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Chapter 6

CYCLOSPORIN A-INDUCED OXIDATIVE STRESS IS NOT THE CONSEQUENCE OF AN INCREASE IN MITOCHONDRIAL MEMBRANE POTENTIAL

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ABSTRACT

Cyclosporin A induces closure of the mitochondrial permeability transition pore. We aimed to investigate whether this closure results in concomitant increases in mitochondrial membrane potential ($\Delta\psi_m$) and the production of reactive oxygen species. Fluorescent probes were used to assess $\Delta\psi_m$ (JC-1, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-benzimidazolyl-carbocyanine iodide), reactive oxygen species [DCF, 5- (and 6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester] and [Ca^{2+}] [Fluo-3, glycine N-[4-[6-[(acetyloxy)methoxy]-2,7-dichloro-3-oxo-3H-xanthen-9-yl]-2-[2-[2-[bis[2-[(acetyloxy)methoxy]-2-oxyethyl]amino]-5-methyl-phenoxy]ethoxy]phenyl]-N-[2-[(acetyloxy)methoxy]-2-oxyethyl]-(acetyloxy)methyl ester] in human kidney cells (HK-2 cells) and in a line of human small cell carcinoma cells (GLC4 cells), because these do not express cyclosporin A-sensitive P-glycoprotein. We used transfected GLC4 cells expressing P-glycoprotein as control for GLC4 cells. NIM811 (N-methyl-4-isoleucinecyclosporin) and PSC833 (SDZ-PSC833) were applied as selective mitochondrial permeability transition pore and P-glycoprotein blockers, respectively. To study the effect of cyclosporin A on mitochondrial function, we isolated mitochondria from fresh pig livers. Cyclosporin A and PSC833 induced a more than two-fold increase in JC-1 fluorescence in HK-2 cells, whereas NIM811 had no effect. None of the three substances induced a significant increase in JC-1 fluorescence in GLC4 cells. Despite this, cyclosporin A, NIM811 and PSC833 induced a 1.5-fold increase in DCF fluorescence ($P < 0.05$) and a two-fold increase in Fluo-3 fluorescence ($P < 0.05$). Studies in isolated mitochondria showed that blockage of mitochondrial permeability transition pores by cyclosporin A affected neither $\Delta\psi_m$, ATP synthesis, nor respiration rate. The mitochondrial permeability transition pore blockers cyclosporin A and NIM811, but also the non-mitochondrial permeability transition pore blocker PSC833, induced comparable degrees of reactive oxygen species production and cytosolic [Ca^{2+}]. Neither mitochondria, effects on P-glycoprotein nor inhibition of calcineurin therefore play a role in cyclosporin A-induced oxidative stress and disturbed Ca^{2+} homeostasis.

INTRODUCTION

Immunosuppressive treatment with cyclosporin A (CsA) is accompanied by accelerated atherosclerosis and fibrosis, which contribute to the development of chronic transplant dysfunction (1). It has been suggested that reactive oxygen species (ROS) play an important underlying role (2–4). Different studies have shown that CsA is able to increase levels of superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide, malondialdehyde, and thiobarbituric acid reactive substances (5,6). Mitochondrial enzymes with antioxidative properties, including superoxide dismutase, catalase, and glutathione peroxidase, become upregulated upon exposure to CsA (7). It is evident that CsA induces oxidative stress, but its origin remains speculative.

Mitochondria represent a major source of intracellular ROS, and play a crucial role in cellular Ca^{2+} homeostasis, which affects various cell signaling pathways (8). The primary function of mitochondria is production of ATP, a process linked to the action of the electron transfer chain. Normally, electrons supplied by metabolic fuel (NADH and $FADH_2$) are transferred along the electron transfer chain to oxygen. Optimally, the terminal enzyme of the electron transfer chain, cytochrome c oxidase, binds oxygen until it has accepted four electrons, when it is released as water. Most of the energy released during the transfer of these electrons is used to pump protons from the mitochondrial matrix towards the inner membrane space, thereby creating a proton gradient. The energy stored in the proton gradient is used to drive the process of oxidative phosphorylation of ADP to ATP. When the intramitochondrial ADP concentration drops (e.g. under conditions of low energy demand), the proton gradient will rise as a consequence of decreased consumption (9–12). This increased proton gradient impairs the flow of electrons along the electron transfer chain, which results in accumulation of electrons along the electron transfer chain (13). This results in an increased likelihood of leakage of electrons from the chain, with increased ROS production as a consequence (14).

One mechanism by which the mitochondrial membrane potential ($\Delta\psi_m$) can decrease is through opening of the mitochondrial permeability transition pore (MPTP) (15–17). CsA is well known as an inhibitor of calcineurin and P-glycoprotein, but it is also a strong inhibitor of the MPTP (18,19). Indeed, it has been suggested that in several cell types CsA prevents opening of the MPTP, thereby leading to an increased $\Delta\psi_m$ (17,20). The CsA analog N-methyl-4-isoleucine-cyclosporin (NIM811) is also known as an inhibitor of MPTP, and to lead to an increase in $\Delta\psi_m$ (21). Fluorescent probes used to assess $\Delta\psi_m$ are pumped out of cells by P-glycoprotein (22). Thus, probe accumulation caused by CsA may result from effects on P-glycoprotein as well as effects on MPTP. The CsA analog SDZ-PSC833 (PSC833) may serve as a useful control substance in this context, because it is an inhibitor of P-glycoprotein rather than MPTP, and is devoid of calcineurin-inhibiting properties (23).

We hypothesized that an increase in steady-state $\Delta\psi_m$ underlies increased ROS production in association with CsA exposure. We set out to investigate the effects of CsA on $\Delta\psi_m$ in relation to the production of ROS, with NIM811 and PSC833 as control.

MATERIALS AND METHODS

Chemicals

The cyclosporin analogs CsA, NIM811 and PSC833 were kindly provided by Novartis (Basel, Switzerland). DNP was obtained from Merck & Co., Inc. (Haarlem, the Netherlands), and sodium succinate, ATP, ADP, KCN and oligomycin were obtained from Sigma-Aldrich Chemie B.V. (Zwijndrecht, the Netherlands). Vincristine sulfate was purchased from Abic Ltd (Netanya, Israel). JC-1, DCF and Fluo-3 were purchased from Molecular probes Europe BV (Leiden, the Netherlands).

Cell culture

Human-derived renal proximal tubular epithelial cells (HK2), obtained from the ATCC (Manassas, VA, USA) were grown in keratinocyte serum-free medium (Gibco-BRL, Breda, the Netherlands), supplemented with 5 $ng \cdot ml^{-1}$ epidermal growth factor, 40 $\mu g \cdot ml^{-1}$ bovine pituitary extract and 20 $\mu g \cdot ml^{-1}$ gentamicin (Centafarm Services, EttenLeur, the Netherlands). The human small cell lung cancer (GLC4) cell line and GLC4 cell line transfected the human multidrug resistance 1 gene were kindly provided by EGE de Vries (Department of Medical Oncology, University Medical Center Groningen, the Netherlands). The cells were grown in RPMI-1640 with 25 mM Hepes and L-glutamine (BioWitthaker, Verviers, Belgium), supplemented with 10% heat-inactivated fetal bovine serum (BioWitthaker) and 20 $\mu g \cdot ml^{-1}$ gentamicin. The multidrug resistance 1-transfected GLC4 cell line expressing P-glycoprotein was grown with a drug pressure of vincristine sulfate (50 nM) until 1 week before the experiments. All cells were grown in 75 cm^2 plastic flasks (Costar, Cambridge, MA) at 37 °C in an atmosphere of 5% CO2.

Immunocytochemical staining of P-glycoprotein

Cytospins of GLC4 and GLC4/P-glycoprotein cells were incubated for 1 min with hematoxylin for staining of nuclei. To assess the localization of P-glycoprotein expression in the membranes, stained cells were evaluated by immunohistochemistry. Monoclonal antibody to P-glycoprotein (C219) (Alexis, San Diego, CA) was used to detect P-glycoprotein expression. An irrelevant antibody was used as isotype control. GLC4 cells without expression of P-glycoprotein did not show P-glycoprotein staining. GLC4 cells expressing P-glycoprotein showed strong and homogeneous membrane-bound staining of P-glycoprotein.

Flow cytometry analyses

Cells at 90% confluence were washed with Hank's buffered saline solution (UMCG, Groningen, the Netherlands) and cultured for 24 h in serum-free medium. After starvation, the cells were resuspended in NaCl/Pi, loaded with a fluorescent probe,

and incubated for 20 min at room temperature in the dark. At the end of the incubation period, cells were washed twice with NaCl/Pi. The loaded cells were incubated for 1 h with different concentration of CsA, NIM811 and PSC833 in an incubator, and analyzed using a FACSCalibur flow cytometer (Becton Dickinson Medical Systems, Sharon, MA).

Isolation of mitochondria from pig liver

Mitochondria were isolated from fresh pig liver using a commercial mitochondria isolation kit (Sigma-Aldrich Chemie B.V.). Briefly, fresh liver tissue (obtained within 1 h of sacrifice) was washed twice with two volumes of extraction buffer (10 mM Hepes, 200 mM mannitol, 70 mM sucrose and 1 mM EGTA, pH 7.5). The liver was cut into small portions and homogenized with 10 volumes of extraction buffer containing 2 mg/ml delipidated BSA (Sigma-Aldrich Chemie B.V.), using a pestle and glass tube. The homogenate was centrifuged at 600 g for 5 min using an Eppendorf centrifuge 5417R, rotor F45-30-11, Eppendorf AG, Hamburg, Germany (all centrifugation carried out using same centrifuge and rotor types). The supernatant was collected and centrifuged at 11 000 g for 10 min. The supernatant was then removed, and the pellet was resuspended in 10 volumes of extraction buffer and centrifuged at 600 g for 5 min. Finally, the supernatant was centrifuged at 11 000 g for 10 min. The supernatant was removed, and the isolated mitochondria were resuspended in respiratory buffer (120 mM KCl, 5 mM K_2PO_4, 3 mM Hepes, 1 mM EGTA, brought to pH 7.2 with 5 mM KH_2PO_4). Mitochondrial protein was estimated by the Bradford method (Bio-Rad Laboratories, Veenendaal, the Netherlands) according to the manufacturer's instructions. To stabilize the mitochondria, respiration buffer was supplemented with 0.2% delipidated BSA (m/v).

Mitochondrial swelling assay

Mitochondria were resuspended in respiration buffer (without EGTA) containing 4 mg mitochondrial protein/ml. The mitochondria were energized with succinate (final concentration 5 mM). The suspension was pipetted into the wells (100 µL per well) of a 96-well polystyrene microtiter plate (Greiner Bio-One B.V., Alphen aan den Rijn, the Netherlands). CsA and its analogs (0.1–10 µM) were added. Five minutes after addition of the CsA and its analogs, $CaCl_2$ (final concentration 1 mM) was added to each well. Immediately after addition of $CaCl_2$, the plate was measured in an EL808 spectrophotometer using a 550 nm filter (Bio-Tek Instruments, Abcoude, the Netherlands). Measurements were repeated every 30 s for a period of 30 min. Measurements were performed at room temperature.

$\Delta\psi_m$ in isolated mitochondria
Isolated mitochondria were resuspended (final protein concentration 100 $\mu g \cdot ml^{-1}$) in 1 mL of respiration buffer. Mitochondria were stained with 0.2 $\mu g \cdot ml^{-1}$ JC-1 probe for 10 min at 37 °C according to the manufacturer's instruction. State III respiration was reached after addition of succinate (final concentration 5 mM) and ADP (final concentration 1 mM). The loaded mitochondria were then suspended in the wells of a 96-well fluorescent plate (Costar) and exposed to 10 µM CsA for 15 min at 37 °C. DNP (final concentration 20 µM) served as negative control. $\Delta\psi_m$ was measured with an excitation wavelength of 485 nm through a 590 nm bandpass filter in a FL500 fluorescent plate reader (Bio-Tek Instruments).

Luminescence monitoring of mitochondrial ATP
Mitochondria (final protein concentration 100 $\mu g \cdot ml^{-1}$) were resuspended in respiration buffer. All experiments were done in state III respiration. DNP (final concentration 20 µM) and state IV respiration served as negative controls. Mitochondria were incubated for 15 min at 37 °C. At the end of the incubation period, ATP synthesis was stopped by freezing the samples in) 196 °C nitrogen. Mitochondrial ATP levels were measured using the Enliten ATP assay (Promega, Leiden, the Netherlands) and a Berthold microplate luminometer (Berthold Detection Systems GmbH, Pforzheim, Germany).

Mitochondrial respirometry
Mitochondria (final concentration 2 $mg \cdot ml^{-1}$) were resuspended in a 1 mL respiration chamber with air-saturated respiration buffer (209 µM O_2). CsA (final concentration 10 µM) was preincubated for 5 min. State III respiration was started after addition of succinate (final concentration 5 mM) and ADP (final concentration 0.5 mM). Mitochondrial respiration was measured at 37 °C with a commercial Clark-type oxygen electrode (U-53002-50; Cole-Parmer, Schiedam, the Netherlands) covered with a high-sensitivity membrane (Yellow Springs Instruments Co., Inc., Ohio, USA), continuously registered with the Oxystat interface and STRATHKELVIN 928 oxygen system software (U-53002-05; Cole-Parmer).

Mitochondrial ROS production
Mitochondrial ROS were measured using the procedure of Garcia-Ruiz et al. (39). Briefly, production of ROS was monitored using the fluorescent probe DCF. Freshly isolated mitochondria (100 $\mu g \cdot ml^{-1}$) were incubated in respiratory buffer (without EGTA) with 1 µM DCF in the absence or presence of 1 mM Ca^{2+}. The effects of CsA (10 µM) were tested under conditions of state III respiration. Antimycin A was used to stimulate maximal ROS production by inhibiting complex III of the electron transfer chain. The mitochondrial suspension was incubated for 15 min at 37 °C. DCF fluorescence was monitored with an excitation

wavelength of 485 nm through a 530 nm bandpass filter in an FL500 fluorescent plate reader.

Statistical analysis

Data were analyzed using PRISM 4 for Windows (GraphPad Software, Inc., San Diego, CA). Two-way ANOVA was used for assessment of dose–response experiments (Figs 2 and 4). Comparisons between different experimental groups were performed with the Newman–Keuls multiple comparison test (Figs 1, 3 and 5). $P < 0.05$ was considered significant. Results are presented as mean (± SEM) unless otherwise mentioned.

RESULTS

Closure of the MPTP and $\Delta\Psi_m$

Human kidney (HK-2) cells are known to express P-glycoprotein (24,25). Both CsA and PSC833 induced a dose-dependent increase in 5,5',6,6'-tetrachloro-1, 1', 3,3'-tetraethyl-benzimidazolyl-carbocyanine iodide (JC-1) fluorescence in these cells (Fig. 1). NIM811, however, did not induce a significant increase in JC-1 fluorescence.

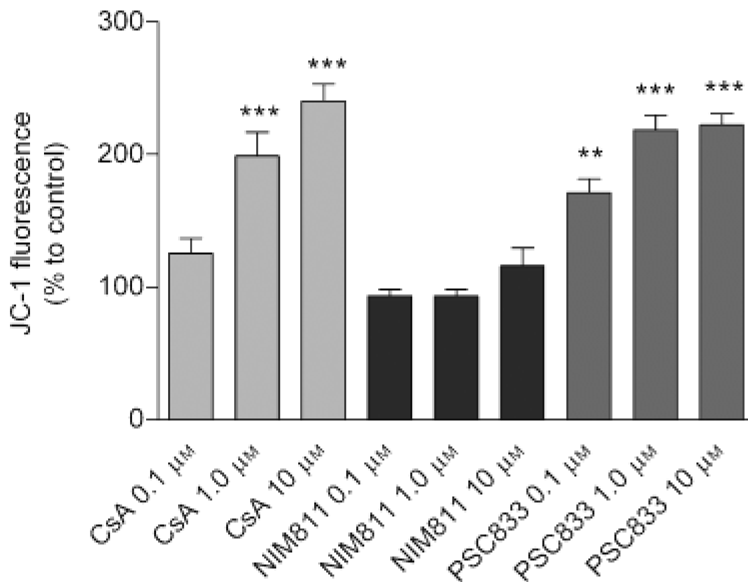

Figure 1. Effect of CsA and its analogs on mitochondrial membrane potential in HK-2 cells. JC-1 probe (5 µg·mL^{-1}) was used to study mitochondrial membrane potential. Data are expressed as mean value ± SEM, and refer to three experiments. *$P < 0.05$ versus control, **$P < 0.01$ versus control, ***$P < 0.001$ versus control by Newman–Keuls multiple comparison test.

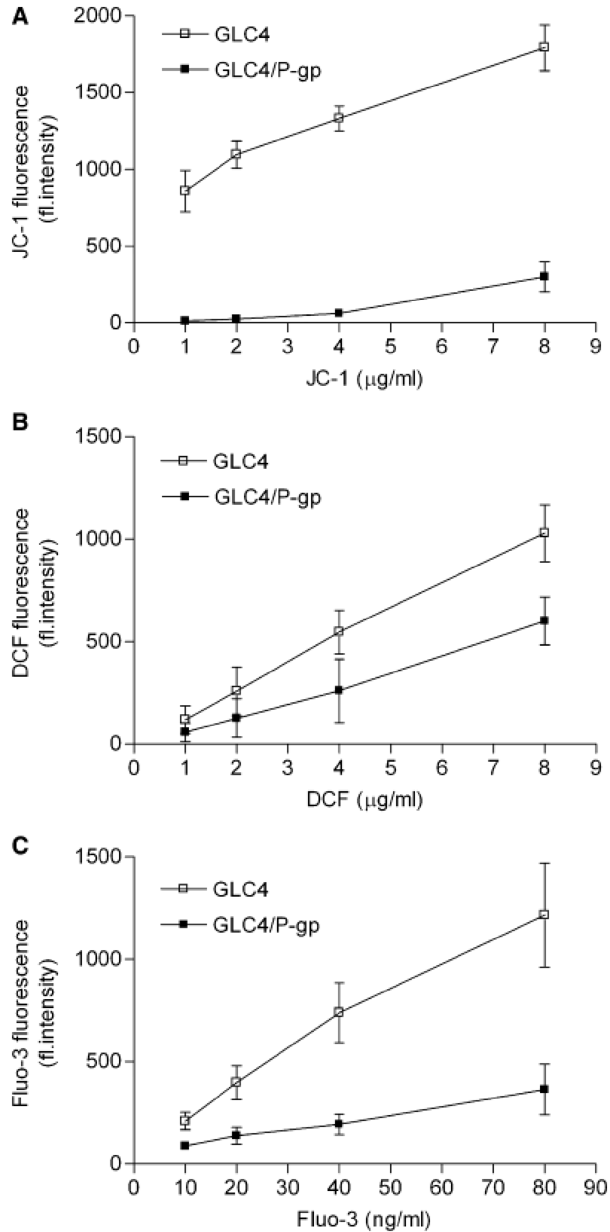

Figure 2. Probe accumulation in GLC4 cells without expression of P-glycoprotein (GLC4) and GLC4 cells with expression of P-glycoprotein (GLC4/P-gp). After loading of cells with probes and subsequent washing, they were kept in culture medium for 1 h, and then measured by flow cytometry. (A) Dose–response curve of JC-1 (mitochondrial membrane potential). (B) Dose–response curve of DCF (intracellular levels of ROS). (C) Dose–response curve of Fluo-3 (intracellular levels of Ca^{2+}). The data presented are from at least three independent experiments, and represent the mean value ± SEM. If no error bar appears, it is hidden by the marker for the mean value.

We used human small cell carcinoma (GLC4) cells and GLC4/P-glycoprotein cells to investigate the effects of CsA and its analogs on $\Delta\Psi_m$. There were no significant increases in JC-1 fluorescence in response to either CsA or its analogs in GLC4 cells. Inhibition of P-glycoprotein by CsA and its analogs, including NIM811, resulted in significant increases in JC-1 fluorescence as compared to GLC4/P-glycoprotein control cells untreated with CsA and its analogs (Fig. 3A).

We also used GLC4 cells to investigate CsA and its analogs in the absence of disturbing effects mediated by inhibition of P-glycoprotein pumps. Analyses with DCF as probe for assessment of ROS production showed, for all three analogs, a significant, more than 1.5-fold, increase in fluorescence (Fig. 3B). Treatment with the antioxidant vitamin E blunted these increases in DCF fluorescence. The Fluo-3 measurements presented in Fig. 3C suggest increases in cytosolic [Ca^{2+}] in response to CsA and its analogs. Both the intracellular Ca^{2+} chelator BAPTA and the extracellular Ca^{2+} chelator EGTA caused significant attenuation of the effects of CsA and its analogs on Fluo-3 fluorescence.

Effects of CsA and its analogs on mitochondrial function

We concluded that experiments in isolated mitochondria were necessary to discern whether mitochondria could be a source of increased ROS production, because we observed ROS production with CsA and both of its analogs even in GLC4 cells that were devoid of P-glycoprotein. To perform these experiments, we used mitochondria that were isolated from fresh liver obtained from pigs. We first confirmed that CsA and NIM811 actually inhibit the MPTP, using the mitochondrial swelling assay. As shown in Fig. 4, isolated mitochondria undergo large-amplitude swelling that is dependent on Ca^{2+}, which is a classical inducer of MPTP opening. Pretreatment of mitochondria with CsA (1 and 10 μM) and NIM811 (10 μM) significantly reduced mitochondrial swelling, whereas CsA (0.1 μM) and PSC833 (10 μM) did not.

Isolated mitochondria

To further examine whether closure of the MPTP results in an increase in $\Delta\Psi_m$, isolated mitochondria were loaded with JC-1. After addition of succinate and ADP, state III respiration was reached. Figure 5A shows that CsA did not result in an increase in JC-1 fluorescence. In response to induction of state IV respiration, however, JC-1 fluorescence increased by 13.5 ± 2.8%. The protonophore 2,4-dinitrophenol (DNP), which dissipates $\Delta\Psi_m$, resulted in a significant (50.7 ± 12.9%, $P < 0.001$) decrease.

Mitochondrial ATP levels were monitored during state III respiration. CsA did not result in an increase in ATP production (Fig. 5A). State IV respiration and DNP were used as negative controls. State IV respiration could not result in ATP production, because there was no supply of ADP. Addition of DNP, an established uncoupler of oxidative phosphorylation, resulted in a decrease in ATP to 28.9 ± 4.5% ($P < 0.01$) as compared to state III.

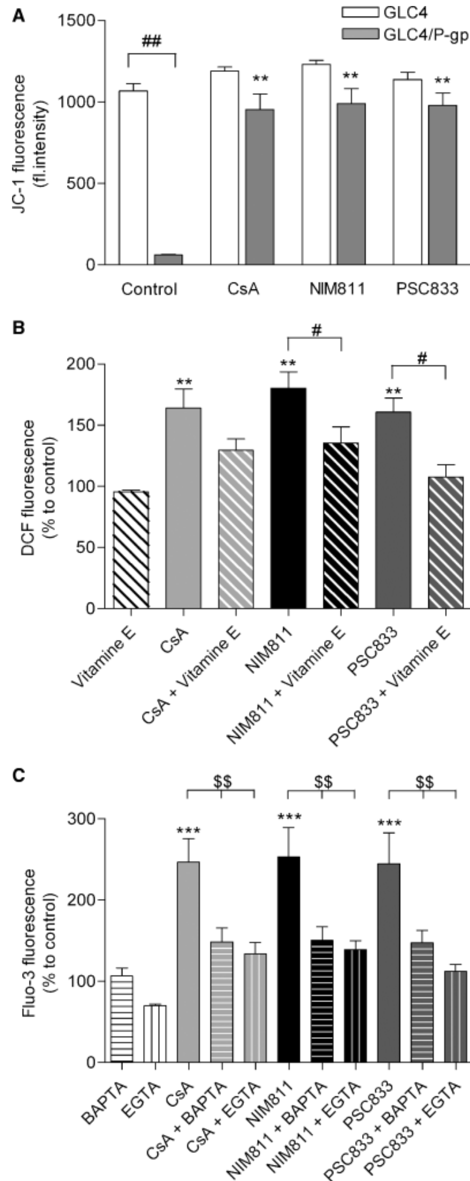

Figure. 3. Effects of CsA (10 μM), NIM811 (10 μM) and PSC833 (10 μM) in GLC4 cells without expression of P-glycoprotein (GLC4) and GLC4 cells expressing P-glycoprotein (GLC4/P-gp). (A) JC-1 (5 μg·mL^{-1}) was used to assess mitochondrial membrane potential. (B) DCF (5 μg·mL^{-1}) was used to detect the generation of ROS. (C) Fluo-3 (50 ng·mL^{-1}) was used to determine Ca^{2+} levels. The data presented are from four independent experiments, and represent the mean value ± SEM. (A) $^{##}P < 0.01$ versus GLC4; $^{**}P < 0.01$ versus control. (B) $^{**}P < 0.01$ versus control; $^{\#}P < 0.05$ versus vitamin E (200 μM) treatment. (C) $^{***}P < 0.001$ versus control; $$$P < 0.01$ versus BAPTA (10 μM) or EGTA (0.1 mM). *P*-values are according to the Newman–Keuls multiple comparison test.

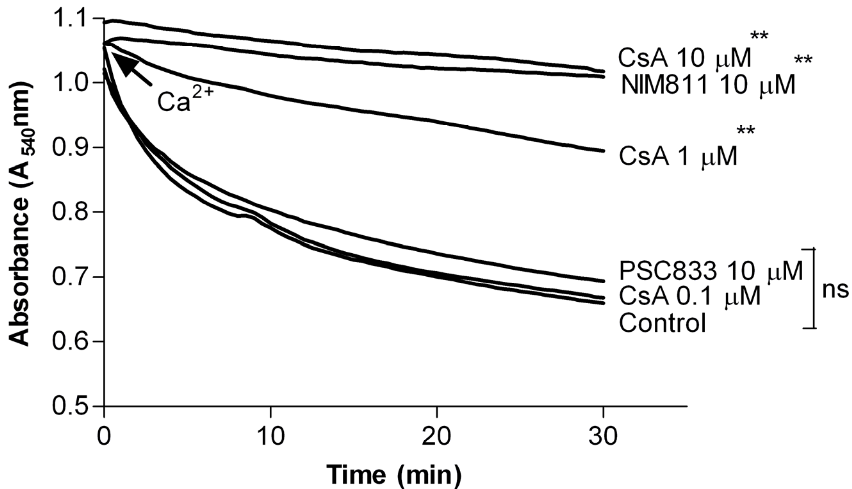

Figure. 4. Effects of different concentrations of CsA and its analogs on the Ca^{2+}-dependent induction of opening of the MPTP. The data are representative of four experiments. A concentration of 1 mM Ca^{2+} was used to induce opening of the MPTP. CsA (1 and 10 μM) and NIM811 (10 μM) caused significant inhibition of mitochondrial swelling. $**P < 0.01$ versus control; ns, not significant by two-way ANOVA.

Oxygen consumption was monitored with sequential addition of succinate, ADP (to induce state III respiration) and CsA, until state IV respiration was reached again, when all ADP was converted to ATP. DNP was then added, followed by KCN (Fig. 5B). Isolated mitochondria were incubated in an oxygraph sample chamber with air-saturated respiration buffer in these experiments. After addition of succinate as metabolic substrate, mitochondria start to respire (4.8 ± 0.5 nmol $O_2 \cdot min^{-1} \cdot mg^{-1}$). Addition of ADP causes a burst of oxygen uptake (15.4 ± 1.8 nmol $O_2 \cdot min^{-1} \cdot mg^{-1}$). The respiratory control index was 3.2 ± 0.3. Addition of CsA during state III respiration did not cause a significant change in oxygen consumption as compared to state III control. DNP was used as positive control. Uncoupling of the mitochondria caused a burst of oxygen uptake (17.8 ± 4.0 nmol $O_2 \cdot min^{-1} \cdot mg^{-1}$). KCN, a blocker of complex IV, was used as negative control. Addition of KCN acutely blocked respiration of the uncoupled mitochondria (2.2 ± 6.8 nmol $O_2 \cdot min^{-1} \cdot mg^{-1}$).

Finally, we examined whether CsA exposure induces changes in ROS production during state III respiration in the presence and absence of 1 mM Ca^{2+}. Mitochondrial ROS production was monitored with DCF in these experiments. Figure 5C shows that addition of Ca^{2+} results in a significant increase in DCF fluorescence. Antimycin A, a blocker of complex III and a well-known inducer of ROS production (26), was used as positive control. Addition of CsA resulted in significant attenuation of DCF fluorescence during state III respiration, both in the absence and in the presence of Ca^{2+}, with no significant difference between the latter two conditions.

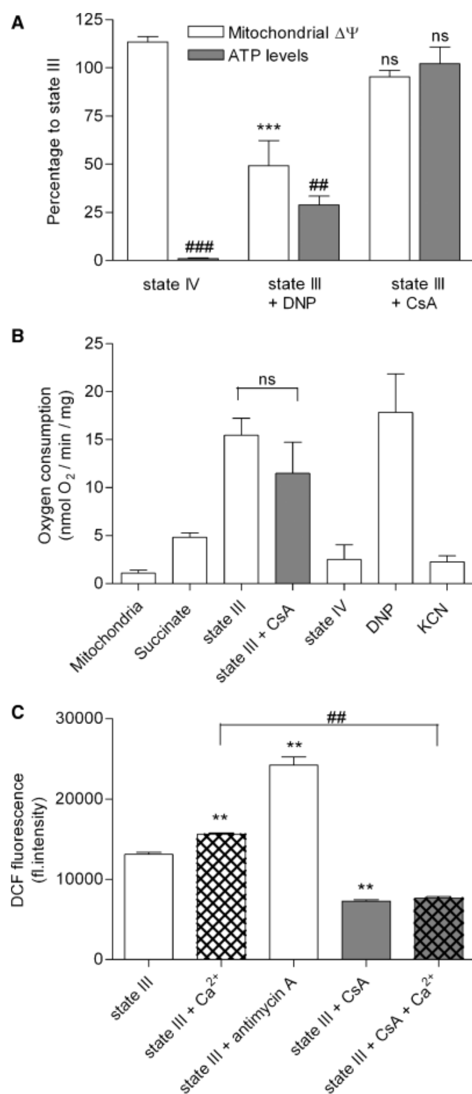

Fig. 5. Effects of 10 µM CsA in isolated liver mitochondria. (A) Mitochondrial membrane potential ($\Delta\Psi_m$) and ATP levels. (B) Respiration rate. (C) ROS. Measurements for assessment of $\Delta\Psi_m$, ATP levels and ROS were performed under different conditions. Measurements of oxygen consumption for assessment of respiration rate represent four experiments in which isolated mitochondria were subsequently exposed to different conditions, starting with respiration medium with mitochondria alone (indicated as mitochondria) and ending with addition of KCN (indicated as KCN). JC-1 (0.2 µg·mL^{-1}) probe was used to monitor mitochondrial membrane potential. Mitochondrial ATP levels were quantified by using a chemiluminescent ATP assay. Mitochondrial respiration rate was measured using an oxygraph. DCF (1 µg·mL^{-1}) was used to quantify ROS. Data are expressed as mean value ± SEM and are representative of four experiments. (A) (mitochondrial $\Delta\Psi_m$) ***$P < 0.001$ versus state III; ns, not significant. (A) (ATP levels) $^{\#\#}P < 0.01$ versus state III; $^{\#\#\#}P < 0.001$ versus state III; ns, not significant. (B) ns, not significant; (C) **$P < 0.01$ versus state III; $^{\#\#}P < 0.01$ versus state III + Ca^{2+}. *P*-values are according to the Newman–Keuls multiple comparison test.

DISCUSSION

In this study, we found that CsA induces increases in the production of ROS and in cytosolic [Ca^{2+}]. In contrast to expectations, we found that these increases cannot be explained by blockage of the MPTP by CsA. We also found that both the MPTP-blocking CsA analog NIM811 and the P-glycoprotein-blocking CsA analog PSC833 induced comparable increases in ROS and cytosolic [Ca^{2+}] to those induced by CsA. All three compounds induced a comparable degree in ROS production in cells devoid of P-glycoprotein. Unlike CsA, both NIM811 and PSC833 do not inhibit calcineurin. Together, these results suggest that neither mitochondria, inhibition of calcineurin nor blockage of P-glycoprotein play a role in the comparable induction of ROS production by CsA and its analogs.

We found that CsA induces a strong increase in JC-1 fluorescence in intact human kidney cells. Studies in human neuroblastoma cells have found the same phenomenon [20,27]. It was suggested by these authors that this action of CsA derives from its inhibition of the MPTP at the level of mitochondria. Our experiments with PSC833, which we confirmed to be a potent inhibitor of P-glycoprotein pumps, but not of the MPTP, showed that it induced comparable increases in JC-1 fluorescence as CsA (Fig. 1). P-glycoprotein is located at the plasma membrane, and is expressed in almost every cell type, including human kidney and neuroblastoma cells [28–30]. This pump is able to excrete many different fluorescent probes [22,31]. Our data imply that the fluorescent probe JC-1 accumulates in cells as a consequence of P-glycoprotein inhibition, resulting in an apparent increase in $\Delta\Psi_m$. We also subsequently tested whether P-glycoprotein pumps are involved in the accumulation of other fluorescent probes, because this might disturb the interpretation of our data concerning these probes. Parallel experiments with the fluorescent probes JC-1, DCF and Fluo-3 in GLC4 cells with and without expression of P-glycoprotein provided evidence that probe accumulation and probe excretion were influenced by the presence of these pumps (Fig. 2A–C). Cells that did not express P-glycoprotein accumulated the probes, resulting in a strong fluorescence signal. We can conclude that P-glycoprotein expression gives rise to false-positive results that do not correspond to the increases in $\Delta\Psi_m$, ROS production and [Ca^{2+}] that the investigated probes were intended to assess. This is a major problem in the interpretation of studies, because many pharmacologic agents can influence the efflux of probes mediated by P-glycoprotein pumps [20,27,32].

In order to investigate the effect of MPTP blockage by CsA in the absence of disturbance by probe efflux effects due to P-glycoprotein pumps, we used GLC4 cells without these pumps. Our study is the first to compare the effect of CsA on intracellular probe accumulation between non-P-glycoprotein-containing cells and the same cells transfected in such a way that they express this protein. We demonstrated that in cells that do not contain P-glycoprotein, neither CsA, NIM811 nor PSC833 was able to induce a significant increase in JC-1 fluorescence (Fig. 3A). This was confirmed at the level of isolated mitochondria (Fig. 5A). CsA blockage of the temporary opening of the MPTP in state III respiration did not result in an increase in JC-1 fluorescence. If closure of the MPTP by CsA altered the flow of electrons along the electron transfer chain, mitochondrial ATP synthesis and

respiration rate would be expected to alter. Our results showed that neither ATP synthesis nor mitochondrial respiration rate was influenced by CsA, which is similar to the observations by Sanchez *et al.* [33]. Strzelecki *et al.* showed that CsA inhibited the process of spontaneous Ca^{2+} discharge in isolated mitochondria [34]. CsA also inhibited the swelling and respiration induced by accumulated Ca^{2+} in these experiments. The authors concluded that CsA at an immunosuppressive level impairs Ca^{2+}-induced membrane permeability. Our findings show that Ca^{2+}-induced increases in mitochondrial ROS production can be prevented by CsA, thereby virtually excluding mitochondria as source of increased ROS production in response to exposure of cells to CsA. Elzinga *et al.* showed that Ca^{2+} uptake by mitochondria isolated from renal cortical cells of rats that had been treated with CsA for 2 weeks was significantly lower than Ca^{2+} uptake by mitochondria isolated from control rats [35]. It was not investigated whether there was an increased concentration gradient as a consequence of prior intramitochondrial Ca^{2+} accumulation. If this was the case, it could be that long-term treatment with CsA results in a net zero effect on mitochondrial ROS production under steady-state conditions *in vivo*.

We showed in experiments in isolated mitochondria that the classic MPTP inducer Ca^{2+} leads to mitochondrial swelling, and that this can be blocked by CsA and NIM811, but not by PSC833. Our experiments in whole cells suggested that both CsA, NIM811 and PSC833 induce an increase in cytosolic [Ca^{2+}] and production of ROS. The discrepancy in the effects of PSC833 between GLC4 cells and isolated mitochondria versus the consistency in the effects of CsA and NIM811 in these experiments suggests that MPTP blockage plays a role neither in the observed increases in cytosolic [Ca^{2+}] nor in increases in ROS production. To further substantiate this suggestion, we performed experiments in the presence and absence of 1 mM Ca^{2+} in isolated mitochondria. We found significant stimulation of ROS production after addition of Ca^{2+} during state III respiration, whereas CsA resulted in a significant attenuation of ROS production.

We observed the same degree of increased ROS production in response to CsA and its analogs NIM811 and PSC833. The increased production was not of mitochondrial origin. It is also unlikely that the calcineurin-inhibiting properties of CsA play a role, because neither NIM811 nor PSC833 inhibit calcineurin. It is furthermore also unlikely that the P-glycoprotein-inhibiting properties of CsA play a role, because CsA, NIM811 and PSC833 all increased ROS production to the same degree in cells that were devoid of P-glycoprotein. ROS-forming candidates that may explain this side-effect of CsA are NADPH oxidase, endoplasmic reticulum cytochrome P450, and glycolate oxidase. Recently, a study by Vetter *et al.* suggested that CsA activates NADPH oxidase and generates release of $O_2^{\cdot -}$ [36]. Other studies have found increased arachidonic acid omega-hydroxylation activity by CsA. The omega-hydroxylation of arachidonic acid is an activity associated with members of the cytochrome P450 family [37,38].

In conclusion, our results showed induction of increased ROS production and cytosolic [Ca^{2+}] by CsA and its analogs. However, mitochondria, involvement of P-glycoprotein and inhibition of calcineurin are unlikely to play a role in CsA-induced oxidative stress and disturbed Ca^{2+} homeostasis. Care must be taken in the use of fluorescent probes in P-glycoprotein-expressing cells when substances with P-glycoprotein-blocking properties, such as CsA, are investigated, because this may

result in false-positive signals. More detailed *in vitro* studies are required to further elucidate the mechanisms responsible for the CsA-induced toxicity.

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