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Cyclosporin A-induced free radical generation is not mediated by cytochrome P-450

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1 Reactive oxygen species (ROS) have been proposed to play a role in the side effects of the immunosuppressive drug cyclosporin A (CsA).

2 The aim of this study was to investigate whether cytochrome P-450 (CYP) dependent metabolism of CsA could be responsible for ROS generation since it has been suggested that CsA may influence the CYP system to produce ROS.

3 We show that CsA (1–10 μM) generated antioxidant-inhibitable ROS in rat aortic smooth muscle cells (RASMC) using the fluorescent probe 2,7-dichlorofluorescin diacetate.

4 Using cytochrome *c* as substrate, we show that CsA (10 μM) did not inhibit NADPH cytochrome P-450 reductase in microsomes prepared from rat liver, kidney or RASMC.

5 CsA (10 μM) did not uncouple the electron flow from NADPH *via* NADPH cytochrome P-450 reductase to the CYP enzymes because CsA did not inhibit the metabolism of substrates selective for several CYP enzymes that do not metabolize CsA in rat liver microsomes.

6 CsA (10 μM) did not generate more radicals in CYP 3A4 expressing immortalized human liver epithelial cells (T5-3A4 cells) than in control cells that do not express CYP 3A4.

7 Neither diphenylene iodonium nor the CYP 3A inhibitor ketoconazole were able to block ROS formation in rat aortic smooth muscle or T5-3A4 cells.

8 These results demonstrate that CYP enzymes do not contribute to CsA-induced ROS formation and that CsA neither inhibits NADPH cytochrome P-450 reductase nor the electron transfer to the CYP enzymes.

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Keywords: Cyclosporin A; cytochrome P-450; metabolism; microsomes; NADPH cytochrome P-450 reductase; reactive oxygen species; smooth muscle cells

Abbreviations: BFC, benzyloxy-4-(trifluoromethyl)-coumarin; CsA, cyclosporin A; CYP, cytochrome P-450; 2,7-DCFH, 2,7-dichlorofluorescin diacetate; DPI, diphenylene iodonium; ERO, ethoxyresorufin; NAC, N-acetyl-L-cysteine; PRO, pentoxyresorufin; RASMC, rat aortic smooth muscle cells; ROS reactive oxygen species; SOD, superoxide dismutase

Introduction

Cyclosporin A (CsA) is the most widely used immunosuppressive drug indicated after organ transplantation and in the therapy of autoimmune diseases (Borel *et al*., 1996). It acts by binding to cyclophilin to inhibit calcineurin phosphatase activity, NF-AT dephosphorylation and IL-2 expression, thus preventing T-lymphocyte proliferation (Borel *et al*., 1996; Rao *et al*., 1997; Aramburu *et al*., 2000). The therapeutic use of this drug, however, is limited by its side effects of which nephrotoxicity and hypertension are clinically the most important (Kahan, 1989; Mason, 1990; Textor *et al*., 1994). Both are likely caused by CsA-induced local vasoconstriction (Lamb & Webb, 1987; Rego *et al*., 1990).

We have shown previously that the vasoconstrictive effect of CsA was due to increased cytosolic free calcium ion concentrations $[Ca^{2+}]_c$ in rat and human aortic smooth muscle cells stimulated with vasoconstrictor hormones such as noradrenalin, vasopressin, angiotensin II, serotonin or endothelin-1 (Lo Russo *et al*., 1996; Avdonin *et al*., 1999). We have also demonstrated that this effect was due to an upregulation of the corresponding vasoconstrictor hormone receptor (the V_1 vasopressin and the AT_1 angiotensin receptor) (Lo Russo *et al*., 1997A, B; Avdonin *et al*., 1999). Recently, we have shown that the upregulation of the V_1 receptor was due to stabilization of its mRNA in rat aortic smooth muscle cells (RASMC) (Cottet-Maire *et al*., 2001). Upregulation of vasoconstrictor hormone receptors would be responsible for an enhanced vasoconstriction under *in vivo* conditions, thus leading, *via* an increase in peripheral resistance, to hypertension and to a decrease in glomerular filtration (Kahan, 1989; Mason, 1990; Textor *et al*., 1994). However, the exact mechanisms by which CsA enhances vasoconstriction have not yet been clarified.

It has been proposed that reactive oxygen species (ROS) could be one of the mediators involved in the toxic actions of CsA (Ahmed *et al*., 1993; Wang & Salahudeen, 1995;

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Stroes *et al*., 1997; Lopez-Ongil *et al*., 1998). Some studies have reported that CsA was able to generate ROS in diverse cellular systems (Perez de Lema *et al*., 1998; Zhong *et al*., 1998; L'Azou *et al*., 1999), but the source has not been identified. ROS have been assigned a role as biological mediators of cellular signals (Lander, 1997). For example, it has been shown that PDGF-induced cell proliferation was dependent on the cellular production of H_2O_2 (Sundaresan *et al*., 1995). In addition, it has been demonstrated that ROS activate several transcription factors, such as nuclear factor-κB (NF-κB) or AP-1 (Schreck *et al*., 1991; Meyer *et al*., 1993; Sen & Packer, 1996; Dalton *et al*., 1999). Therefore, ROS could play a role as initial triggers for the direct or indirect modulation of several second messengers, among which calcium, necessary for vasoconstriction.

Cytochrome P-450 3A (CYP 3A) enzymes are thought to be predominantly, if not exclusively, responsible for the metabolism of CsA (Kronbach *et al*., 1988; Combalbert *et al*., 1989) and the CYP system is also known to be a source of ROS. It has already been shown that ROS can arise from the decay of oxygenated CYP intermediates produced during the catalytic mechanism of mixed-function oxidation (Kuthan *et al*., 1978; Kuthan & Ullrich, 1982; Bondy & Naderi, 1994; Paolini *et al*., 1996; Coon *et al*., 1998). Also, isolated liver microsomes were shown to produce ROS during NADPH-dependent electron transfer (Prough & Masters, 1973; Rashba-Step & Cederbaum, 1994; Perret & Pompon, 1998). Therefore, it is not surprising that a number of studies have suggested that CYP-dependent metabolism of CsA could be involved in ROS formation (Inselmann *et al*., 1991; Serino *et al*., 1993; 1994). Besides, it has been shown that CsA produced ROS in rat liver microsomes (Ahmed *et al*., 1993; 1995). However, concentrations of CsA used for these experiments largely exceeded therapeutical concentrations in patients treated with CsA (maximal 0.1 to 1 μM). Moreover, because the solubility of CsA in aqueous solutions is limited (about 10 μM in 0.1% ethanol), several studies used surfactants/detergents or higher concentrations of solvents. CYP enzymes are very sensitive to both detergents and solvents, and microsomal CYP enzymes are already inhibited at detergent concentrations as low as 0.01% (own results, not shown) and at solvent concentrations above 1% (Busby *et al*., 1999; Buetler *et al*., 2000).

Several authors have proposed that ROS generation and lipid peroxidation could be due to an inhibition of the NADPH cytochrome P-450 reductase by CsA in liver microsomes (Ahmed *et al*., 1993; 1995; Serino *et al*., 1993; 1994). In fact, uncoupling of the microsomal electron transfer from NADPH *via* NADPH cytochrome P-450 reductase to the CYP enzymes may enhance ROS generation (Rashba-Step & Cederbaum, 1994). All this evidence points to a possible interference of CsA with the CYP system, leading to ROS formation. Our previous studies also suggest that CsA metabolism may lead to ROS formation (Nguyen *et al*., 1999).

In this study we thoroughly investigated whether CsA had a direct effect on the CYP system in rat liver microsomes. Furthermore, we tested whether CYP-dependent metabolism of CsA was capable of producing ROS in rat aortic smooth muscle cells and immortalized human liver epithelial cells expressing CYP 3A4.

Methods

Chemicals and buffers

CsA was a gift from Novartis Pharma (Basel, Switzerland). Dulbecco's modified Eagle's medium (DMEM) and foetal calf serum (FCS) were from Life Technologies (Basel, Switzerland). Ciproxin was from Bayer Pharma AG (Zürich, Switzerland). Pasadena Foundation for Medical Research Medium #4 (PMFR-4) was from Biofluids, Inc. (Rockville, MD, U.S.A.) and 2,7-dichlorofluorescin diacetate (2,7-DCFH) was from Molecular Probes (Juro Supply AG, Lucerne, Switzerland). Ethoxyresorufin (ERO), pentoxyresorufin (PRO), D, L-α-tocopherol, resveratrol, N-acetyl-L-cysteine (NAC) and cytochrome *c* (type VI) were from Sigma (Buchs, Switzerland). 7-Benzyloxy-4-(trifluoromethyl)-coumarin (BFC) was from Gentest and ketoconazole was from Biomol and were purchased from Anawa Trading Corporation (Wangen, Switzerland). Silymarin was from Calbiochem (Juro Supply AG, Lucerne, Switzerland). Diphenylene iodonium (DPI) was purchased from Alexis Corporation (Läufelfingen, Switzerland). PCR buffer, Q-solution and Taq DNA polymerase were from Qiagen (Basel, Switzerland) and dNTPs were purchased from Promega (Catalys AG, Wallisellen, Switzerland). Stock solutions of chemicals were prepared in ethanol, buffer or DMSO. CsA stock solutions were prepared in ethanol at a concentration of 10^{-2} M and diluted to 10 μM for experiments, thus resulting in EtOH concentration of 0.1% maximally. In all fluorescence experiments, 0.1% EtOH or DMSO served as control and were set at 100%. The physiological salt solution (PSS) contained (in mM) NaCl 145, KCl 5, $MgCl_2$ 1, $CaCl_2$ 1, HEPES 5, and glucose 10, adjusted to pH 7.4.

Cultures of smooth muscle cells

Rat aortic smooth muscle cells (RASMC) were prepared from aortae of male Wistar Kyoto rats (200–300 g) as described (Lo Russo *et al*., 1996). RASMC were cultured in DMEM supplemented with essential amino acids, vitamins, 0.001% ciproxin and 10% FCS, and kept at 37°C in a humidified atmosphere of 5% CO_2 in air. For studies with CsA, cells were used at confluence (after 7 to 9 days of culture) between passages 6 and 11.

Twenty-four hours before experiments, culture media were replaced with fresh DMEM without FCS. Treatment of RASMC with CsA and other agents was always performed in serum-free DMEM.

CYP 3A4-expressing human liver epithelial cells (T5-3A4)

The T5-3A4 cell line, a non-tumorigenic SV40 large T-antigen-immortalized human liver epithelial cell line (THLE) (Pfeifer *et al*., 1993) transfected with an expression vector containing a human CYP 3A4 cDNA (Macé *et al*., 1997) and the control T5-neo cell line were seeded on fibronectin/collagen coated flasks in PMFR-4 serum free low calcium medium with the following additives: 2 mM L-glutamine, 50 μg ml^{-1} gentamycin, 1.75 μM insulin, 0.2 μM hydrocortisone, 5 ng ml^{-1} EGF, 10 μg ml^{-1} transferrin, 500 nM phosphoethanol/ethanolamine, 50 nM triiodothyronine,

15 μg ml^{-1} bovine pituitary extract, 0.33 nM retinoic acid and 3% factor free serum.

Preparation of microsomes

Male Sprague-Dawley rats weighing 200–230 g were killed by decapitation. Livers and kidneys were removed and either shock frozen in liquid nitrogen to be stored at −80°C or used freshly. Liver microsomes were prepared according to the method described by van der Hoeven and Coon (van der Hoeven & Coon, 1974) with some modifications. All steps were carried out at 4°C in the presence of protease inhibitors (0.1 μg ml^{-1} aprotinin, 1 μM leupeptin, 5 μM phenylmethanesulfonylfluoride and 0.1 μM pepstatin). Livers were homogenized in 50 mM Tris, 0.25 M sucrose, pH 7.4 and centrifuged at 10,000 × g. The supernatant was subsequently centrifuged at 100,000 × g and the pellet was resuspended in 0.1 M sodium pyrophosphate, 1 mM EDTA, pH 7.25. After another centrifugation at 100,000 × g, the pellet was resuspended in 0.1 M sodium phosphate, pH 7.4. The protein concentration was determined by the Bradford method (Bradford, 1976) using bovine serum albumin as standard. Microsomal aliquots adjusted to 2 mg ml^{-1} were snap-frozen in liquid nitrogen and stored at −80°C. The procedure was the same for kidneys whereas the preparation of microsomes from RASMC was as follows. Confluent cell layers were washed twice with PSS, cells were scraped off and collected in 50 mM Tris, 0.25 M sucrose, pH 7.4. The cell suspension was homogenized manually in a dounce homogenizer with a glass piston. The ensuing steps were as described for liver.

Spectrophotometric analysis of microsomal cytochrome P-450 content

The P-450 content in hepatic, kidney and SMC microsomes was determined by the method of Omura and Sato (Omura & Sato, 1964) from the reduced CO difference spectrum, using an extinction coefficient of 91 mM^{-1} cm^{-1}. Spectra were recorded on an Uvikon 810 spectrophotometer (Biotek Kontron AG, Zürich, Switzerland) between 500 nm and 400 nm.

Measurements of ROS by 2,7-DCFH

Confluent cultures, seeded in 24 or 96-well plates, were incubated in DMEM without FCS or in PMFR-4 in the presence of 5 μM 2,7-DCFH and CsA. After incubation, the medium was replaced with PSS and cellular fluorescence (λ_ex = 485 nm and λ_em = 520 nm) was determined at 37°C in a microtiter plate reader (Fluostar Galaxy, BMG Labtechnologies, Offenburg, Germany).

NADPH-cytochrome P-450 reductase

NADPH cytochrome P-450 reductase activity was assayed essentially as described by Masters *et al.* (1967) using a Cary 50 DW-2 split beam dual-wavelength spectrophotometer (Varian, SAS Strumenti Scientifici, Padova, Italy). Briefly, the assays were performed at 25°C in a 50 mM potassium phosphate buffer containing 0.1 mM EDTA, 40 μM cytochrome c, 10 μg ml^{-1} of microsomal protein and test compounds. The reaction was initiated by the addition of 96 μM NADPH and the rate of cytochrome c reduction was followed for 5 min. The amount of reduced cytochrome c was calculated using an extinction coefficient of 21 mM^{1} cm^{-1} (Massey, 1959) for the difference in absorbance between reduced (λ = 550 nm) and oxidized (λ = 541 nm) cytochrome c.

In vitro *cytochrome P-450 metabolism*

Microsomal alkoxy-O-dealkylation activities of 7-ethoxy- and 7-pentoxyresorufin were measured by the fluorimetric method of Burke *et al.* (1985) with excitation and emission wavelengths set at 530 nm and 585 nm, respectively. Reactions were carried out in 1 ml fluorimeter cuvettes at 37°C in a SPEX spectrofluorometer (Yobin Yvon, Stanford, U.K.). The reaction mixture containing liver microsomal protein (50 μg ml^{-1}), 5 μM substrate and test compounds in 0.1 M phosphate buffer was equilibrated for 3 min at 37°C. The reaction was initiated by the addition of 25 μl NADPH (10 mM in phosphate buffer) and resorufin formation was measured for 6 min. To calculate the specific activity, the fluorescence was calibrated by the addition of 10 μl of resorufin (10 μM in DMSO) and data were calculated to represent the amount of product formed per min per nmol P-450 in the reaction mixture.

Rates of 7-Benzyloxy-4-(trifluoromethyl)-coumarin (BFC) O-dealkylation were determined as described above. Fluorimeter excitation and emission wavelengths were set to 410 nm and 538 nm, respectively (Henderson *et al.*, 1999), and BFC was used at a concentration of 50 μM. To calculate the specific activity, the fluorescence was calibrated by the addition of 5 μl of 5 μM 7-hydroxy-4-trifluoro-methylcoumarin and data were calculated to represent the amount of product formed per min per nmol P-450 in the reaction mixture.

Isolation of total RNA, DNase treatment and reverse transcription

Total RNA was isolated from T5-3A4 and T5-neo cells cultured in 10 cm diameter Petridishes using the Trizol® reagent (Life Technologies, Basel, Switzerland). Isolation, DNase treatment and reverse transcription were performed as described (Cottet-Maire *et al.*, 2001).

Polymerase chain reaction

The CYP 3A4 forward-primer (5′-GCTCTGTCCGATCTG-GAG-3′), the CYP3A4 reverse-primer (5′-AACCTCATGC-CAATGCAG-3′), the actin forward-primer (5′-TGCGCTTGGACCTGGCT-3′) and the actin reverse-primer (5′-ACGCACGATTTCCCTCT-3′) were synthesized by Microsynth (Windisch, Switzerland). Amplification reactions were conducted in a final volume of 50 μl containing 45 μl of a Master Mix (PCR buffer, Q-Solution, 200 μM dNTP mix, 1.25 units of Taq DNA polymerase and 2.5 mM $MgCl_2$), 1 μmol of corresponding forward and reverse primers and 400 ng of cDNA in a PCR Sprint thermal cycler (BioConcept, Allschwil, Switzerland). The reaction were heated for 2 cycles to 98°C for 1 min, 60°C for 2 min and 72°C for 2 min and then cycled 28 times through 1 min denaturation step at 94°C, a 1 min annealing step at 60°C and a 2 min extension

step at 72°C and finally one elongation step of 10 min at 72°C. After amplification, PCR products were separated on a 2% agarose gel containing ethidium bromide for UV detection of the DNA bands.

Data analysis

Results are presented as the means of at least three independent experiments with vertical bars indicating standard errors of the mean (s.e.mean). IC_{50} values were calculated by non-linear regression using the software InPlotPrism (GraphPad Software, San Diego, CA, U.S.A.). Statistical evaluation was performed using one-way ANOVA followed by Newmann Keuls or Dunnett post-tests. Differences with a value of $P<0.05$ were considered significant.

Results

ROS formation by CsA in RASMC

Because the oxidation of 2,7-DCFH has been used by many laboratories to detect cellular radical formation (Rosenkranz *et al*., 1992; Hempel *et al*., 1999; Ischiropoulos *et al*., 1999; Wang & Joseph, 1999), we used this approach to measure ROS in RASMC. A concentration-response curve of CsA-induced ROS formation with CsA concentrations ranging from 0.1 μM to 10 μM, revealed that concentrations as low as 1 μM CsA were able to generate significant amounts of ROS (Figure 1A).

Kinetic analysis showed that CsA-induced ROS formation increased transiently within the first hour after CsA addition. After 45 min (Figure 1B) and up to 2 h (not shown), CsA induced DCF-fluorescence started to decrease. A maximal 2.5 fold increase above control levels was observed. However, increases between 2 and 4 fold were observed in individual experiments. The control values did not change significantly between 10 and 60 min (not shown).

To ensure that the CsA-induced DCF signal represents genuine ROS formation we tested whether antioxidants could block this signal. Table 1 shows the pIC_{50} values of N-acetyl-L-cysteine (NAC, tested at concentrations ranging from 10 μM to 1 mM), α-tocopherol, silymarin and resveratrol (tested at concentrations ranging from 30 nM to 100 μM). Because all antioxidants tested inhibited the CsA induced signal, we conclude that this assay represents a valid measure of CsA-induced generation of radicals in RASMC.

Implication of the cytochrome P-450 system in CsA-induced ROS formation

To investigate whether ROS are formed by the interaction of CsA with the CYP system, we first tested whether CsA was able to directly inhibit microsomal NADPH cytochrome P-450 reductase. As shown in Figure 2, CsA did not inhibit cytochrome *c* reduction in microsomes prepared from RASMC, rat liver and rat kidney, whereas DPI, a flavoprotein inhibitor, inhibited this activity concentration-dependently with an IC_{50} between 0.6 and 1 μM.

Secondly, we tested if CsA interfered with the electron transfer from NADPH *via* NADPH cytochrome P-450 reductase to the CYP enzymes. For this purpose, using rat liver microsomes, we tested whether CsA inhibited the activity of two CYP enzymes that are not implicated in CsA metabolism, CYP 1A and CYP 2B, using ERO (Figure 3A) and PRO (Figure 3B) as substrates, respectively. The results shown in Figure 3 demonstrate that CsA had no significant effect on ethoxyresorufin-O-deethylase (EROD) and pentoxyresorufin-O-dealkylase (PROD) activities, indi-

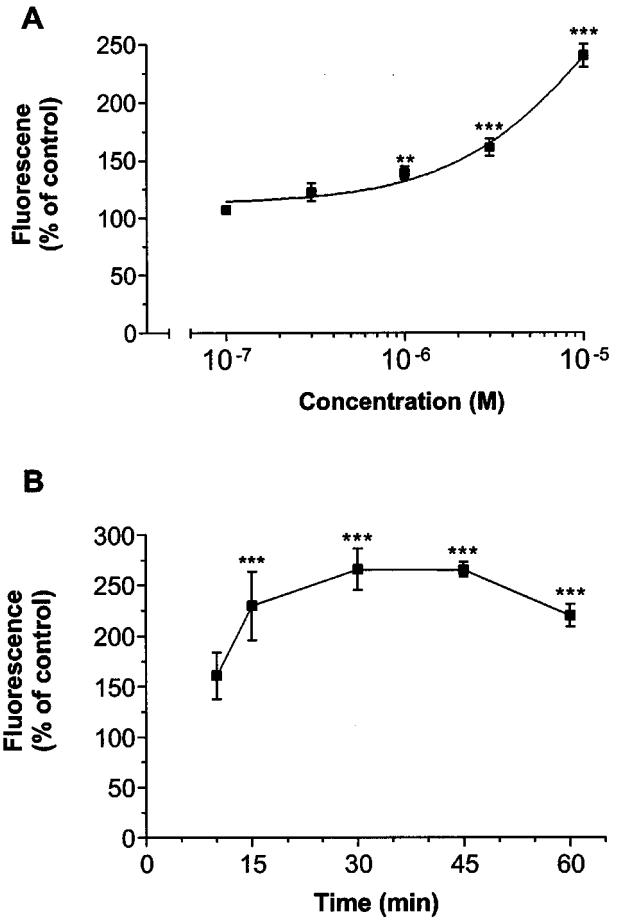

Figure 1 CsA-induced generation of ROS in intact RASMC. Confluent monolayers of RASMC were exposed to CsA (0.1–10 μM) for 1 h at 37°C (A) and to CsA (10 μM) at 37°C between 10 and 60 min (B) and ROS formation was detected with the fluorescent indicator 2,7-DCFH. Each data point represents the mean ± s.e.mean of at least five independent experiments done in quadruplicate. Asterisks indicate values significantly different from control at $P<0.01$ (**) or at $P<0.001$ (***).

Table 1 Inhibition of CsA-induced ROS formation by antioxidants

Antioxidants	*pIC_{50}* (−log M)
N-Acetyl L-cysteine	4.73 ± 0.34
α-Tocopherol	6.07 ± 0.11
Silymarin	5.58 ± 0.12
Resveratrol	5.20 ± 0.15

The pIC_{50} values of antioxidants on CsA (10 μM) induced ROS formation in RASMC. Fluorescence was measured after 1 h at 37°C as detected by DCF fluorescence. Values represent the mean ± s.e.mean of at least seven independent experiments done in quadruplicate.

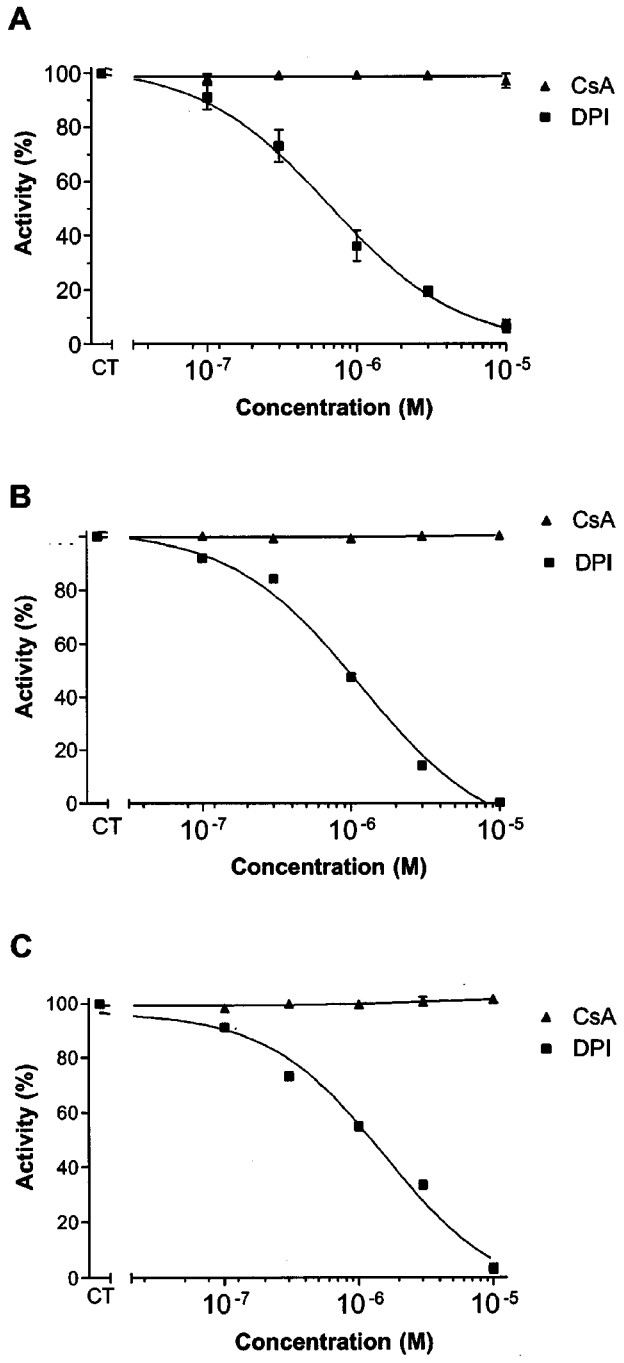

Figure 2 Effect of CsA on NADPH cytochrome P-450 reductase. NADPH reductase activity was assessed using cytochrome *c* as substrate in microsomes prepared from RASMC (A), rat liver (B) and rat kidney (C). Microsomes were incubated in 0.1 M phosphate buffer containing 40 μM cytochrome *c* and either CsA or DPI. The reaction was initiated by the addition of fresh NADPH (final concentration 96 μM). Activity is expressed in per cent of control using either 0.1% EtOH or 0.1% DMSO with control set at 100%. Data are means$\pm$s.e.mean of 3–4 experiments done in triplicate.

cating that CsA did not uncouple electron transfer between the reductase and the P-450 enzymes. DPI was again used as a positive control and inhibited both EROD and PROD activities, *via* inhibition of NADPH CYP-reductase, with an IC_{50} between 0.6 and 1 μM.

Finally, we investigated the interaction of CsA with the CYP 3A enzyme using the CYP 3A substrate BFC in rat liver microsomes. As shown in Figure 4, CsA inhibited CYP 3A activity concentration-dependently and significantly at concentrations above 3 μM, indicating a slight competition of CsA for BFC metabolism. Ketoconazole, a competitive CYP 3A inhibitor, concentration-dependently inhibited the metabolism of BFC (Figure 4). Furthermore, as expected, the NADPH cytochrome P-450 reductase inhibitor DPI inhibited CYP 3A activity with an IC_{50} of 0.5 μM.

Effect of CYP 3A inhibitors on CsA-induced ROS formation

To investigate whether the specific interaction of CsA with the CYP 3A enzyme may lead to ROS formation, we determined the effect of CYP inhibitors on CsA-induced ROS formation in RASMC. Neither DPI nor the CYP 3A inhibitor ketoconazole were able to block CsA induced ROS formation, as shown in Figure 5.

Specific interaction of CsA with the CYP 3A enzyme

To confirm that ROS are not formed by the specific interaction of CsA with CYP 3A, we determined CsA-induced ROS formation in immortalized human liver epithelial cells expressing human CYP 3A4 (T5-3A4). In these T5-3A4 cells, CsA did not generate more radicals than in T5-neo control cells that do not express CYP 3A4 (Figure 6), and neither DPI nor the CYP 3A4 inhibitor ketoconazole had any effect on the DCF signal.

Detection of CYP 3A4 mRNA in T5-cells

To check for the presence of CYP 3A4 mRNA in T5-3A4 cells, reverse transcriptase-polymerase chain reaction (RT–PCR) with CYP 3A4 specific primers was performed. As shown in Figure 7, a band migrating at the expected position of the CYP 3A4 amplification product (484 bp) was detected only in RNA prepared from T5-3A4, but not from T5-neo cells. In contrast, ß-actin, used as a positive control, showed a band at the expected position of 100 bp in both RNA preparations.

Discussion

The results of the present study demonstrate that ROS were formed by CsA in RASMC; however, they were neither produced by CYP-dependent metabolism of CsA nor by non specific interactions of CsA with CYP systems. To verify that the DCF-signal was due to CsA-induced radical generation we used various antioxidants to block this signal. NAC (Chen *et al*., 1995), α-tocopherol (Martin *et al*., 1996), silymarin (Zhao *et al*., 2000), and resveratrol (Martinez & Moreno, 2000) were all able to block CsA induced radical formation with $IC_{50's}$ between 1–20 μM. These results confirm that CsA indeed is capable of producing ROS.

The further focus of this work was, therefore, to investigate whether ROS are formed during cytochrome P-450 mono-oxygenase-dependent metabolism of CsA. The microsomal mono-oxygenase system includes the various CYP enzymes, NADPH cytochrome P-450 reductase and cytochrome b_5.

Because the literature contains indications that CsA may inhibit NADPH cytochrome P-450 reductase (Cunningham *et al*., 1984; Augustine & Zemaitis, 1986; Isogai *et al*., 1993), we investigated the effect of CsA on NADPH cytochrome P-450 reductase. Should CsA inhibit the reductase, the electron flow from NADPH cytochrome P-450 reductase to the CYP

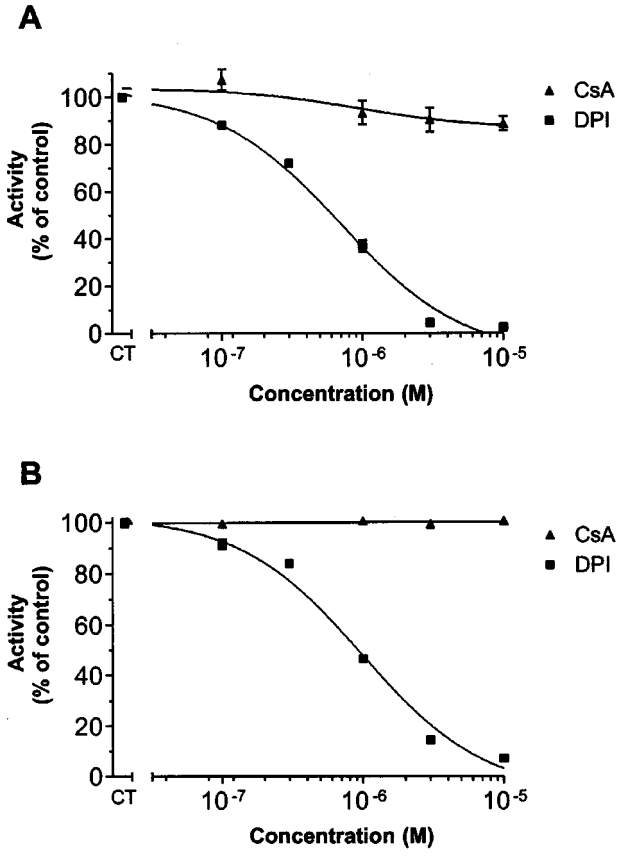

Figure 3 Effect of CsA and DPI on the microsomal O-dealkylation of ERO (A) and PRO (B). Reaction mixtures contained rat liver microsomes (50 μg ml^{-1}), 5 μM substrate and test compounds in 0.1 M phosphate buffer. The reaction was initiated by addition of NADPH (final concentration 250 μM). Activities are expressed in per cent of control. Data are means$\pm$s.e.mean from 3–5 experiments done in tri- or quadruplicate.

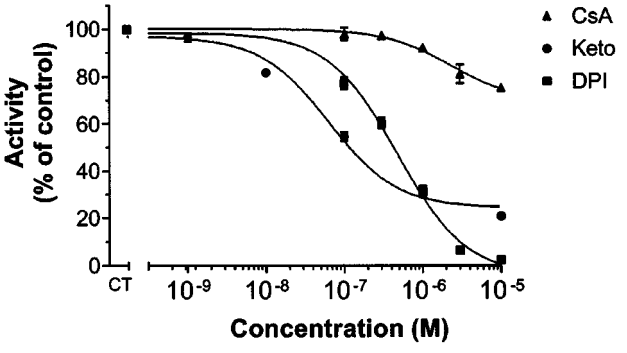

Figure 4 Effect of CsA, DPI and ketoconazole on the microsomal O-dealkylation of BFC. Reaction mixtures contained rat liver microsomes (50 μg ml^{-1}), 50 μM substrate and test compounds in 0.1 M phosphate buffer. The reaction was initiated by addition of NADPH (final concentration 250 μM). Activities are expressed in per cent of control of specific activity in pmol/nmol cytochrome P-450/min. Data are means$\pm$s.e.mean from 3–5 experiments done in tri- or quadruplicate.

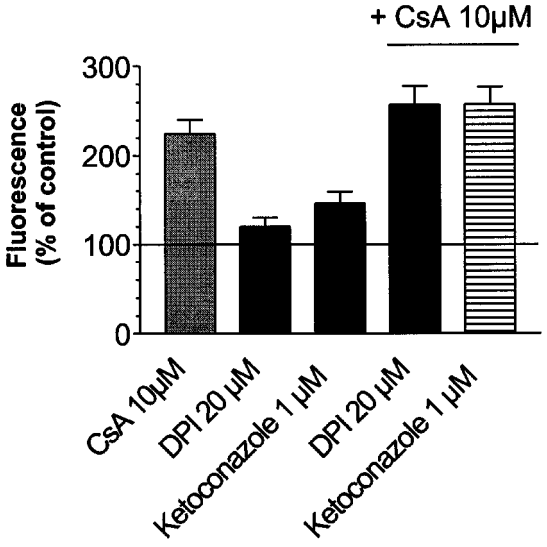

Figure 5 Effect of cytochrome P-450 inhibitors on CsA-induced ROS formation in RASMC. DCF fluorescence was monitored in RASMC after a 1 h incubation of all compounds in culture medium at 37°C. The effect of DPI (20 μM) and ketoconazole (1 μM) alone and on CsA-induced ROS formation was assessed. Data are means$\pm$s.e.mean from at least five experiments done in quadruplicate. The values for the inhibitors were not significantly different from CsA alone at $P<0.05$.

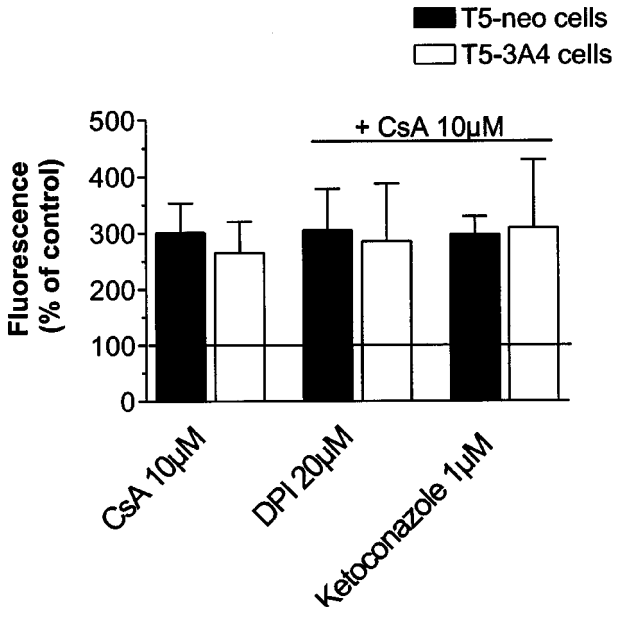

Figure 6 Effect of cytochrome P-450 inhibitors on CsA-induced ROS formation in immortalized human liver epithelial cells. DCF fluorescence was monitored either in T5-3A4 cells or in control cells that do not express human CYP 3A4 (T5-neo) after 1 h incubation of all compounds in culture medium at 37°C. The effect of DPI (20 μM) and ketoconazole (1 μM) on CsA-induced ROS formation was assessed. Data are means$\pm$s.e.mean from at least five experiments done in quadruplicate. The values for the inhibitors in both types of cells were not significantly different from CsA alone at $P<0.05$.

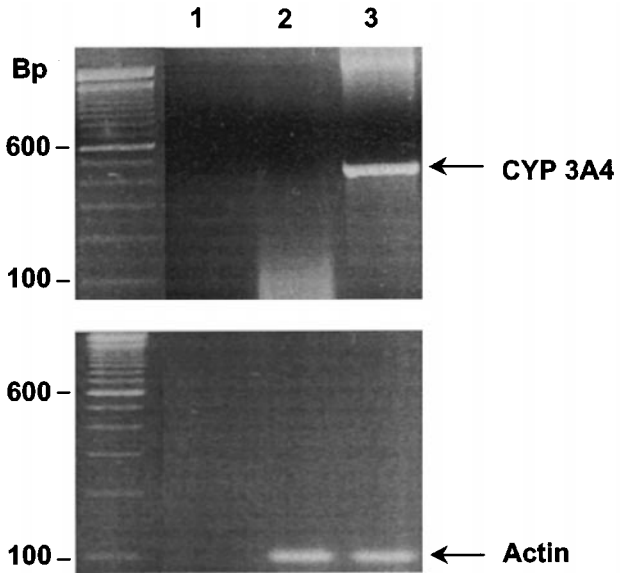

Figure 7 Detection of CYP 3A4 (upper panel) and β-actin (lower panel) mRNA in T5-cells by RT–PCR. The photographs show electrophoretic analysis of PCR reaction products without cDNA (lane 1), with cDNA derived from total RNA extracted from confluent T5-neo cells (lane 2) and T5-3A4 cells (lane 3). The DNA standards are on the left. The location of the band corresponding to 484 bp specific for CYP 3A4 and 100 bp specific for β-actin are indicated. Data are representative of three independent experiments.

enzymes would be attenuated or blocked. However, our results show that, in microsomes prepared from rat liver, kidney and aortic smooth muscle cells, CsA did not inhibit the reduction of the model substrate cytochrome *c*, indicating that NADPH cytochrome P-450 reductase is not directly inhibited by CsA.

Another possibility is that CsA may interfere with the electron transfer from NADPH cytochrome P-450 reductase to the CYP enzymes. This 'uncoupling' could also lead to formation of ROS. For this, we determined the influence of CsA on the metabolism of CYP enzymes that do not metabolize CsA (CYP 1A and CYP 2B). Because CsA did not inhibit the activity of these two CYP enzymes we conclude that CsA did not uncouple electron transfer between the reductase and the CYP enzymes.

These results are in disagreement with published *in vivo* experiments. In rats treated with CsA (25 or 50 mg kg^{-1} per day) either the activity of the hepatic NADPH cytochrome P-450 reductase and/or the P-450 content were decreased significantly after 9 or 10 days, respectively (Augustine & Zemaitis, 1986; Isogai *et al*., 1993). Cunningham *et al*. (1984) showed that in CsA-treated rats (25 mg kg^{-1} per day) NADPH cytochrome P-450 reductase activity was reduced, but there was no significant change in P-450 content after 21 days. In addition, the same group and others showed that CsA was able to decrease the activity of CYP enzymes using specific *in vivo* assays (Augustine & Zemaitis, 1989; Mayer *et al*., 1989; Isogai *et al*., 1993).

It is known that oxidative stress decreases protein and messenger RNA (mRNA) expression of CYP enzymes, with resulting changes in hepatic metabolism (Renton & Knickle, 1990; Gallagher *et al*., 1995). The decrease in the CYP components in the *in vivo* experiments is probably not due to a direct effect of CsA on these components, but rather that CsA-induced ROS are responsible for the downregulation of CYP enzymes. This is also consistent with our findings that acute CsA treatment does neither inhibit NADPH cytochrome P-450 reductase nor CYP enzyme activities.

The CYP 3A substrate BFC was used to test the interaction of CsA with the CYP 3A enzyme. As expected, a slight competition occurred between the two CYP 3A substrates; however, the affinity of BFC for the CYP 3A enzyme seems to be considerably higher than that of CsA, since the maximal inhibition of BFC metabolism by 10 μM CsA was 25%. Higher concentrations of CsA could not be tested because of the limited solubility of CsA in aqueous solutions. The solubility of CsA can be increased by using surfactants/detergents or higher concentrations of solvents. However, it has been shown that CYP enzymes are inhibited at solvent concentrations above 1% (Busby *et al*., 1999). In control experiments various detergents were tested (Tween 80, Triton X-100 and Emulgen) on ERO, PRO and BFC metabolism in rat liver microsomes (not shown). These tests showed that CYP activities were inhibited at detergent concentrations as low as 0.01%. Unfortunately, some authors published work using excessively high solvent concentrations in experiments with microsomes. Indeed, Ahmed *et al*. (1993; 1995) showed that 500 μM CsA was able to induce lipid peroxidation in rat and human liver microsomes which could be decreased by antioxidants. They also showed that 500 μM CsA was capable to produce ROS in rat liver microsomes using the adrenochrome reaction. Because CsA concentrations were excessively high, neither lipid peroxidation nor ROS generation may be caused by CsA itself, but may rather be an effect of the high solvent concentrations (10%) used. Similar, most likely artefactual, results were reported by Serino *et al*. (1993; 1994). In rat liver microsomes, with concentrations of CsA above 100 μM (5% ethanol) they showed increased production of malondialdehyde, the end product of lipid peroxidation. Another group measured lipid peroxidation in rat liver and renal microsomes treated with CsA dissolved in ethanol/Tween 80 (5 : 1) using final concentrations of CsA from about 10 to 250 μM (Inselmann *et al*., 1990) or even up to 833 μM (Inselmann *et al*., 1991). However, because 0.01% Tween completely abolished CYP activities in our experiments (not shown), the lipid peroxidation observed could not originate from CYP-generated ROS as suggested by these authors. To avoid generation of artefacts, we used CsA at a maximal concentration of 10 μM, solvent concentrations of maximally 0.1% and no detergents.

DPI completely inhibited BFC metabolism which was not the case with ketoconazole where inhibition was maximally 80%. This is most likely due to the fact that a small part of BFC is also metabolized by other CYP enzymes as suggested by the supplier (Gentest, Woburn, MA, U.S.A.). The observation that DPI completely inhibited BFC metabolism is consistent with the fact that inhibition of the flavin-containing NADPH cytochrome P-450 reductase inhibits metabolism of all CYP substrates.

Because neither ketoconazole nor DPI had a measurable effect on CsA-induced ROS formation, we conclude that CYP enzymes do not contribute to CsA-induced ROS formation in RASMC. The fact that DPI did not inhibit basal ROS production indicates that flavoproteins do not contribute substantially to basal ROS formation.

Our results in RASMC were confirmed using SV40 large T-antigen immortalized human liver epithelial cells expressing

human CYP 3A4 (T5-3A4). Our results show that CsA generated at least as much ROS in this cell line as in the corresponding control cell line (T5-neo) not expressing CYP 3A4. Moreover, neither DPI nor ketoconazole were able to decrease CsA-induced ROS formation in either cell line. The presence of CYP 3A4 mRNA in T5-3A4 cells was confirmed by RT–PCR.

Our previous results suggested that CYP-dependent CsA metabolism may lead to ROS formation (Nguyen *et al*., 1999). However, signal-to-noise ratios were very low and no clear concentration-dependency could be established using the previous method. With increases of only 30% above background using 1 μM CsA, inhibition was difficult to quantitate in our earlier study because of low signal-to-noise ratios. Therefore, in the current study we used 10 μM CsA that generated a robust DCF signal.

In conclusion, the results of the present study demonstrate that CsA is capable of generating ROS in RASMC, but these ROS do neither arise from cytochrome P-450 dependent metabolism of CsA nor from inhibition of NADPH cytochrome P-450 reductase or from interference with the electron flow to the CYP enzymes.

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References

AHMED, S.S., NAPOLI, K.L. & STROBEL, H.W. (1995). Oxygen radical formation during cytochrome P450-catalyzed cyclosporine metabolism in rat and human liver microsomes at varying hydrogen ion concentrations. *Mol. Cell. Biochem.,* **151,** 131–140.

AHMED, S.S., STROBEL, H.W., NAPOLI, K.L. & GREVEL, J. (1993). Adrenochrome reaction implicates oxygen radicals in metabolism of cyclosporine A and FK-506 in rat and human liver microsomes. *J. Pharmacol. Exp. Ther.,* **265,** 1047–1054.

ARAMBURU, J., RAO, A. & KLEE, C.B. (2000). Calcineurin: from structure to function. *Curr. Top. Cell. Regul.,* **36,** 237–295.

AUGUSTINE, J.A. & ZEMAITIS, M.A. (1989). A comparison of the effects of cyclosporine (CsA) on hepatic microsomal drug metabolism in three different strains of rat. *Gen. Pharmacol.,* **20,** 137–141.

AUGUSTINE, J.A. & ZEMAITIS, M.A. (1986). The effects of cyclosporin A (CsA) on hepatic microsomal drug metabolism in the rat. *Drug Metab. Disp.,* **14,** 73–78.

AVDONIN, P.V., COTTET-MAIRE, F., AFANASJEVA, G.V., LOKTIONOVA, S.A., LHOTE, P. & RUEGG, U.T. (1999). Cyclosporine A up-regulates angiotensin II receptors and calcium responses in human vascular smooth muscle cells. *Kidney Int.,* **55,** 2407–2414.

BONDY, S.C. & NADERI, S. (1994). Contribution of hepatic cytochrome P450 systems to the generation of reactive oxygen species. *Biochem. Pharmacol.,* **48,** 155–159.

BOREL, J.F., BAUMANN, G., CHAPMAN, I., DONATSCH, P., FAHR, A., MUELLER, E.A. & VIGOURET, J.M. (1996). In vivo pharmacological effects of ciclosporin and some analogues. *Adv. Pharmacol.,* **35,** 115–246.

BRADFORD, M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.,* **72,** 248–254.

BUETLER, T.M., COTTET-MAIRE, F., KRAUSKOPF, A. & RUEGG, U.T. (2000). Does cyclosporin A generate free radicals? *Trends Pharmac. Sci.,* **21,** 288–290.

BURKE, M.D., THOMPSON, S., ELCOMBE, C.R., HALPERT, J., HAAPARANTA, T. & MAYER, R.T. (1985). Ethoxy-, pentoxy- and benzyloxyphenoxazones and homologues: a series of substrates to distinguish between different induced cytochromes P-450. *Biochem. Pharmacol.,* **34,** 3337–3345.

BUSBY, JR. W.F., ACKERMANN, J.M. & CRESPI, C.L. (1999). Effect of methanol, ethanol, dimethyl sulfoxide, and acetonitrile on in vitro activities of cDNA-expressed human cytochromes P-450. *Drug Metab. Dispos.,* **27,** 246–249.

CHEN, Q., OLASHAW, N. & WU, J. (1995). Participation of reactive oxygen species in the lysophosphatidic acid-stimulated mitogen-activated protein kinase kinase activation pathway. *J. Biol. Chem.,* **270,** 28499–28502.

COMBALBERT, J., FABRE, I., FABRE, G., DALET, I., DERANCOURT, J., CANO, J.P. & MAUREL, P. (1989). Metabolism of cyclosporin A. IV. Purification and identification of the rifampicin-inducible human liver cytochrome P-450 (cyclosporin A oxidase) as a product of P450IIIA gene subfamily. *Drug Metab. Dispos.,* **17,** 197–207.

COON, M.J., VAZ, A.D.N., MCGINNITY, D.F. & PENG, H.M. (1998). Multiple activated oxygen species in P450 catalysis–Contributions to specificity in drug metabolism. *Drug Metab. Disp.,* **26,** 1190–1193.

COTTET-MAIRE, F., AVDONIN, P.V., ROULET, E., BUETLER, T.M., MERMOD, N. & RUEGG, U.T. (2001). Upregulation of vasopressin V 1A receptor mRNA and protein in vascular smooth muscle cells following cyclosporin A treatment. *Br. J. Pharmacol.,* **132,** 909–917.

CUNNINGHAM, C., GAVIN, M.P., WHITING, P.H., BURKE, M.D., MACINTYRE, F., THOMSON, A.W. & SIMPSON, J.G. (1984). Serum cyclosporin levels, hepatic drug metabolism and renal tubulotoxicity. *Biochem. Pharmacol.,* **33,** 2857–2861.

DALTON, T.D., SHERTZER, H.G. & PUGA, A. (1999). Regulation of gene expression by reactive oxygen. *Ann. Rev. Pharmacol. Toxicol.,* **39,** 67–101.

GALLAGHER, E.P., BUETLER, T.M., STAPLETON, P.L., WANG, C., STAHL, D.L. & EATON, D.L. (1995). The effects of diquat and ciprofibrate on mRNA expression and catalytic activities of hepatic xenobiotic metabolizing and antioxidant enzymes in rat liver. *Toxicol. Appl. Pharmacol.,* **134,** 81–91.

HEMPEL, S.L., BUETTNER, G.R., O'MALLEY, Y.Q., WESSELS, D.A. & FLAHERTY, D.M. (1999). Dihydrofluorescein diacetate is superior for detecting intracellular oxidants: Comparison with 2′,7′-dichlorodihydrofluorescein diacetate, 5(and 6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate, and dihydrorhodamine 123. *Free Rad. Biol. Med.,* **27,** 146–159.

HENDERSON, G.L., HARKEY, M.R., GERSHWIN, M.E., HACKMAN, R.M., STERN, J.S. & STRESSER, D.M. (1999). Effects of ginseng components on c-DNA-expressed cytochrome P450 enzyme catalytic activity. *Life Sci.,* **65,** L209–L214.

INSELMANN, G., BARTH, A., ENGEMANN, R. & HEIDEMANN, H.T. (1991). Cyclosporin-A-induced lipid peroxidation in human liver microsomes and its influence on cytochrome P-450. *Eur. J. Clin. Invest.,* **21,** 461–465.

INSELMANN, G., HANNEMANN, J. & BAUMANN, K. (1990). Cyclosporine A induced lipid peroxidation and influence on glucose-6-phosphatase in rat hepatic and renal microsomes. *Res. Commun. Chem. Pathol. Pharmacol.,* **68,** 189–203.

ISCHIROPOULOS, H., GOW, A., THOM, S.R., KOOY, N.W., ROYALL, J.A. & CROW, J.P. (1999). Detection of reactive nitrogen species using 2,7-dichlorodihydrofluorescein and dihydrorhodamine 123. In *Methods in Enzymology: Oxygen Radicals in Biological Systems, Part D*. ed. Packer, L. pp. 367–373. San Diego: Academic Press.

ISOGAI, M., SHIMADA, N., KAMATAKI, T., IMAOKA, S. & FUNAE, Y. (1993). Changes in the amounts of cytochromes P450 in rat hepatic microsomes produced by cyclosporin A. *Xenobiotica,* **23,** 799–807.

KAHAN, B.D. (1989). Cyclosporine. *New Engl. J. Med.,* **321,** 1725–1738.

KRONBACH, T., FISCHER, V. & MEYER, U.A. (1988). Cyclosporine metabolism in human liver: identification of a cytochrome P-450III gene family as the major cyclosporine-metabolizing enzyme explains interactions of cyclosporine with other drugs. *Clin. Pharmacol. Therapeut.,* **43,** 630 – 635.

KUTHAN, H., TSUJI, H., GRAF, H. & ULLRICH, V. (1978). Generation of superoxide anion as a source of hydrogen peroxide in a reconstituted monooxygenase system. *FEBS Lett.,* **91,** 343 – 345.

KUTHAN, H. & ULLRICH, V. (1982). Oxidase and oxygenase function of the microsomal cytochrome P450 monooxygenase system. *Eur. J. Biochem.,* **126,** 583 – 588.

LAMB, F.S. & WEBB, R.C. (1987). Cyclosporine augments reactivity of isolated blood vessels. *Life Sci.,* **40,** 2571 – 2578.

LANDER, H.M. (1997). An essential role for free radicals and derived species in signal transduction. *FASEB J.,* **11,** 118 – 124.

L'AZOU, B., MEDINA, J., FRIEAUFF, W., CORDIER, A., CAMBAR, J. & WOLF, A. (1999). In vitro models to study mechanisms involved in cyclosporine A-mediated glomerular contraction. *Arch. Toxicol.,* **73,** 337 – 345.

LO RUSSO, A., PASSAQUIN, A.C., ANDRÉ, P., SKUTELLA, M. & RUEGG, U.T. (1996). Effect of cyclosporin A and analogues on cytosolic calcium and vasoconstriction: possible lack of relationship to immunosuppressive activity. *Br. J. Pharmacol.,* **118,** 885 – 892.

LO RUSSO, A., PASSAQUIN, A.-C., COX, C. & RUEGG, U.T. (1997A). Cyclosporin A potentiates receptor-activated $[Ca^{2+}]_c$ increase. *J. Rec. Signal Transd. Res.,* **17,** 149 – 161.

LO RUSSO, A., PASSAQUIN, A.C. & RUEGG, U.T. (1997B). Mechanism of enhanced vasoconstrictor hormone action in vascular smooth muscle cells by cyclosporin A. *Br. J. Pharmacol.,* **121,** 248 – 252.

LOPEZ-ONGIL, S., HERNANDEZ-PERERA, O., NAVARRO-ANTOLIN, J., PEREZ DE LEMA, G., RODRIGUEZ-PUYOL, M., LAMAS, S. & RODRIGUEZ-PUYOL, D. (1998). Role of reactive oxygen species in the signalling cascade of cyclosporine A-mediated up-regulation of eNOS in vascular endothelial cells. *Br. J. Pharmacol.,* **124,** 447 – 454.

MACÉ, K., AGUILAR, F., WANG, J.S., VAUTRAVERS, P., GOMEZ-LECHON, M., GONZALEZ, F.J., GROOPMAN, J., HARRIS, C.C. & PFEIFER, A.M. (1997). Aflatoxin B_1-induced DNA adduct formation and p53 mutations in CYP450-expressing human liver cell lines. *Carcinogenesis,* **18,** 1291 – 1297.

MARTIN, A., WU, D., BAUR, W., MEYDANI, S.N., BLUMBERG, J.B. & MEYDANI, M. (1996). Effect of vitamin E on human aortic endothelial cell responses to oxidative injury. *Free Radical Biology & Medicine,* **21,** 505 – 511.

MARTINEZ, J. & MORENO, J.J. (2000). Effect of resveratrol, a natural polyphenolic compound, on reactive oxygen species and prostaglandin production. *Biochem. Pharmacol.,* **59,** 865 – 870.

MASON, J. (1990). Pharmacology of cyclosporine (sandimmune). VII. Pathophysiology and toxicology of cyclosporine in humans and animals. *Pharmacol. Rev.,* **41,** 423 – 434.

MASSEY, V. (1959). The microestimation of succinate and the extinction coefficient of cytochrome c. *Biochim. Biophys. Acta,* **34,** 225 – 256.

MASTERS, B., WILLIAMS, C. & KAMIN, H. (1967). The Prepararion and Properties of Microsomal TPNH-Cytochrome c Reductase from Pig Liver. In *Methods in Enzymology*. pp. 565 – 573. San Diego: Academic Press.

MAYER, R.D., BERMAN, S., COCKETT, A.T. & MAINES, M.D. (1989). Differential effects of cyclosporin on hepatic and renal heme, cytochrome P-450 and drug metabolism. Possible role in nephrotoxicity of the drug. *Biochem. Pharmacol.,* **38,** 1001 – 1007.

MEYER, M., SCHRECK, R. & BAEUERLE, P.A. (1993). H2O2 and antioxidants have opposite effects on activation of NF-kappa B and AP-1 in intact cells: AP-1 as secondary antioxidant-responsive factor. *EMBO J.,* **12,** 2005 – 2015.

NGUYEN, N.S.D., COTTET, F., BUETLER, T.M., LO RUSSO, A., KRAUSKOPF, A.S., ARMSTRONG, J., VICKERS, A.E.M., MACÉ, K. & RUEGG, U.T. (1999). Metabolism-dependent stimulation of reactive oxygen species and DNA synthesis by cyclosporin A in rat smooth muscle cells. *Free Radic. Biol. Med.,* **27,** 1267 – 1275.

OMURA, T. & SATO, R. (1964). The Carbon Monoxide-binding Pigment of Liver Microsomes I. Evidence for its Hemoprotein Nature. *J. Biol. Chem.,* **239,** 2370 – 2378.

PAOLINI, M., POZZETTI, L., PEDULLI, G.F., CIPOLLONE, M., MESIRCA, R. & CANTELLI-FORTI, G. (1996). Paramagnetic resonance in detecting carcinogenic risk from cytochrome P450 overexpression. *J. Investig. Med.,* **44,** 470 – 473.

PEREZ DE LEMA, G., ARRIBAS, I., PRIETO, A., PARRA, T., DE ARRIBA, G., RODRIGUEZ-PUYOL, D. & RODRIGUEZ-PUYOL, M. (1998). Cyclosporin A-induced hydrogen peroxide synthesis by cultured human mesangial cells is blocked by exogenous antioxidants. *Life Sci.,* **62,** 1745 – 1753.

PERRET, A. & POMPON, D. (1998). Electron shuttle between membrane-bound cytochrome P450 3A4 and b5 rules uncoupling mechanisms. *Biochemistry,* **37,** 11412 – 11424.

PFEIFER, A.M., COLE, K.E., SMOOT, D.T., WESTON, A., GROOPMAN, J.D., SHIELDS, P.G., VIGNAUD, J.M., JUILLERAT, M., LIPSKY, M.M. & TRUMP, B.F. (1993). Simian virus 40 large tumor antigen-immortalised normal human liver epithelial cells express hepatocyte characteristics and metabolize chemical carcinogens. *Proc. Natl. Acad. Sci. USA,* **90,** 5123 – 5127.

PROUGH, R.A. & MASTERS, B.S. (1973). Studies on the NADPH oxidase reaction of NADPH-cytochrome c reductase. I. The role of superoxide anion. *Annals N.Y. Acad. Sci.,* **212,** 89 – 93.

RAO, A., LUO, C. & HOGAN, P.G. (1997). Transcription factors of the NFAT family: Regulation and function. *Annu. Rev. Immunol.,* **15,** 707 – 747.

RASHBA-STEP, J. & CEDERBAUM, A.I. (1994). Generation of reactive oxygen intermediates by human liver microsomes in the presence of NADPH or NADH. *Mol. Pharmacol.,* **45,** 150 – 157.

REGO, A., VARGAS, R., SUAREZ, K.R., FOEGH, M.L. & RAMWELL, P.W. (1990). Mechanism of cyclosporin potentiation of vasoconstriction of the isolated rat mesenteric arterial bed: role of extracellular calcium. *J. Pharmacol. Exp. Ther.,* **254,** 799 – 808.

RENTON, K.W. & KNICKLE, L.C. (1990). Regulation of hepatic cytochrome P-450 during infectious disease. *Can. J. Physiol. Pharmacol.,* **68,** 777 – 781.

ROSENKRANZ, A.R., SCHMALDIENST, S., STUHLMEIER, K.M., CHEN, W., KNAPP, W. & ZLABINGER, G.J. (1992). A microplate assay for the detection of oxidative products using 2′,7′-dichlorofluorescin-diacetate. *J. Immunol. Methods,* **156,** 39 – 45.

SCHRECK, R., RIEBER, P. & BAEUERLE, P.A. (1991). Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-kappa B transcription factor and HIV-1. *EMBO J.,* **10,** 2247 – 2258.

SEN, C.K. & PACKER, L. (1996). Antioxidant and redox regulation of gene transcription. *FASEB J.,* **10,** 709 – 720.

SERINO, F., GREVEL, J., NAPOLI, K.L., KAHAN, B.D. & STROBEL, H.W. (1993). Generation of oxygen free radicals during the metabolism of cyclosporine A: A cause-effect relationship with metabolism inhibition. *Mol. Cell Biochem.,* **122,** 101 – 112.

SERINO, F., GREVEL, J., NAPOLI, K.L., KAHAN, B.D. & STROBEL, H.W. (1994). Oxygen radical formation by the cytochrome P450 system as a cellular mechanism for cyclosporine toxicity. *Transplant. Proc.,* **26,** 2916 – 2917.

STROES, E.S., LUSCHER, T.F., DE GROOT, F.G., KOOMANS, H.A. & RABELINK, T.J. (1997). Cyclosporin A increases nitric oxide activity in vivo. *Hypertension,* **29,** 570 – 575.

SUNDARESAN, M., YU, Z.X., FERRANS, V.J., IRANI, K. & FINKEL, T. (1995). Requirement for generation of H_2O_2 for platelet-derived growth factor signal transduction. *Science,* **270,** 296 – 299.

TEXTOR, S.C., CANZANELLO, V.J., TALER, S.J., WILSON, D.J., SCHWARTZ, L.L., AUGUSTINE, J.E., RAYMER, J.M., ROMERO, J.C., WIESNER, R.H. & KROM, R.A. (1994). Cyclosporine-induced hypertension after transplantation. *Mayo Clin. Proc.,* **69,** 1182 – 1193.

VAN DER HOEVEN, T.A. & COON, M.J. (1974). Preparation and properties of partially purified cytochrome P-450 and reduced NADPH-cytochrome P-450 reductase from rabbit liver. *J. Biol. Chem.,* **249,** 6302 – 6310.

WANG, C. & SALAHUDEEN, A.K. (1995). Lipid peroxidation accompanies cyclosporine nephrotoxicity: effects of vitamin E. *Kidney Int.,* **47,** 927 – 934.

WANG, H. & JOSEPH, J.A. (1999). Quantifying cellular oxidative stress by dichlorofluorescein assay using microplate reader. *Free Radic. Biol. Med.,* **27,** 612 – 616.

ZHAO, J., LAHIRI-CHATTERJEE, M., SHARMA, Y. & AGARWAL, R. (2000). Inhibitory effect of a flavonoid antioxidant silymarin on benzoyl peroxide-induced tumor promotion, oxidative stress and inflammatory responses in SENCAR mouse skin. *Carcinogenesis,* **21,** 811 – 816.

ZHONG, Z., ARTEEL, G.E., CONNOR, H.D., YIN, M., FRANKENBERG, M.V., STACHLEWITZ, R.F., RALEIGH, J.A., MASON, R.P. & THURMAN, R.G. (1998). Cyclosporin A increases hypoxia and free radical production in rat kidneys: prevention by dietary glycine. *Am. J. Physiol.,* **275,** F595 – F604.

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