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ORIGINALS

Cyclosporin a causes oxidative stress and mitochondrial dysfunction in tubular renal cells

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SUMMARY

Reactive oxygen species (ROS) have been implicated in cyclosporin A (CsA) nephrotoxicity. As mitochondria are one of the main sources of ROS in cells, we evaluated the role of CsA in mitochondrial structure and function in LLC-PK1 cells. We incubated cells with CsA 1 µM for 24 hours and studies were performed by flow cytometry and confocal microscopy. We studied mitochondrial NAD(P)H content, superoxide anion (O2.-) production (MitoSOX Red), oxidation of cardiolipin of inner mitochondrial membrane (NAO) and mitochondrial membrane potential [DIOC2(3)]. We also analyzed the intracellular ROS synthesis (H2DCF-DA) and reduced glutation (GSH) of cells. Our results showed that CsA decreased NAD(P)H and membrane potential, and increased O2.- in mitochondria. CsA also provoked oxidation of cardiolipin. Furthermore, CsA increased intracellular ROS production and decreased GSH content. These results suggest that CsA has crucial effects in mitochondria. CsA modified mitochondrial physiology through the decrease of antioxidant mitochondrial compounds as NAD(P)H and the dissipation of mitochondrial membrane potential and increase of oxidants such O2.-. Also, CsA alters lipidic structure of inner mitochondrial membrane through the oxidation of cardiolipin. These effects trigger a chain of events that favour intracellular synthesis of ROS and depletion of GSH that can compromise cellular viability. Nephrotoxic cellular effects of CsA can be explained, at least in part, through its influence on mitochondrial functionalism.

Key words: ***Cyclosporin A. Mitochondria. NAD(P)H. Superoxide anion. Cardiolipin. Reactive oxygen species.***

LA CICLOSPORINA A ORIGINA ESTRÉS OXIDATIVO Y DISFUNCIÓN MITOCONDRIAL EN CÉLULAS TUBULARES RENALES

RESUMEN

Estudiamos el efecto de la ciclosporina A (CsA) sobre la estructura y función mitocondrial en células LLC-PK1. Las células se incubaron durante 24 horas con

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CsA 1 μM y se analizó la producción de anión superóxido, contenido de NAD(P)H, oxidación de cardiolipina y potencial de membrana mitocondrial; además se estudió la formación de radicales libres y el contenido de glutatión reducido intracelular. Nuestros resultados demuestran que la CsA provocó un aumento del anión superóxido mitocondrial de modo paralelo al descenso de NAD(P)H; además, se produjo oxidación de la cardiolipina de la membrana interna y un descenso del potencial de membrana mitocondrial. Finalmente, observamos un aumento de la producción de radicales libres intracelulares y un descenso del glutatión reducido. En conclusión, la CsA produce modificaciones importantes en la fisiología y estructura mitocondrial con aumento de la síntesis de especies reactivas de oxígeno y descenso de la capacidad antioxidante, hechos que podrían justificar la toxicidad celular de la droga.

Palabras clave: ***Ciclosporina A. Mitocondria. Especies reactivas de oxígeno. Anión superóxido. Cardiolipina.***

INTRODUCTION

Cyclosporin A (CsA) is an immunosuppressive drug widely use for the prevention of transplant rejection as well as for the treatment of several autoimmune diseases. [1,2] Its main side effect is nephrotoxicity that may be acute or chronic. [3] Acute toxicity usually occurs within the first treatment weeks and is manifested as renal function worsening being reversible in most of the cases by reducing the drug dose.[4] Chronic toxicity produces progressive renal function impairment and, at the histological level, arteriolar lesions, interstitial fibrosis, and tubular atrophy may be seen.[5, 6]

The pathophysiologic mechanisms of CsA-induced nephrotoxicity have been deeply studied, and several mediators, such as the renin-angiotensin-aldosterone system, the platelet activator factor, endothelin, and eicosanoids have been implicated.[7-9] We have shown that CsA increases glomerular synthesis and thromboxane urinary excretion in rats, suggesting that this eicosanoid may have a relevant role in renal impairment.[10]

There are other studies implicating reactive oxygen species (ROS) in CsA-induced nephrotoxicity.[11-14] Our group has showed that isolated glomeruli from CsA-treated rats showed an increase in the synthesis of superoxide anion ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2), and that this phenomenon occurred in parallel with renal function worsening observed in the animals.[10, 11] Besides, when we administered the antioxidant vitamin E (Vit E) in the diet, renal function deterioration was prevented as well as the increase of glomerular ROS glomerular.[10, 11] In experiments done on human mesangial cells, we confirmed that CsA increases in vitro synthesis of ROS and that pre-incubation with vit E neutralizes this effect.[12, 13]

Our hypothesis postulates that CsA increases ROS production that likely through membrane lipids peroxidation conditions the release of vasoconstricting agents such as thromboxane A_2, finally leading to renal function worsening.[10-16]

However, the ultimate mechanisms by which ROS increase is produced in response to CsA treatment have not been established so far. Since mitochondria constitute one of the main sources of ROS within the cells, our objective has been to investigate the effects of CsA on mitochondrial structure and function in pig proximal tubular cells (LLC-PK1) and the relationship with the production of ROS.

MATERIAL AND METHODS

Cellular cultures

LLC-PK1 cells cultivated in 6- or 24-well plaques (depending on whether they were analyzed by flow cytometry or my confocal microscopy) were used up to 80% of confluence. Cells were grown up in an incubator at 37º C and 5% CO_2, with RPMI culture media and 10% of fetal calf serum (FCS) and antibiotics (ampicillin 125 mg/mL, cloxacillin 40 mg/mL, and gentamycin 125 mg/mL). When confluence was reached, the culture growth was stopped by replacing the media by another of the same composition but 0.5% FCS. The cells were exposed for 24 hours to CsA diluted in RPMI (from a solution of 10^{-2} M CsA in ethanol) to obtain a final concentration of 1 mM. Control cells were only exposed to ethanol. The results are expressed as Arbitrary Fluorescence Units (AFU).

Estimation of mitochondrial NAD(P)H content

The NAD(P)H amount is related with the redox state of the complex I of the mitochondrial electron transporting chain and its oxidation reflects the release of electrons to oxygen molecules leading to a breakdown of the normal flow of the respiratory chain and of the synthesis of superoxide anion ($O_2^{\cdot-}$) molecules. The cells emit blue auto-fluorescence proportionally to their content in NAD(P)H molecules when they are excited with ultraviolet laser (360 nm). [17] In our experiments, CsA-treated cells were tripsinized and resuspended in 0.5% RPMI 0.5% and acquired in a sorter (Facscan Plus, Becton Dickinson). The autofluorescence was analyzed by using a filter with a 450 nm gate after excitation with an ultraviolet laser.

Mitochondrial production of the superoxide anion ($O_2^{\cdot-}$)

$O_2^{\cdot-}$ is the main free radical produced by mitochondria. Its synthesis was determined by confocal microscopy (Olympus XL) using the fluorescent probe MitoSOX Red (Molecular Probes, Leiden, Holland). The probe freely crosses through cellular membranes and is specifically oxidized by $O_2^{\cdot-}$.[18] LLC-PK1 cells were directly incubated in the plaques with 1 mL Hanks solution (HBSS, $CaCl_2$ 140 mg/dL, $MgCl_2$-$6H_2O$ 10 mg/dL, and $MgSO_4$-$7H_2O$ 10 mg/dL) and MitoSOX Red 5 mM for 10 minutes, and were washed twice. They were analyzed by exciting them with a 530-nm laser and determining the emission fluorescence with a 580-nm filter.

Mitochondrial lipid peroxidation

Cardiolipin (CL) is the main lipid component of the internal mitochondrial membrane and contributes to maintain its structure.[19,20] The cells were incubated with 1 mL of RPMI with 0.5% FCS in the presence of cardiolipin-avid fluorochrome NAO (10-N-nonyl acridin orange, Molecular Probes), at a concentration of 100 nM for 10 minutes, at 37º C. After washing two times and excitation with 488-nm laser, the fluorescence at 530 nm by flow cytometry.

Study of the mitochondrial membrane potential ($\Delta\psi$ m)

$\Delta\psi$ m is the consequence of the electron passage through the respiratory chain and its interruption leads to its decrease. $DIOC_2(3)$ is a cationic lipophilic substrate (3,3'-diethyloxacarbocyanine iodide, Molecular Probes) that is used to detect changes in the membrane potential.[21] It freely diffuses to the cellular cytosol and preferentially accumulates in the mitochondria with active membrane potential, emitting fluorescence at 530 nm and at 575 nm; its fluorescent emission decreases when there is impairment in the mitochondrial membrane potential. The suspension of LLC-PK1 cells was incubated with 0.5 mL RPMI and 0.5% FCS containing $DIOC_2(3)$ at a final concentration of 50 nM for 15 minutes at 37º C and 5% CO_2. After washing twice, the cells were analyzed by flow cytometry using the correspondent fluorescence detectors.

Intracellular free radical production

H_2DCF-DA (2',7'-dichlorodihydrofluorescein diacetate, Molecular Probes) was used, which is a non-fluorescent probe that freely diffuses into the cell due to its lipophilic properties. Once in the cytosol, esterases hydrolyze its ester bound yielding a hydrophobic substance (H_2DCF) that cannot escape the cytosolic compartment. The oxidized molecule (DCF) is highly fluorescent, so that oxidants may be identified within the cytosol by measuring the fluorescence emitted by the cells after being excited with a 488-nm laser and detected by the corresponding detectors at 530-nm fluorescence emission.[22]

Estimation of the cellular content of reduced gluthathione (GSH)

GSH is one of the main cellular antioxidants. The compounds of the bimane family are thiol-derived reactants,[23] essentially non-fluorescent until they go through the plasma membrane by passive diffusion and produce blue fluorescent compounds when they bind to intracellular gluthathione molecules and to thiol-containing proteins. In our experiments, we have used mono-bromobimane (mBBr, Molecular Probes) as the substrate at a final concentration of 20 mM in 1 mL of RPMI. The cells were incubated with the reactant in water bath at 37º C for 10 minutes and after washing them twice they were analyzed by confocal microscopy after being excited with 405-nm ultraviolet laser.

Statistical analysis

Numerical results are presented as mean ± one standard deviation of at least three experiments. The means were compared by parametric (Student's t test)

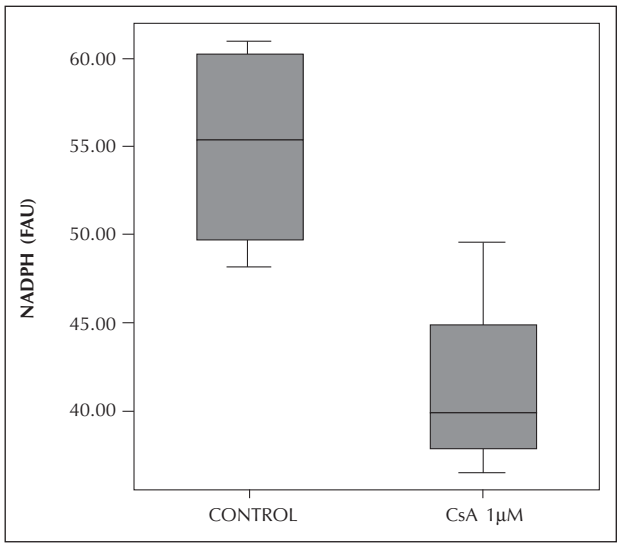

Fig. 1.—NADP(H) levels measured as auto-fluorescence values of LLC-PK1 cells. Mean auto-fluorescence decreased by 24.6% in CsA-treated cells.

or non- parametric (Friedman's test) tests and p values < 0.05 were considered to be statistically significant.

RESULTS

CsA produced a decrease in cellular NAD(P)H. The values of the average fluorescence were 55 ± 12.3 and 41.5 ± 11.3 (AFU) for the control and 1 mM CsA, respectively ($p < 0.05$). The data from four experiments are summarized in Figure 1.

On the other hand, CsA produced an increase in cell fluorescence (39.3 ± 2.9 AFU) as compared to control cells (18.3 ± 2.4 AFU) after incubation with MitoSOX Red, indicating an increase in mitochondrial $O_2^{\cdot-}$ production (Figure 2).

CsA-treated cells showed fluorescence at 530 nm after incubation with NAO , 159.2 ± 116 AFU, whereas control cells showed fluorescence at 239.1 ± 60.4 AFU (Figure 3). At the histogram, two cellular populations may be seen, with high and low (M1) fluorescence. The M1 subpopulation representing the cells with high content in oxidized cardiolipin was 80% among CsA-treated cells and 47% among control cells (Figure 3).

CsA induced a decrease in green fluorescence (223.6 ± 125.5 AFU, Figure 4b) emitted after incubating the cells with $DIOC_2(3)$, as compared with control cells (481.3 ± 170.8 AFU, Figure 4a). Besides, two cellular populations may be differentiated by green fluorescence: those having lower values (left lower corner) represent 60% of the cells treated with the drug and 27% of the control cells. Finally, CsA promoted the occurrence of a third population that represented about 15% of the whole (labeled with a dotted line) that shows intermediate green fluorescence; these cells also show decreased red fluorescence, which may indicate higher susceptibility for depolarizing.

The experiments with DCFH-DA revealed a fluorescence increase in the cells treated with the drug (4211 AFU per cell, Figure 5b) as compared to controls (1997 AFU per cell, Figure 5a) due to substrate oxidation because of an increase in intracellular free radicals production. Finally, staining with mono-bromobimane (mBrB) showed higher content of reduced thiols (GSH) in control cells (2863 AFU per cell, Figure 5c) as compared CsA-treated cells (631 AFU per cell, Figure 5d).

DISCUSSION

It has been shown that CsA-induced nephrotoxicity is partly due to production of ROS.[10,11,14,16] The molecular mechanisms by which CsA indices ROS production are not well understood, although it has been considered that their production could be promoted by either the drug's action on several systems such as cytochrome P450, NADPH oxidase or xanthine oxidase, or either the result of decreased cellular antioxidant systems.[24] Since mitochondria are one of the main physiologic sources of ROS, our study was conceived with the aim of defining the possible role of mitochondria in CsA-induced nephrotoxicity. In fact, under normal conditions, it is estimated that 1-2% of the oxygen of the respiratory chain is turned into $O_2^{\cdot-}$.[25] However, mitochondria have a complex antioxidant system that can detoxify these ROS,[25] among which there are enzymes (such as dismutase superoxide, gluthathione peroxidase, catalase, gluthathione reductase or gluthathione NADP trans-hydrogenase) and other smaller molecules (such as gluthathione, NAD(P)H, and vitamins E and C) that combined constitute an effective antioxidant system.[26] The action of these compounds is integrated so that the $O_2^{\cdot-}$ released in the respiratory chain is metabolized by the mitochondrial dismutase superoxide (SOD-Mn) to hydrogen peroxide, and this, in turn, is detoxified by intra- and/or extramitochondrial gluthathione peroxidase.[27-29] Gluthathione peroxidase is kept in it is reduced state by gluthathione, which in turn is reduced by NADH and NAD(P)H by the NADP-trans-hydrogenase.[27]

We have shown that CsA produces an increase in mitochondrial production of $O_2^{\cdot-}$ and this increase parallels the decrease in NADPH, an essential mole-

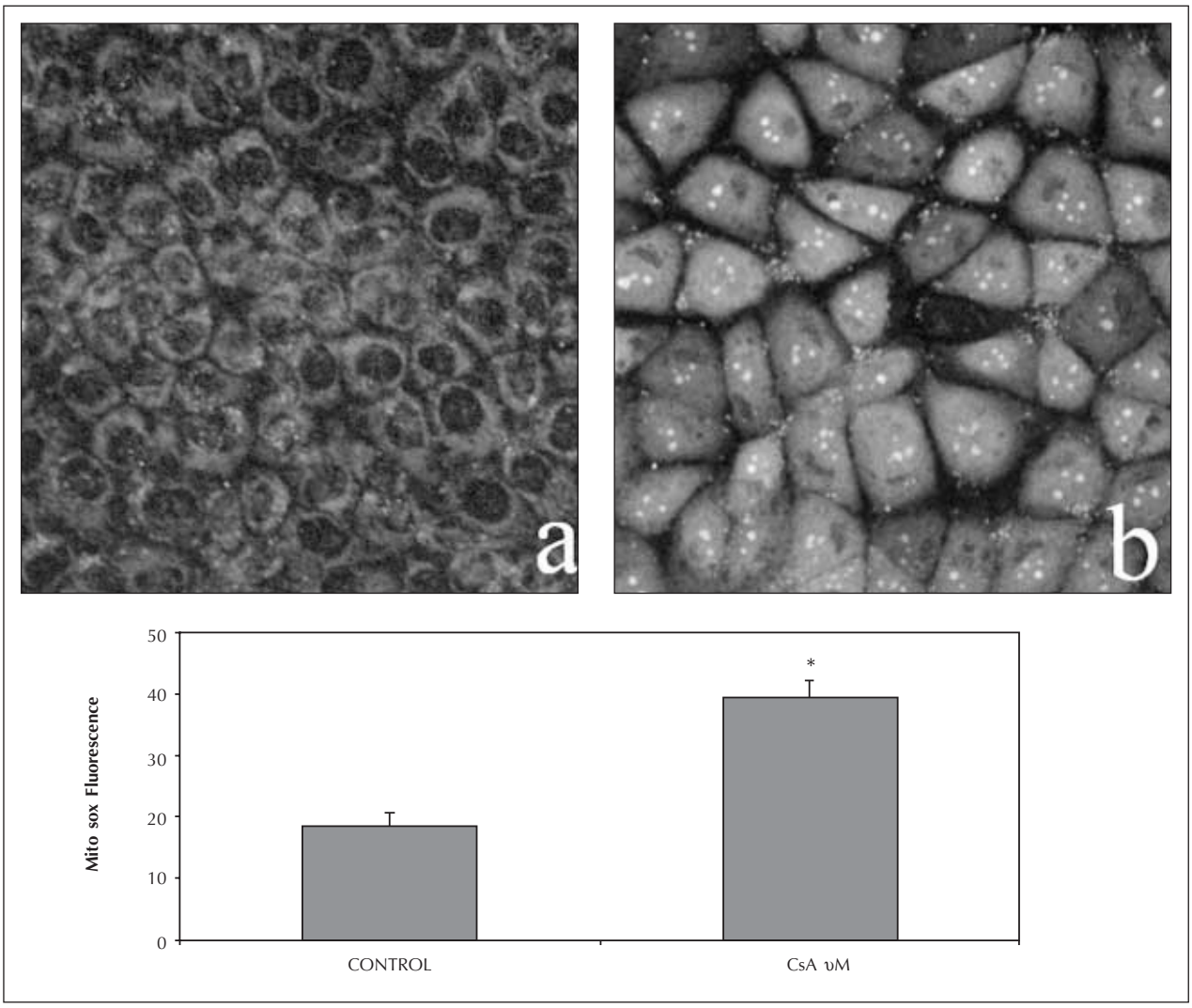

Fig. 2.—CsA-treated cells (1 μM) (b) showed greater fluorescence (2.1 fold) than controls (a) ($p < 0.05$), as it may be observed in confocal microscopy images (upper panel) and bar diagram (lower panel).

cule in the maintenance of the reduced state of complex I of the mitochondrial respiratory chain, which decrease is related with different models of cellular death.[30,31] Experiments done on isolated mitochondria have suggested that NADPH decrease could be the result of oxidation of the enzymes implicated in its synthesis, such as mitochondrial dehydrogenase or citrate.[32] $O_2^{\cdot -}$ increase has also been described by other authors in tubular and endothelial cells.[24,33,34] Although the ultimate mechanisms by which this effect is produced are not well known, it has been suggested that the enzyme NADPH oxidase could be activated.[33]

We have also shown that CsA increased intracellular ROS in the same way as has been observed by other authors.[24,35,36] Besides, it produced a decreased in the cellular content of reduced thiols, a phenomenon that may be critical for cellular metabolism since many proteins contain thiol groups and their oxidation, with creation of disulfide bounds, may induce changes in the tridimensional structure and functionality.[37] On the other hand, under normal conditions, GSH keeps the enzyme gluthathione-peroxidase in a reduced state[27,29] and this fact contributes to decrease the antioxidant capacity of the cell.

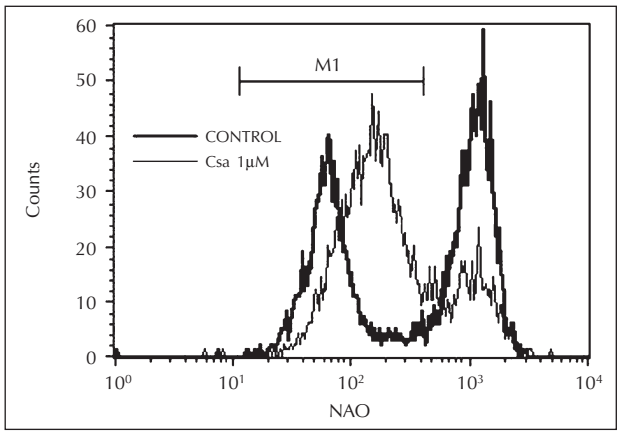

Fig. 3.—Analysis of mitochondrial cardiolipin content. CsA decreases green fluorescence from NAO as compared with control cells by 1.7 fold ($p < 0.05$) and increases the percentage of cells with high content in oxidized cardiolipin (M1).

The consequences of $O_2^{\cdot -}$ increase may be very harmful for cells since it is highly reactive and it may easily oxidize adjacent molecules.[25] CL is the main lipid constituent of inner mitochondrial membrane and contributes to its stabilization by interacting with the complexes and proteins of the respiratory chain.[38-40] Its high content in unsaturated fatty acids and its proximity to the mitochondrial respiratory chain complexes make of it particularly susceptible to oxidative damage by ROS.[41-44] Our experiments have shown that CsA altered the fluorescence emission of NAO, a substrate that specifically binds to CL, suggesting the oxidation of the latter. We have also observed an increase in the percentage of cells with high content of oxidized cardiolipin. This may have very important consequences on mitochondrial physiology and structure. One of the earliest effects is derived from the fact that CL binds with less affinity to cytochrome C, thus favoring the its release to the intermembranous space.[45] On the other hand, CL oxidation affects several complexes of the mitochondrial respiratory chain, altering the electronic flow through them. [20] Since there exists a mechanism coupled to electron transport that consists in proton translocation from the mitochondrial matrix to the intermembranous space, the electrochemical gradient, which is essential for mitochondrial ATP synthesis, will not be generated and the cellular energy metabolism will also be compromised.[19]

Our results, using $DIOC_2(3)$, corroborate this hypothesis and demonstrate that CsA produced a decrease of the mitochondrial membrane potential ($\Delta\psi$ m) and an increase in the percentage of depolarized cells. Similar findings have been described by other authors in different cellular models.[24,36] Thus, it has been shown that in human monocytes CsA produces a depolarization of the mitochondrial membrane and an increase in the percentage of depolarized cells through time in parallel with the increase in ROS production.[36] CsA also produced an increase in the release of mitochondrial cytochrome c to the cytosol, suggesting that it opens the so-called mitochondrial permeability transition pores (MPTP).[36] These are not completely defined structures that seem to be created

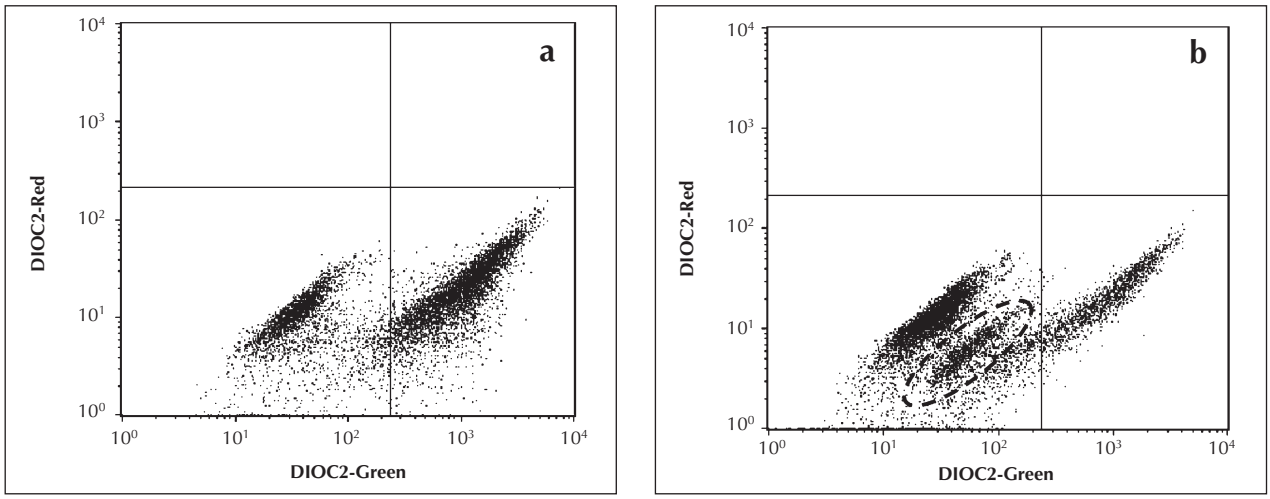

Fig. 4.—Evaluation of the mitochondrial membrane potential. After labeling with DIOC2 (3), CsA-treated cells (b) experienced a 2.2 fold decrease in the mean fluorescence value down to 530 nm (green) as compared with controls (a) ($p < 0,05$). The dotted line shows the subpopulation with intermediate fluorescence at 530 nm and decreased fluorescence at 575 nm (red) (cells with higher tendency to depolarize).

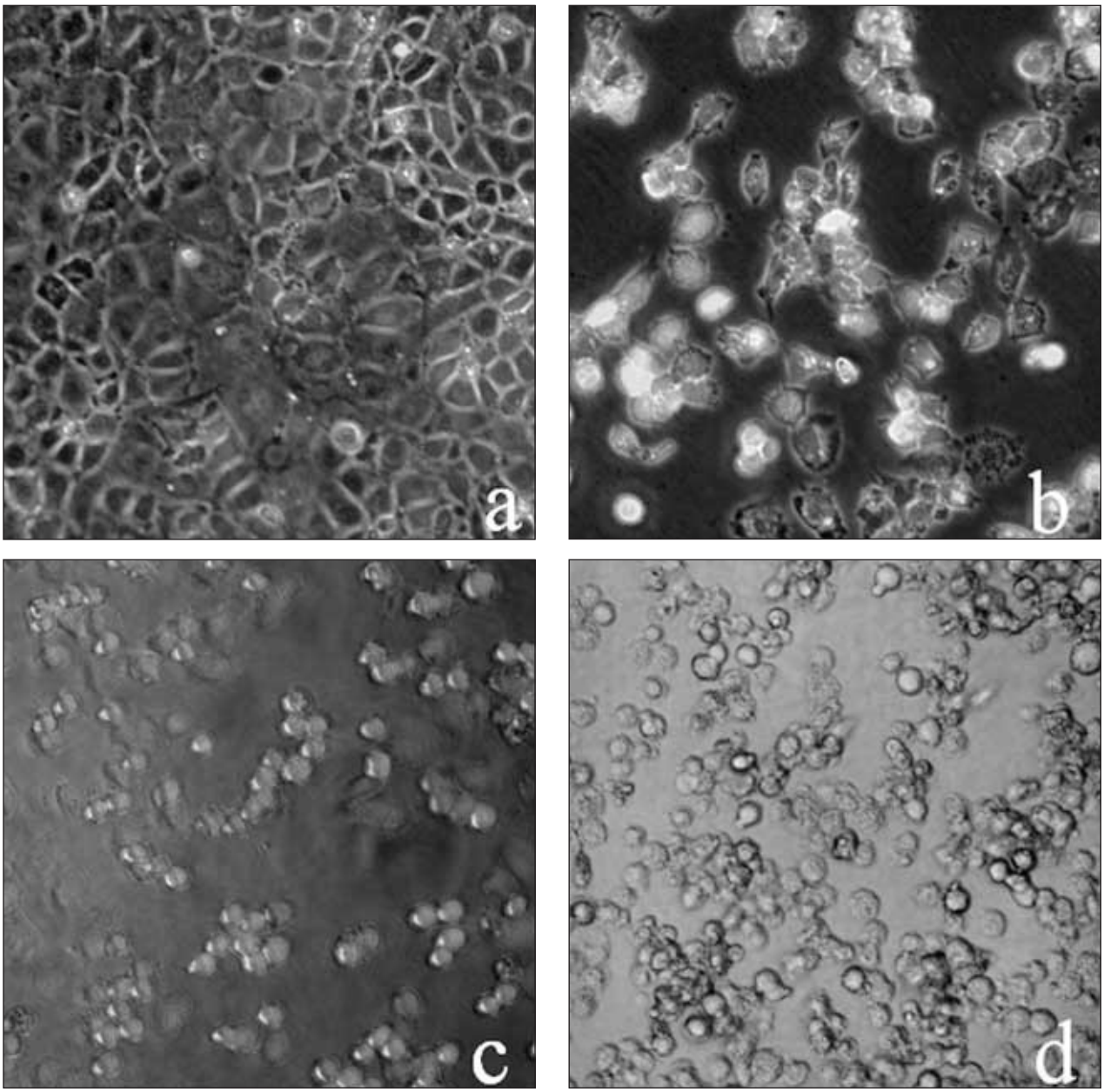

Fig. 5.—Intracellular free radicals. The upper panel shows that CsA (b) produced an increase of the fluorescence emitted by DCFH-DA as compared with control cells (a). The lower panel shows that the staining with mono-bromobimane (mBrB) revealed higher content in reduced thiols in control cells (a) as compared with CsA-treated cells (b).

at sites of contact of the inner and outer mitochondrial membranes.[37, 46-48] It has been described that its opening may condition the release of mitochondrial pro-apoptotic factors such as the cytochrome c. [37, 45, 48] Thus, it has been shown that besides inducing a depolarization of the mitochondrial membrane, CsA also increases the activity of caspase 3 with the occurrence of markers suggestive of cellular apoptosis (positivity for anexin and DNA fragmentation).[24, 36] Moreover, the opening of the pores would originate a decoupling of the respiratory chain and the interruption of the electron transfer because of cytochrome c release and added increase in $O_2^{\cdot-}$ production.[49] At higher doses, CsA may even produce a disruption of

the plasma membrane, LDH release and, finally, cellular necrosis.[36, 50]

Previous studies have shown that CsA produces cellular apoptosis, although the ultimate mechanisms are not completely understood.[51] A plausible hypothesis is that CsA-induced mitochondrial damage with the opening of the MPTPs, mitochondrial depolarization, cardiolipin oxidation, and eventual release of cytochrome c to the cytoplasm has a relevant role in giving way to apoptosis.[45]

In summary, our results suggest that CsA produces an increase in ROS within the mitochondria that, in turn, is affected by this increase, leading to inner membrane cardiolipin oxidation and impairment of the membrane potential; such phenomena may have their consequences on the mitochondrial structure (creation of MPTP and release of cytochrome c) and function (impairment of the respiratory chain and ATP synthesis).[52] In turn, mitochondrial damage may condition the start of events leading to cellular apoptosis or necrosis.

Although it has not been yet clearly shown in humans that CsA- or other drugs-induced nephrotoxicity may be reverted by exogenous antioxidants, our experiments open new lines of knowledge that may help better knowing the role of ROS in renal cells toxicity.

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