods

Materials

LLC-PK1 cells were obtained from ATCC (Rockville, MD, USA). Cyclosporin A, ethanol, RPMI 1640 medium, Hank's Balanced Salt Sodium, bongkrekic acid, ionomycin calcium salt, cobalt (II) chloride hexahydrate and antibodies anti-VDAC/Porin (rabbit polyclonal IgG) were purchased from Sigma-Aldrich (St. Louis, MO, USA). D-L Alphatocopherylacetate (Vit E) wasobtained from Roche(Basel, Switzerland). Foetal calf serum (FCS) and trypsin EDTA solution 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′-tetraethylbenzimidazolycarbocianine iodide), Alexa Fluor 488 antibody anti-cytochrome c mouse IgG, Vybrant apoptosis assay kit #2 and AO (acridine

orange) came from Molecular Probes (Leiden, Netherlands). Calcein-AM was purchased from Invitrogen (Paisley, Scotland, United Kingdom). Antibodies against Bax (rabbit polyclonal IgG), cytochrome c (mouse monoclonal IgG) and β-Tubulin (rabbit polyclonal IgG) were acquired from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Goat antirabbit IgG H&L horseradish peroxidase conjugated and goat anti-mouse IgG H&L peroxidase conjugated were a gift from Chemicon International (CA, USA), and acrylamide/bisacrylamide from Bio-Rad Laboratories (Hercules, CA, USA). The oligonucleotides sequences used caspase-6 (Hs00154250_m1, AB) and housekeeping gene 18S rRNA (TaqMan® Ribosomal RNA Control Reagents) were from Applied Biosystems (Foster City, CA, USA). All other reagents were of analytical grade.

Cell cultures

Pig renal tubular epithelial cells (LLC-PK1) were cultured in RPMI 1640 medium supplemented with 10% decomplemented FCS, 2 mM L-glutamine, sodium bicarbonate 2 g/l and antibiotics (ampicillin 125 µg/ml, gentamicin 40 µg/ml and cloxacillin 125 µg/ml). Experiments were performed when cultures have reached a 90% confluence. For the flow cytometry and confocal microscopy procedures, 6-well and 24-well plates (Corning Inc, NY, USA) respectively, were used to grow the cells in a 5% CO_2 atmosphere at 37 °C. The number of cells in a 6 well plate was 600,000–700,000 and in a 24 well plate 100,000– 150,000 (approximately 25,000 cells/cm^2). When cellular confluence was reached and 24 h before treatment, the medium was replaced with a fresh medium containing 0.5% FCS.

Treatments and experimental design

CsA was dissolved in ethanol to prepare a 0.01 M stock solution and in 0.5% RPMI to obtain the desired final concentration. In previous experiments, we checked that ethanol does not affect the cultures. Vit E was dissolved in RPMI to prepare a 0.01 M stock solution and added to the cultures at a final concentration of 0.1 mM 30 min before CsA, and antioxidant treatment continues during exposure to CsA.

Experiment dose and time response to measure superoxide anion ($O2U^{-}$) synthesis were performed after 12, 24 and 48 h of incubation with several concentrations of CsA (0.1 µM, 1 µM and 10 µM) . When doses of 10 µM were used for 24–48 h, we observed a high percentage of nonviable cells; for example, with 10 µM for 48 h a 60% of cells were necrotic (studied by two methods, propidium iodide and trypan blue dye). Other authors (Vetter et al.,

2003) observed in the same cellular type that a dose of CsA 1 μM for 24 h increased O_2^{U-} and cGMP levels.
Most of the experiments were done with CsA 1 μM. Control cells were incubated only with 0.5% FCS in RPMI.

Flow cytometry and confocal microscopy

For flow cytometry assays, supernatants were collected in order to recover the cells that were detached during treatment. After washing with 0.9% saline solution, adhered cells were harvested with 2.2 mM EDTA in trypsin, mixed with supernatant and labelled as it will be described. Flow cytometry experiments were performed in a FACScan (BD Biosciences, San Diego, CA, USA) with a 488 nmline laser and FL1 (530/30 nm band-pass filter), FL2 (585/42 nm bandpass filter) and FL3 (670 nm long pass filter) detectors. At least 10,000 cells were examined in eachassay usingCellQuest program. Results were expressed in Mean Fluorescence Intensity (MFI).
Confocal microscopy assays were done by direct labelling of the adhered cells in plates. All images were taken using a ×20 objective and amplified with different zooms. Data for each experiment were obtained after counting the cells in at least three fields per well. Images were then analyzed via the Olympus FV 1000 software (Olympus Fluoview, version 1.6, Tokyo, Japan). After subtracting the background, total fluorescence of each field was divided by the number of cells (400–500 approximately), and the results were then presented as mean intensity fluorescence per cell (MFI/cell).

Superoxide anion (O_2U^-) production

O_2U^- is the main free radical produced in the mitochondria and was measured in LLC-PK1 cells using the cell-permeable probe MitoSOX Red. The reduced probe is taken up by cells and in the presence of O_2U^- is oxidized and becomes fluorescent, emitting a red fluorescence after 488 nm laser excitation.
After treatment with the drug, cells were incubated for 10 min with MitoSOX Red reagent 5 μM in 0.5 ml of Hank's Balanced Salt Solution with calcium and magnesium ($CaCl_2$ 140 mg/l, $MgCl_2$–$6H_2O$ 10 mg/l and $MgSO_4$–$7H_2O$ 10 mg/l) at 37 °C.
After washing twice, adhered cells were observed after excitation with the 488 nm laser line and centering at 580 nm the band-pass filter(maximum excitation/emissionofMitoSOX reagent: 510/580 nm). The images and values of fluorescence were obtained by confocal microscopy as MFI/cell.

Permeability transition pore (PTP) opening

The opening of PTP was estimated by calcein-AM labelling combined with $CoCl_2$ in order to detect exclusively the mitochondrial calcein fluorescence. Mitochondrial fluorescence is detected while PTP remains closed and $CoCl_2$ does not penetrate into the mitochondria. The opening of PTP connects mitochondrial matrix with cytosolic compartment and intramitochondrial calcein fluorescence is quenched by $CoCl_2$ that can freely diffuse now inside organelles.
After treatment with the drug for 24 h, the cells were incubated with 1 μM calcein-AM and 0.1 M $CoCl_2$ in Hank's Balanced Salt Solution with 140 mg/l $CaCl_2$ for 20 min at 37 °C. After washing, green fluorescence into mitochondria was analyzed by confocal microscopy and flow cytometry. Ionomycin (500 μM) was used as a positive control for PTP opening. For specific inhibition of PTP opening, experiments adding bongkrekic acid (50 μM) before CsA treatment were performed.

Mitochondrial membrane potential (ΔΨm) assay

MitoProbe JC-1 Assay was used. JC-1 is a cationic dye and an indicator of mitochondrial membrane potential (ΔΨm) (Reers et al., 1995). When live cells are incubated with JC-1, the dye penetrates the plasma membrane of cells as monomers and the uptake of JC-1 into mitochondria is driven by the ΔΨm. Functional mitochondria are polarized and JC-1 is rapidly taken up. This uptake raises the concentration of JC-1 leading to the formation of aggregates (J-aggregates) within mitochondria. Excited by a 488 nm laser, J-aggregates provoke a red spectral shift emission (590 nm). JC-1 does not accumulate in depolarized mitochondria but it remains in the cytoplasm as monomers which emit green fluorescence (525 nm). Thus, in healthy cells, JC-1 fluorescence is seen in both the green (FL1) and red (FL2) channels, and a loss of red fluorescence indicates depolarized mitochondria. The red/green fluorescence ratio is, therefore, dependent on ΔΨm. LLC-PK1 cells were stained with 2 μM JC-1 in 1 ml of RPMI 0.5% FCS for 15 min at 37 °C in 5% CO_2, washed twice and examined by flow cytometry and confocal microscopy. Red and green fluorescences were collected through appropriated filters and the red/ green ratio was calculated as an index of ΔΨm.

Cardiolipin oxidation and mitochondrial swelling

NAO presents high affinity by cardiolipin, a polyunsaturated acidic glycerophospholipid exclusively found in the inner mitochondrial membrane. Mitochondrial uptake of NAO is not dependent on membrane potential and it binds to cardiolipin regardless of the mitochondrial polarization state. When NAO

is bound to cardiolipin it emits fluorescence at 630 nm after 488 nm laser excitation, and the fluorescence is related to mitochondrial cardiolipin content providing a peroxidation index. Therefore, a decreased red fluorescence is related to decreased mitochondrial cardiolipin content as a consequence of peroxidation processes (Mileykovskaya et al., 2001).
After CsA treatment for 24 h, LLC-PK1 cells were detached and labelled with 100 nM NAO for 10 min at 37 °C in 0.5 ml of 0.5% FCS RPMI solution. After washing twice, cells were acquired in the FL2 channel of the flow cytometer. Moreover, cultures were directly labelled in wells with the dye in the same conditions, and confocal microscopy images were taken to visualize mitochondrial morphology.

Determination of Bax and cytochrome c proteins

Isolation of cytosolic and mitochondrial cellular fractions. We used the Mitochondria Isolation Kit for Cultured Cells (Pierce Biotechnology Inc., Rockford, IL) according to the manufacturer's instructions with the following modifications: after 24 h treatment with CsA, approximately 2×10^7 cells were trypsinized, washed and centrifuged. The pellet was resuspended in 400 µl of Mitochondria Isolation Reagent A and incubated on ice for 2 min. Afterwards, cells were mechanically homogenized on ice. 400 µl of Mitochondria Isolation Reagent C was added and the mixture was centrifuged at 700 g for 10 min at 4 °C. Supernatant was transferred to a tube and centrifuged at 3000 g for 15 min at 4 °C. Supernatant (cytosolic fraction) was recovered and the pellet containing mitochondrial fraction, was washed in 250 µL of Mitochondria Isolation Reagent C. Mitochondrial lysis was performed by incubation in 100 µl CHAPS 2% in TBS for 1 min and the mixture was centrifuged at 13,000 rpm for 5 min.
Mitochondrial proteins are in the supernatant. In both mitochondrial and cytosolic fractions, proteins were quantified at 280 nm (1 Absorbance Unit=1 mg/ml) in a spectrophotometer (BTS Reader510, Organon Teknika).

Western blot.

All protein samples were processed by SDS-PAGE and transferred to nitrocellulose membranes overnight at 4 °C. After blocking, the membrane was incubated with the indicated primary antibody (anti-cytochrome c and anti-Bax) and then with secondary antibody.
Membranes containing proteins of the cytosolic fraction were incubated with antibody anti-β-Tubulin, which is a major cytoskeleton component. Membrane containing proteins of the mitochondrial fraction were incubated with antibody anti-VDAC/Porin, which is the main component of the outer mitochondrial membrane. Samples were finally detected with enhanced ECL-plus

chemiluminescence (ImmunoStar HRP substrate kit, Bio-Rad, 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, Maryland, USA).

Determination of caspase 6 using real-time PCR (RT-PCR).

Total RNA was isolated by using Ultraspec reagent (Biotech, Texas, USA). This method is based on the principle of formulation of a 14 M solution of guanidine salts and urea as denaturing agents in conjunction with phenol and detergents. One-step RT-PCR was performed using MultiScribe reverse transcriptase and hexamers mix from the High-Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA, USA) and 2 μg of total RNA. This cDNA was mixed with specific primers (250 nM final concentration) and TaqMan MGB probes (900 nM final concentration). MGB probes contain: a reporter dye (6-FAM) linked to the 5′ end of the probe, a minor groove binder (MGB) that increases the melting temperature without increasing probe length and a non-fluorescent quencher (NFQ) at the 3′ end of the probe. Constitutive 18S ribosomal RNA was used as endogenous control (Applied Biosystems).

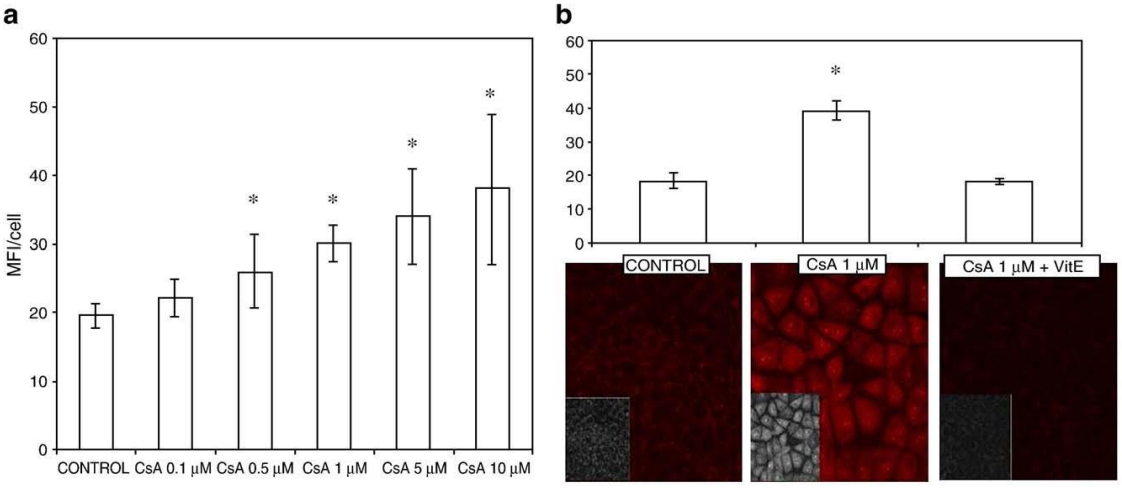

Fig. 1. Superoxide anion production as determined by MitoSOX staining in Confocal Microscopy. (a) Dose-response experiments with CsA showing the increase of MitoSOX Red fluorescence induced by the drug. (b) Upper panel and lower panel (confocal microscope images) show the increase in superoxide production induced by CsA; Vit E pretreatment kept superoxide at similar levels than control cells. Phase contrast images (insets in confocal images) show the cell density in the fields. Results are expressed in mean fluorescence intensity (MFI/cell). $*p \leq 0.05$

Experiments were performed according to the manufacturer's instructions in the TaqMan Gene Expression Assays kit. RT-PCR and PCR were performed on an ABI7000 Real Time cycler (Applied Biosystems, Foster City, CA, USA).
The threshold cycle number of cDNA amplification reactions (Ct) was determined using the ABI Prism 7000 SDS software. Levels of caspase-6 mRNA expression were normalized to 18S RNA levels using the formula: ΔCt=Ct (caspase-6)−Ct (18S RNA). Data were expressed in arbitrary units with respect to the ΔCt value for control cells using the mathematic algorism known as “Comparative Method of Ct or ΔΔCt Method” [ΔΔCt=ΔCt (control)−ΔCt (problem sample)]. The relative amount of caspase-6 expression was obtained using the expression: $2^{-\Delta\Delta Ct}$.

Measurement of cellular apoptosis by Annexin V and acridine orange (AO).

To quantify apoptosis, cells were trypsinized, washed and resuspended in 100 µl Annexin-binding buffer from Vybrant apoptosis assay kit #2 (Molecular Probes, Oregon, USA) and then labelled with Alexa Fluor 488-Annexin V and propidium iodide following the manufacturer's instructions. Finally, cell samples were placed in 14 µl-chambers (ThermoShendon, Pittsburgh, PA, USA) for confocal microscopy studies.
To determine the rate of apoptosis, cells from three microscopy fields for each sample were counted and Alexa Fluor 488-labelled cells were considered apoptotic.
A second assay, with the DNA-binding fluorochrome AO, was performed to quantify apoptosis by detection of nuclear condensation and fragmentation. AO is a cell-permeable dye that gets into all cells, intercalates among DNA molecules and appeared with a homogenous fluorescence. The nuclear condensation (feature of apoptotic cells), causes an intensification of this fluorescence and loss of homogeneous pattern (Menaker et al., 2004).
Cells were stained for 5 min with 10 µM AO in PBS for 10 min at 37 °C and 5% CO_2, washed twice, loaded in chambers and counted after 488 nm laser excitation and 519 nm filter detection. Apoptotic cells were identified by counting 500 cells at multiple randomly selected fields by confocal microscopy.

DNA fragmentation assay.

DNA fragmentation was detected by agarose gel electrophoresis. Cells were trypsinized, centrifuged and incubated with 50 µl STE and 275 µl TE (Tris–EDTA pH 8) and 62 µl SDS 10% overnight at 37 °C. Afterwards, 5 µg/ml RNAse A was added and incubated for 1 more hour, and after was mixed with 75 µl AcK (1.33 M, final concentration). The fragmented DNA was extracted with an equal volume

of phenol:$CHCl_3$:isoamylic alcohol (24:1:1). 2 V ethanol were added and DNAwas leftprecipitatingovernightat−20 °C. After washing twice with ethanol, we resuspended DNA in 50 µl TE. After heating at 65.5 °C for 5 min,DNAwasprocessed byelectrophoresis at 100 V through a 2%agarosegel containing 0.1 mg/mlof ethidium bromide inTBE buffer. Oligonucleosomal DNA fragments were visualized under ultraviolet illumination and photographed on a Polaroid film. In summary, PTP opening, ΔΨm and cardiolipin oxidation were analyzed by FC and CM. $O2^-$ production and cellular apoptosis were studied by CM.

Analysis of data

All experiments were performed at least five times, except Western blot experiments that were performed in triplicate. Results were expressed as mean±SD and were studied by analysis of variance with Scheffé correction in group comparisons or Kruskal– Wallis test using SPSS 12.0 software (SPSS Inc, Chicago, Il, USA). The level of significance was set at $p \leq 0.05$.

Results

CsA increased ROS production in LLC-PK1 cells and Vit E inhibited this effect

The increase in MitoSOX Red fluorescence caused by CsA was dosedependent and was evident with doses of CsA greater than 0.5 µM (Fig. 1a). Vit E pretreatment inhibited the increase in MitoSOX Red fluorescence induced by CsA 1 µM as was shown by confocal images
(Fig. 1b).

CsA allowed permeability transition pore opening

CsA decreased mitochondrial calcein fluorescence as shown in both confocal microscopy (Fig. 2, upper panel) and flow cytometry (Fig. 2, lower panel) experiments and Vit E avoided this decrease. Ionomycin (500 µM) was used as a positive control of PTP opening and it showed similar fluorescence than higher concentrations of CsA (10 µM) (data not shown). Bongkrekic acid (50 µM) produced specific inhibition of permeability transition and avoided mitochondrial fluorescence loss (data not shown).

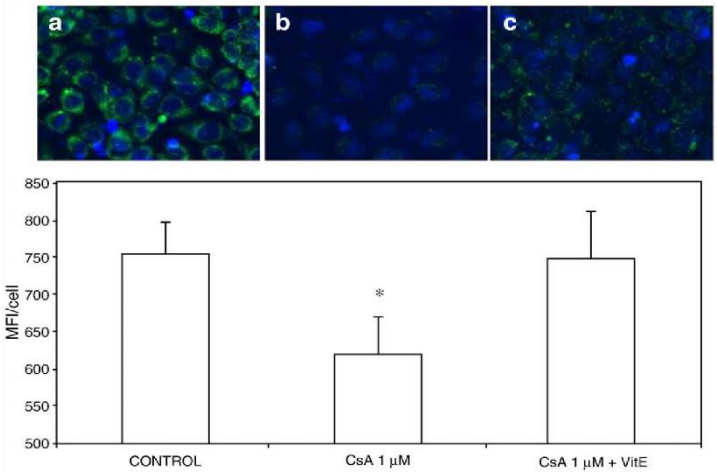

Fig. 2. Permeability Transition Pore (PTP) opening was caused by CsA. Upper panel shows confocal microscopy and lower panel flow cytometry experiments in calcein labelled cells. CsA decreased mitochondrial calcein fluorescence with respect to control or Vit E pretreated cells. MFI: Mean fluorescence intensity. ∗p≤0.05.

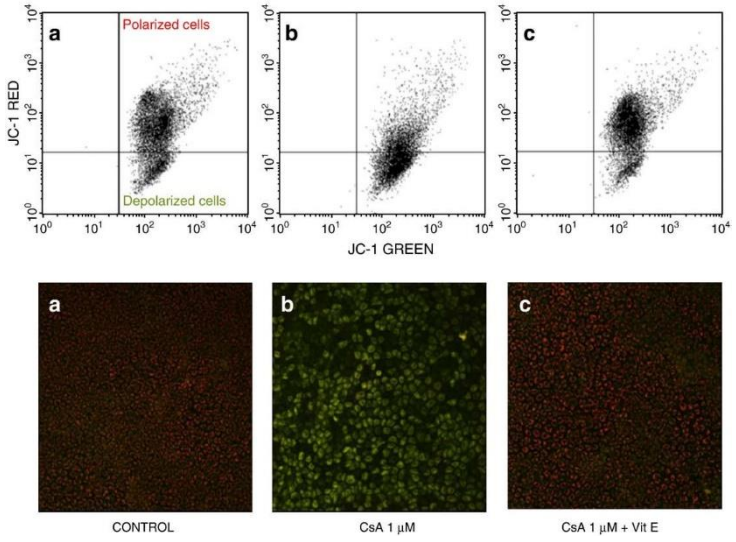

Fig. 3. Mitochondrial membrane potential (ΔΨm) was determined by flow cytometry and confocal microscopy with JC-1-labelling. Upper panel: flow cytometry plots for JC-1 labelling. CsA provoked the loss of ΔΨm as shown in the decrease in the red/green ratio fluorescence of JC-1 in CsA-treated cells (plot b; 0.5±0.2; p≤0.05) with respect to control cells (plot a, normalized ratio=1) or cells pretreated with Vit E (plot c; 1.2±0.3; ∗p≤0.05). Cells with polarized mitochondria are located in the upper right quadrant, and cells containing non-polarized mitochondria in the lower right quadrant. CsA increased the percentage of depolarized cells compared to control or Vit E pretreated cells. Lower panel: confocal microscopy images showing lack of J-aggregates formation. Control cells (a) showed high red fluorescence because of the formation of J-aggregates. In CsA-treated cells (b), the JC-1 red/green ratio was reduced and JC-1 molecules remained as green cytoplasmic monomers. Vit E pretreatment (c) reversed the effects of CsA and the pattern of fluorescence was similar to control cells.

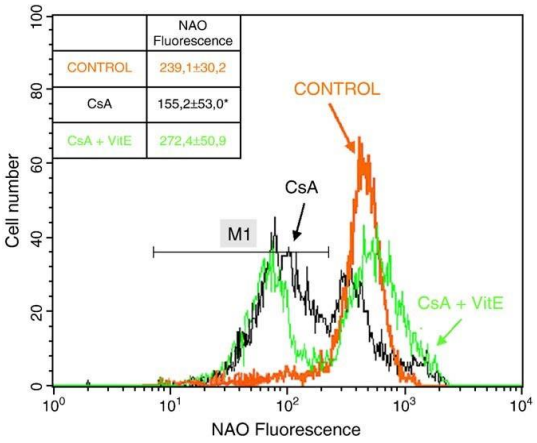

Fig. 4. Cardiolipin peroxidation determined by flow cytometry and NAO-labelling. FC experiments show that CsA decreased mean fluorescence with respect to control or Vit E pretreated cells. Mean values of fluorescence are included in the inset. M1 represents cells with reduced NAO fluorescence. ∗p≤0.05.

CsA decreased mitochondrial membrane potential and Vit E pretreatment reverted this effect

Flow cytometry experiments with JC-1 staining (Fig. 3, upper panel) showed a drop in the red/green ratio in cells treated with CsA 1 μM compared to control cells; Vit E pretreated cells had similar ratio than control cells. In addition, a greater proportion of depolarized cells was observed among the CsA-treated cells with respect to control or Vit E pretreated cells. Confocal microscopy experiments (Fig. 3, lower panel) confirmed these data and a reduction of red fluorescence in CsA-treated cells was observed compared to control cells. Vit E pretreatment increased the red fluorescence values to levels similar to control cells.

CsA caused peroxidation of cardiolipin and this effect was inhibited by Vit E pretreatment

In flow cytometry experiments (Fig. 4), treatment with CsA 1 μM led to a decrease in the mean NAO green fluorescence emitted by LLC-PK1 cells and Vit E avoided this effect (data of fluorescence are included in the inset table). The proportion of cells with reduced NAO fluorescence (Marker M1), was increased by CsA to 65–75% compared to control (5–10%) and Vit E pretreated cells (25–30%).
NAO-labelling showed that the appearance of mitochondria changed after CsA treatment. Control or Vit E pretreated cells had a nodular or linear appearance.

However, cells treated with CsA showed a diffuse distribution of the dye suggesting the presence of abnormalities in mitochondria described as swelling (Fig. 5).

Distribution of Bax and cytochrome c in cytosol and mitochondria after CsA treatment

Western blot revealed that CsA caused an increase in mitochondrial Bax. This effect was parallel to a decrease in cytosolic Bax. Vit E avoided the increase of mitochondrial Bax induced by CsA (Fig. 6). We also showed that cytochrome c in control cells is mainly located in the mitochondria. However, after CsA treatment cytochrome c appeared in cytosolic fraction of cells. Vit E pretreatment maintained the mitochondrial localization of cytochrome c (Fig. 6).

CsA provoked apoptosis in LLC-PK1 cells and Vit E inhibited this effect

CsA increased the expression of caspase-6 and Vit E inhibited this effect. CsA increased the expression of caspase-6. Vit E pretreatment inhibited this effect and cells had similar mRNA expression than control cells (Fig. 7). Levels of caspase-6 gene expression were obtained after normalizing with 18S rRNA as an endogenous reference. Data were calculated using the mathematical expression $2^{-\Delta\Delta Ct}$ in attention of the “Comparative Method of Ct or ΔΔCt Method”.

Annexin V/propidium iodide experiments. Annexin V and propidium iodide labelling showed how CsA treatment increased the proportion of apoptotic (green) and necrotic (red) cells with respect to control or Vit E pretreated cells (Fig. 8).

DNA fragmentation assay. CsA induced fragmentation of DNA with the appearance of typical ladder pattern suggestive of cellular apoptosis. However this pattern did not appear when cells were pretreated with Vit E (Fig. 9a). Furthermore, AO labelling (Fig. 9b) allowed the identification of cells with apoptotic bodies that were increased after CsA 1 μM (N70%) treatment compared to control or Vit E treated cells (b15%).

Discussion

We have previously demonstrated that glomeruli of rats treated with CsA have an increase of ROS synthesis (Parra et al.,1998a,1998b). This increase was also seen in cultured rat mesangial cells incubated with CsA (Parra Cid et al., 2003). As these effects were dose and time-dependent and the antioxidant Vit E inhibited cellular damage, we hypothesized that the nephrotoxic effect of CsA

could be mediated by ROS (Perez de Lema et al., 1997; Parra et al., 1998a, 1998b; Parra Cid et al., 2003).

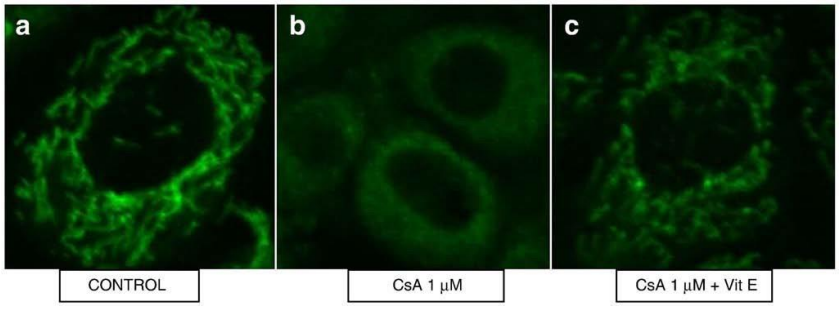

Fig. 5. Confocal microscopy of cells labelled with NAO. The images reveal the arrangement and organization of mitochondria in control cells, which appear as chains of nodular structures (a). Cells treated with CsA showed mitochondria with margins not well defined which reflect the phenomenon of swelling (b). The appearance of cells pretreated with Vit E before adding CsA was similar in control cells (c).

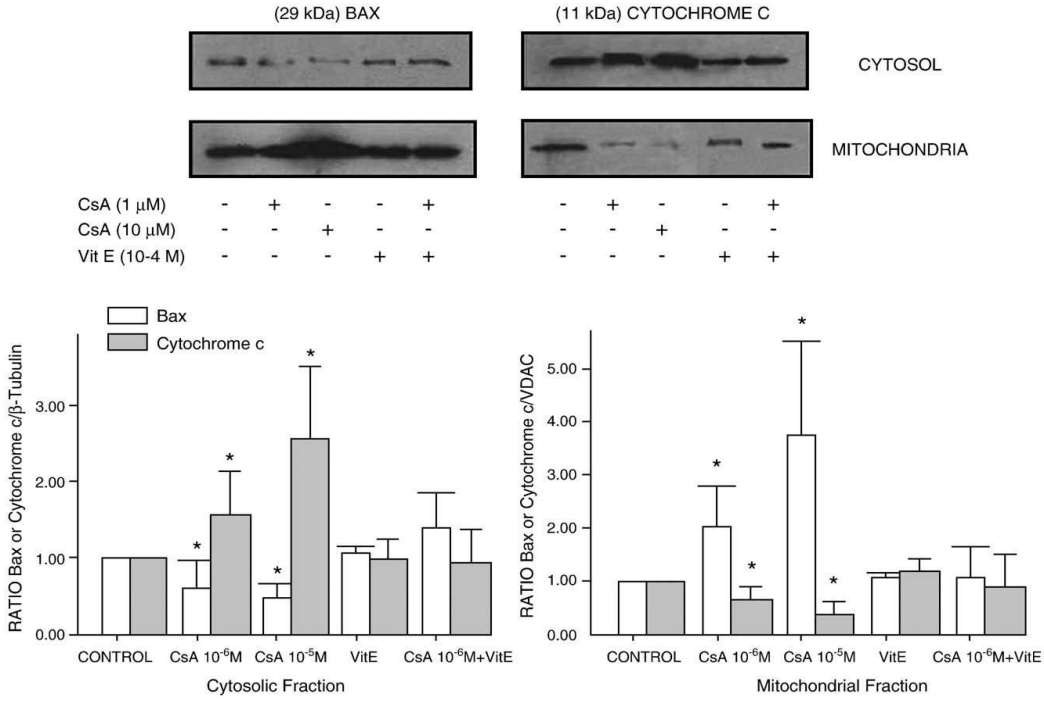

Fig. 6. Western of Bax and cytochrome c. A representative Western blot for Bax and cytochrome c in cytosolic and mitochondrial subcellular fractions is shown. Histogram with bars represents the mean±SD (n=3) of the ratio protein (Bax or cytochrome c) to β-Tubulin in cytosolic fraction or VDAC/Porin in mitochondrial fraction. Experiments showed that after CsA treatment, Bax increased and cytochrome c decreased in mitochondrial fraction. However, cytosol showed an increase of cytochrome c and decrease of Bax. Vit E avoided

these effects. $*p \leq 0.05$ versus control cells.

The molecular mechanisms involved in ROS generation by CsA are not well defined. It has been suggested that ROS can be produced through activation of NAD(P)H oxidase, xantine–oxidase system or mitochondrial pathways (Jeon et al., 2005). In addition, CsA has been shown to alter the expression levels of antioxidant enzymes, decreasing the activity of catalase and glutathione peroxidase in kidney tissue (Durak et al., 1998).

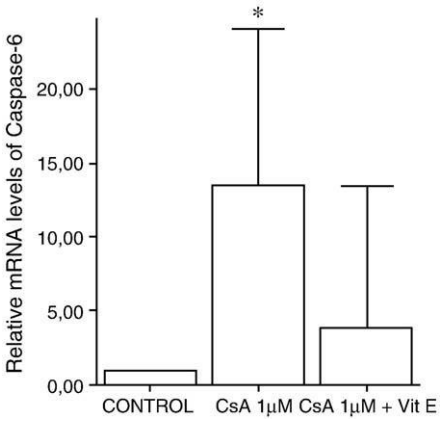

Fig. 7. PCR of caspase-6. Quantitative real-time PCR analysis of caspase-6 expression. Data were calculated using the "Comparative Method of Ct or ΔΔCt Method". Relative quantification was done with 18S RNA as an endogenous control by means of the expression $2^{-\Delta\Delta Ct}$. CsA increased the expression of caspase-6. This effect was inhibited by Vit E. Data were expressed as the number of copies of RNAm with respect to control cells. $*p \leq 0.05$ versus control cells.

One of the main sources of intracellular ROS is mitochondria and we studied the specific production of O_2U^- by mitochondria in LLCPK1 cells using MitoSOX Red. We have observed that CsA increased the mitochondrial synthesis of O_2U^- in a dose and time-dependent manner. The preincubation of cells with Vit E inhibited this effect suggesting that the antioxidant can avoid nephrotoxic effects of CsA by scavenging O_2U^-. Our data were supported by other authors that have proved that selective inhibitors of mitochondrial electron transport decreased the generation of ROS induced by CsA in MDCK cells (Jeon et al., 2005).
Several groups have identified apoptosis as a mechanism involved in CsA nephrotoxicity, both in vivo and in vitro (Healy et al., 1998; Ortiz et al., 1998a; Hortelano et al., 2000; Justo et al., 2003; Jeon et al., 2005; Roy et al., 2006). As mitochondria-mediated apoptosis is a common mechanism used by cells, we hypothesize that the increase of ROS and apoptosis mediated by CsA could be related.

The O_2U^- is a highly reactive molecule that can oxidize other molecules (Paradies et al., 2002; Raymond et al., 2003; Iverson and Orrenius, 2004; Kagan et al.,

2004). One of its main targets is cardiolipin, an acidic glycerophospholipid present only in the inner mitochondrial membrane (Kagan et al., 2004). Cardiolipin is an essential phospholipid for eukaryotic energy metabolism and plays a key role in the maintenance of mitochondrial structure and function (Haines and Dencher, 2002; McMillin and Dowhan, 2002; Iverson and Orrenius, 2004). This role has been attributed to its close relationship with respiratory chain proteins, including cytochrome c (Kagan et al., 2004). Cardiolipin is especially vulnerable to oxidative attack because of its unsaturated carbon chains and their proximity to ROS-producing sites in the mitochondrial inner membrane (Iverson and Orrenius, 2004; Orrenius, 2007; Valko et al., 2007).

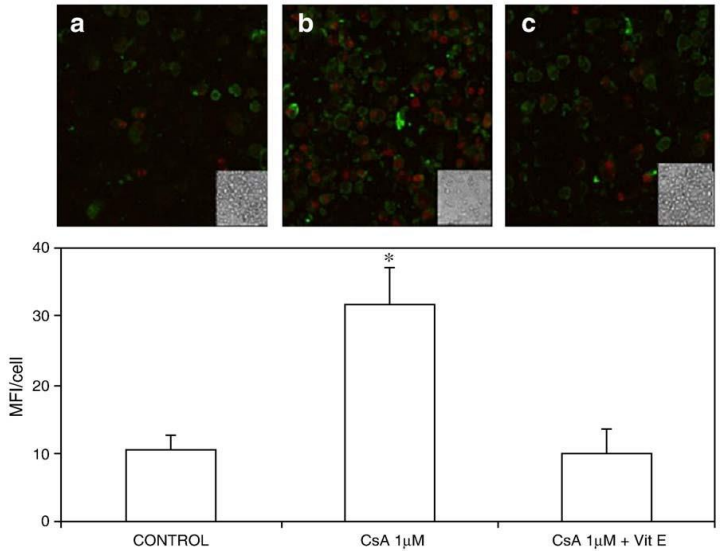

Fig. 8. Apoptotic rate determined by Annexin V–propidium iodide (confocal microscopy). Upper panel shows that CsA treatment (b) triggered apoptosis and increases the number of cells labelled with Annexin V, with respect to control (a) or Vit E pretreated cells (c). Phase contrast images (insets in confocal images) show the cell density in the microscopic fields. Lower panel reflects the percentage of apoptotic cells. $*p \leq 0.05$ versus control cells.

Our experiments indicate that CsA increases the peroxidized cardiolipin content of mitochondria and the proportion of cells with high amounts of peroxidized cardiolipin increased after CsA treatment. Vit E pretreatment decreased the peroxidation of cardiolipin and the proportion of cells with higher percentages of peroxidized cardiolipin suggesting that Vit E inhibition of ROS avoided the cardiolipin peroxidation process.

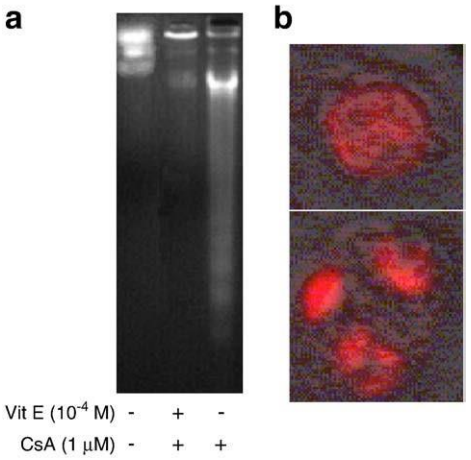

Fig. 9. DNA fragmentation assay. a. DNA ladder formation after treatment with CsA was detected by gel electrophoresis. Vit E treated cells did not show appreciable effects on DNA ladder formation. b. CsA increased the number of cells with apoptotic bodies (AO labelling, confocal microscopy).

The oxidation of cardiolipin may have important consequences (Haines and Dencher, 2002; McMillin and Dowhan, 2002; Petrosillo et al., 2003), like the release of cytochrome c into intermembrane mitochondrial space. Furthermore, the loss of cytochrome c and the damage to other components of the electronic transport system may further contribute to the increase in O_2U^- (Jezek and Hlavata, 2005; Brady et al., 2006; Zorov et al., 2006).

On the other way, the alteration of mitochondrial structure secondary to cardiolipin oxidation can induce the decrease of ΔΨm (Justo et al., 2003). In agreement with previously published results, our experiments using JC-1 indicate that CsA induces a decrease in the potential of the mitochondrial membrane, and Vit E inhibited this effect. It has been reported that CsA diminished the mitochondrial membrane potential in murine MCT cells and this effect was accompanied by the release of mitochondrial cytochrome c, caspase activation and induction of cellular apoptosis (Justo et al., 2003).

Our experiments have been focused in the mitochondrial effects of CsA, and we have demonstrated that CsA induced O_2U^- synthesis in LLC-PK1 cells. Our hypothesis suggests that O_2U^- mitochondrial production altered the structure (oxidation of cardiolipin) and function of the mitochondria. Especially, the loss of ΔΨm, uncouples electron transfer chain and oxidative phosphorylation, resulting in new O_2U^- production and inefficient ATP synthesis (Green and Kroemer, 2004). In summary, an early increase of O_2U^- that is not neutralized by mitochondrial antioxidant defences, could produce mitochondrial alterations that maintain a vicious circle with new synthesis of O_2U^- and mitochondrial and cellular damages. The decrease in ATP synthesis, which depends on the

generation of an electrochemical gradient in mitochondria, can worsen many cellular metabolic processes (Kahan, 1989; Paradies et al., 2002).

A phenomenon closely related to the increase of oxidative stress and loss of mitochondrial membrane potential is the opening of mitochondrial permeability transition pores (PTP) (Kowaltowski et al., 2001; Kim et al., 2003; Bernardi et al., 2006; Skulachev, 2006). It has been suggested that PTP plays an important role in cellular apoptosis (Gagne et al., 2002; Green and Kroemer, 2004; Orrenius et al., 2007; Rasola and Bernardi, 2007; Schwarz et al., 2007; Tsujimoto and Shimizu, 2007). PTP pores form at contact sites between the mitochondrial inner and outer membranes and involve several proteins including the voltage-dependent anion channel (VDAC), the adenine nucleotide translocase (ANT) and cyclophilin D. There are many data indicating that PTP can operate in two modes, a regulated mode that is activated by calcium and inhibited by CsA and magnesium, and an unregulated mode that is calcium-independent and insensitive to CsA and magnesium (He and Lemasters, 2002). Several studies have shown that oxidant chemicals favoured the behaviour of mitochondrial unregulated pore. It has been described that CsA can prevent PTP formation through its binding to cyclophilin D, and further interaction with other proteins (He and Lemasters, 2002; Raymond et al., 2003; Rasola and Bernardi, 2007). However, this effect of CsA is a concentration dependent phenomenon as it has been shown in MDCK and H9C2 cells (Chen et al., 2002; Jeon et al., 2005). Furthermore, some experiments 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).

Another mechanism that can induce damage in outer mitochondrial membrane is mediated by the proapoptotic Bcl-2 family proteins such as Bax and Bak (Wei et al., 2001; Kuwana et al., 2002; Iverson and Orrenius, 2004). Bax is located predominantly in the cytosolic compartment in a monomeric form. After apoptotic stimuli, a significant fraction of Bax protein forms multimers and translocates to the outer mitochondrial membrane, where it induces directly the formation of pores (Wei et al., 2001; Rasola and Bernardi, 2007). Also, it has been hypothesized that Bax can interact with VDAC to form specific channels in the outer mitochondrial membrane (Gagne et al., 2002; Green and Kroemer, 2004; Antignani and Youle, 2006; Kinnally and Antonsson, 2007).

In summary, it is possible that several types of mitochondrial membrane pores can coexist although their regulation and the potential interactions among them are at present not well understood (Antignani and Youle, 2006; Dejean et al., 2006; Kinnally and Antonsson, 2007).

The opening of mitochondrial pores can cause an osmotic disbalance between the mitochondrial matrix and the intermembrane space and swelling of the

mitochondrial matrix (Skulachev, 2006; Kaasik et al., 2007). As a result, the mitochondrial cristae straighten and the outer membrane can be broken since its area is smaller than that of the inner membrane (Sesso et al., 2004). This fact can induce the release of proteins from the intermembrane space to cytosol that, in turn, may generate persistent pore opening due to the disruption of respiration leading to ROS production and attenuated production of ATP (Iverson and Orrenius, 2004).

Our experiments with calcein-AM suggest that CsA induced the formation of pores in mitochondrial membrane. We also demonstrated in cells treated with CsA that cytosolic Bax migrated to the mitochondria, as was shown after studying subcellular fractions in LLC-PK1 cells. Vit E pretreatment avoided the mitochondrial Bax apposition.

We cannot conclude if CsA induced permeabilization of mitochondrial membranes as a result of the opening of PTP, through the action of the proapoptotic member Bax or even through the rupture of the outer mitochondrial membrane in the process of swelling (Belizario et al., 2007; Kaasik et al., 2007). However, this permeabilization is one of the crucial events in the process of controlled death of cells (Sugioka et al., 2004). These processes, and probably the mechanism of swelling, lead to the release of intermembrane proteins such as cytochrome c, apoptosis-inducing factor (AIF) and endonuclease G to cytosol (Leist and Jaattela, 2001; Kim et al., 2003). We confirmed that, after treatment with CsA, an increase of cytosolic cytochrome c was observed. Vit E pretreatment avoided this increase and cytochrome c remained intramitochondrial.

Several independent groups have shown that CsA induced apoptosis in tubular cells in a dose and time-dependent manner (Healy et al., 1998; Ortiz et al., 1998a; Hortelano et al., 2000; Justo et al., 2003; Iverson and Orrenius, 2004; Jeon et al., 2005; Roy et al., 2006). It has been suggested that apoptosis mediated by CsA does not involve the extrinsic pathway because although CsA increased the expression of Fas, apoptosis was not blocked by inhibition of caspase 8 or by anti-FasL antibodies (Ortiz et al., 1998b; Justo et al., 2003). Our results agree with previous reports and also suggest that Baxmediated mitochondrial injury contributed to apoptotic pathway in CsA-induced tubular toxicity.

We demonstrated increased expression of the executioner caspase-6 after CsA treatment of cells. The antioxidant Vit E avoided this increase, suggesting that ROS contributed to apoptosis in cells (Fuentes-Prior and Salvesen, 2004).

Our results with confocal microscopy confirmed that the percentage of apoptotic cells increased after CsA treatment in LLC-PK1 cells. As it has been mentioned, CsA-induced apoptosis has also been demonstrated in other cellular types (Chen et al., 2002; Jeanmart et al., 2002; Justo et al., 2003; Jeon et al., 2005; Roy et al., 2006). These results were also corroborated with the analysis of DNA

fragmentation after CsA. Vit E pretreatment avoided apoptosis in our cells suggesting that ROS have an important role in the genesis of this process.
In conclusion, our results suggest that CsA induces several consequences in mitochondrial structure and function in LLC-PK1 cells. Although it may be difficult to establish the sequence of events, the fact that the antioxidant Vit E inhibited the O_2U^- formation and mitochondrial alterations, suggests that CsA initially increases mitochondrial O_2U^- production. The increase of intramitochondrial ROS can oxidize several molecules, such as cardiolipin, at the inner mitochondrial membrane, triggering cytochrome c release to the intermembrane space. CsA induces PTP and Bax-mediated pores in mitochondrial membrane allowing the release of proteins to cytosol and triggering the activation of caspases and in turn the process of programmed cell death.

Conflict of interest None.

Acknowledgments

This work was supported by a grant from the Junta de Comunidades de Castilla-La Mancha (04065-00). J. Pérez de Hornedo was a fellow of the Fundación Francisco Cobos. S. Ramírez Rubio holds fellowship awards from the Junta de Comunidades de Castilla-La Mancha (2006/02, GC0/5000). M. Calvino Fernández was supported by a Contrato de Apoyo a la Investigación en el SNS from Instituto de Salud Carlos III (CA07/00157). S. Benito Martínez was supported by an Ayuda para la Incorporación de Jóvenes Investigadores a Grupos de Investigación de Castilla-La Mancha from FISCAM (MV2007JI/18).

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