Is the Beneficial Effect of Calcium Channel Blockers Against Cyclosporine A Toxicity Related to a Restoration of ATP Synthesis?

Marie D. Salducci,[1] Anne M. Chauvet-Monges,[1] Bertrand M. Dussol,[2] Yvon F. Berland,[2] and Aimé D. Crevat[1]

Received July 7, 1994; accepted November 30, 1994

ATP synthesis inhibited by Cyclosporine A is restored by calcium channel blockers: nifedipine, verapamil, bepridil, diltiazem. ATP synthesis was estimated using liver mitochondria by measuring the rate of respiration during state 3 and a measure of the yield of ATP synthesis, the P/O ratio. The study of calcium fluxes through mitochondrial membrane indicates that calcium channel blockers counteract the mitochondrial calcium storage induced by cyclosporine A. If the restoration of ATP synthesis observed in vitro also occurred in vivo, the increase in ATP pool might contribute to a better functioning of the Ca^{2+} extrusion pumps of the cells, thereby maintaining the cytosolic calcium concentration (Ca_i) in the normal range. The nephrotoxicity of cyclosporine A appears to be due to a vasoconstrictive effect related to an increased Ca_i. This result may account for the reduction of clinical cyclosporine A toxicity by calcium channel blockers. Verapamil appears to be the most efficient in restoring ATP synthesis.

KEY WORDS: cyclosporine A; calcium channel blockers; ATP synthesis; mitochondria model; nephrotoxicity.

INTRODUCTION

During chronic administration limitations in the clinical use of the immunosupressor cyclosporine A (Cys A) include its nephrotoxicity and arterial hypertension (1). The nephrotoxicity is mainly due to a direct intrarenal vasoconstriction (2). The two main clinical side effects of Cys A suggest the drug increases the cytosolic calcium concentration Ca_i since classically this is the initial event of vasoconstriction. Such a vasoconstriction may be explained by previous in vitro results showing that Cys A enhanced mitochondrial calcium uptake and storage (3) by blocking the mitochondrial calcium release mechanisms (4,5,6). Increased mitochondrial calcium inhibits ATP synthesis (7). We demonstrated that in presence of Ca^{2+}, Cys A inhibits ATP synthesis in vitro (8). If such an in vitro effect occurs in vivo, the ATP depletion due to the mitochondrial calcium overload induced by Cys A will lead to impaired functioning of the ATP dependent calcium pump. Hence, during chronic administration, Cys A should increase cytosolic calcium concentration. Experimental results supported this hypothesis since an increase in Ca_i has been observed in glomerular mesengial cells treated with Cys A (9). Therefore the inhibition of ATP synthesis by Cys A in an in vitro mitochondrial model (8) is consistent with the vasoconstrictive effect of Cys A.

Clinical cyclosporine A toxicity may be partially reversed or prevented by concomitant administration of calcium channel blockers (CCB) (10). This protection is probably not due to the inhibition of calcium entry into the cell (11), but it may result from CCB counteracting the deleterious effect of Cys A on ATP synthesis. This hypothesis is supported by preliminary results we obtained with nifedipine and verapamil with the in vitro mitochondrial model (8). It was therefore of interest to study the mechanism of the action of CCB on in vitro ATP synthesis inhibited by Cys A. In the present paper we report the results obtained with four CCB: nifedipine, verapamil, bepridil, and diltiazem.

MATERIAL AND METHODS

Drugs

Cyclosporine A was a gift from Sandoz Laboratory. Verapamil, nifedipine, bepridil and diltiazem were purchased from Sigma.

Mitochondrial Preparations

Mitochondria were obtained from rat liver by using a modified version of the technique described by Johnson and Lardy (12). Briefly, male Wistar rats (170-220g) were cervically stunned and decapitated. Mitochondrial isolation procedure was carried out at 4°C. Liver tissue (6–10 g) was rapidly excised, blotted, washed and minced in extraction buffer (0.25 M saccharose, 10 mM tris-HCl, 1 mM EGTA, pH 7.8 adjusted with HCl). Tissue was suspended in the buffer and homogenized. The suspension was centrifuged at 600g for 10 min and the pellet discarded. The supernatant was then centrifuged at 15000g for 5 min. The brown mitochondrial pellet was gently homogenized in 20ml of washing buffer (0.25 M saccharose, 10 mM tris-HCl, pH 7.8 adjusted with HCl). The suspension was centrifuged twice at 15000g for 5 min. The pellet was then homogenized with 100µl of respiration buffer (0.25M saccharose, 4 mM KH_2PO_4, 1 µM rotenone, pH 7.2 adjusted with KOH). Protein contents were measured using Biorad Protein Assay (Biorad Laboratory). All the experiments with Cys A were performed after incubation of mitochondria with the drug during 10 min at 4°C. Since Cys A was dissolved in ethanol, controls were done after incubation in the same conditions with the same quantity of ethanol.

ATP Synthesis Measurements

Oxygen consumption was measured in a water-jacketed reaction chamber (1.8 ml) at 25°C by using a Clark microelectrode fitted to a Gilson oxygraph. Mitochondria (1.5 mg of protein) were suspended in respiratory medium. Sodium succinate was used as substrate (6 mM final concentration); oxidative phosphorylation was started by adding ADP to a final concentration of 0.10 mM.

Oxygen consumption rate during state 3, V_3, corre-

[1] Laboratory of Biophysics, Faculty of Pharmacy, Marseilles, France.

[2] Nephrology Department, Hospital Ste-Marguerite, Marseilles, France.

0724-8741/95/0400-0518$07.50/0

sponds to the ATP synthesis. State 4 is reached when all the ADP is phosphorylated (Fig. 1). Respiratory control is the ratio of the oxygen uptake rate in state 3 to that state 4. The mitochondria exhibited a respiratory control between 3.4 and 4.0 because the respiratory medium did not contain bovine serum albumin and EGTA (Ethylene Glycol-bis(aminoethylether)-*N,N,N',N'*-Tetraacetic acid) so as not to modify the calcium concentration in the medium.

P/O corresponds to the number of ADP molecules added in the medium per oxygen atom consumed during the phosphorylation. This ratio represents the yield of ATP synthesis.

V_3 and P/O were measured in presence of 50 nmoles of Ca^{2+} in the reaction chamber, using mitochondria incubated with or without Cys A 1 μM. Experiments were then performed in the same conditions in presence of various doses of CCB (0.05 μM to 1.00 μM).

For each drug, five to seven experiments were performed. Each series was conducted with mitochondria from the same extraction. Measurements were performed in duplicate.

The corrective effect of CCB on the action of Cys A on V_3 and P/O was expressed as percentage of restoration calculated as follows [V_3 or P/O = (ϕ)]:

$$\% \text{ Restoration } (\phi) = \frac{(\phi)\,(\text{CysA} + Ca^{2+} + \text{CCB}) - (\phi)\,(\text{CysA} + Ca^{2+})}{(\phi)\,(Ca^{2+}\ \text{control}) - (\phi)\,(\text{CysA} + Ca^{2+})} \times 100$$

Results in the tables are means ± SD.

Calcium Fluxes

Calcium movements were monitored by recording Ca^{2+} variations in extramitochondrial medium in a water-jacketed reaction chamber (3.8 ml) equipped with a specific Orion 9320 calcium electrode fitted to a PD2 Sefram recorder via a SA720 Orion ionometer. As shown in Figure 3a, when mitochondria (2 mg of mitochondrial protein) were added, Ca^{2+} concentration in extramitochondrial medium rapidly decreased because of the uptake of calcium into mitochondria. The equilibrium between Ca^{2+} entry and release led to a dynamic steady state. After all the oxygen in the medium had been consumed, anaerobiosis led to a Ca^{2+} release. In presence of high concentration of calcium this release may occur before anaerobiosis.

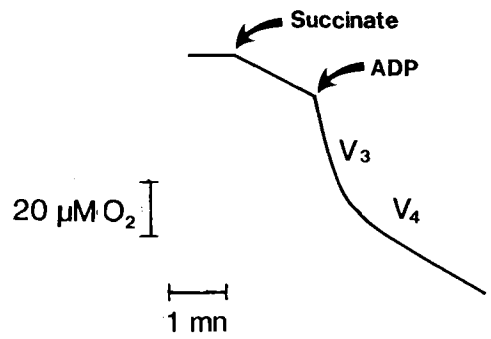

Fig. 1. Schematic representation of mitochondrial respiration. Arrows indicate the addition of reagents.

First, we measured steady state duration (SSD). Controls were done in presence of 100 nmoles of Ca^{2+} per mg of mitochondrial protein. Cyclosporine A prolongs SSD (3). Thus, we determined the effect of the four CCB (0.25 μM to 10 μM) on Cys A effect (0.2 μM). For these measurements we used higher concentrations of calcium and CCB than in the other experiments so as to improve accuracy. Experiments were performed in triplicate.

The effect of CCB on CysA-induced Ca^{2+} overload corresponds to a diminution of SSD, and was expressed as a percentage calculated as follows:

$$\% \text{ Dimution (SSD)} = \frac{(\text{SSD})\,(\text{CysA} + Ca^{2+}) - (\text{SSD})\,(\text{CysA} + Ca^{2+} + \text{CCB})}{(\text{SSD})\,(\text{CysA} + Ca^{2+}) - (\text{SSD})\,(Ca^{2+}\ \text{control})} \times 100$$

Results in the table are means ± SD.

Second, we performed experiments in presence of Ruthenium Red (RR), a classical inhibitor of the Ca^{2+} mitochondrial carrier. Experiments were performed in presence of 1.0 μM of Cys A and 12.5 nmoles of Ca^{2+} per mg of mitochondrial protein. When the steady state was reached, RR was added (1 nmole per mg of mitochondrial protein). The same experiments were also performed in presence of various doses of CCB (0.1 and 0.5 μM). Controls were done with RR only. Experiments were performed in duplicate.

RESULTS

Using the liver mitochondrial model, we previously showed that Cys A in presence of Ca^{2+} decreases V_3 and the P/O ratio (8). In the present paper we chose Cys A and Ca^{2+} concentrations which decreased the two parameters by about 25% (see Method).

Results in Table IA and IB show that four CCB restored V_3 and P/O in a dose dependent manner. As an example Figure 2 shows the restorative effect of verapamil on these parameters.

For V_3 the maxima of restoration observed were 70%–75% for nifedipine, bepridil and diltiazem and reached 95% for verapamil, all at a concentration of 1.0 μM. For P/O the maxima of restoration were 75%–80% for nifedipine and verapamil and only about 60% for bepridil and diltiazem. More-

Table IA. Restoration by Calcium Channel Blockers of V3 Inhibited by Cys A (1.0 μM)

Concentration, μM	V_3 Restoration, %			
	Verapamil (n = 6)	Nifedipine (n = 5)	Bepridil (n = 6)	Diltiazem (n = 7)
0.05	17 ± 5	15 ± 2	35 ± 6	25 ± 5
0.10	33 ± 5	29 ± 6	40 ± 6	32 ± 7
0.20	45 ± 4	43 ± 5	49 ± 6	54 ± 5
0.40	73 ± 6	49 ± 2	55 ± 2	60 ± 5
1.00	94 ± 5	75 ± 5	75 ± 6	69 ± 4

Results are expressed as percentages of restoration. Each value represents the mean ± SD.

Table IB. Restoration by Calcium Channel Blockers of P/O Inhibited by Cys A (1.0 μM)

Concentration, μM	P/O Restoration, %			
	Verapamil (n = 6)	Nifedipine (n = 5)	Bepridil (n = 6)	Diltiazem (n =7)
0.05	17 ± 3	23 ± 4	33 ± 4	20 ± 4
0.10	33 ± 4	38 ± 7	49 ± 4	46 ± 6
0.20	42 ± 5	43 ± 5	55 ± 6	60 ± 5
0.40	62 ± 5	50 ± 4	57 ± 7	33 ± 7
1.00	81 ± 7	75 ± 5	63 ± 5	33 ± 6

Results are expressed as percentages of restoration. Each value represents mean ± SD.

over these maxima were obtained for a concentration of 1 μM except for diltiazem, which showed a maximum at 0.20 μM, followed by a decrease.

Figure 3 presents the recordings of calcium fluxes through mitochondrial membrane in presence of Cys A and bepridil. Cys A increased the SSD (Fig. 3a and 3b). Bepridil corrected this effect in a dose dependent manner (Figure 3c, 3d and 3e). The four CCB gave similar results; the percentages of diminution of SSD are given in Table II. The percentages increased with the concentrations of verapamil, nifedipine, and bepridil. Diltiazem exhibited a particular behavior on SSD: as in the case of P/O restoration, its effect is described by a bell-shaped curve with a maximum (29%) corresponding to a concentration of 5.00 μM.

When added during the steady state, RR classically induced a Ca^{2+} release from mitochondria. Cys A inhibited this efflux; the four CCB partially corrected this effect. Figure 4 shows the action of diltiazem in presence of Cys A on the RR-induced calcium efflux.

DISCUSSION

Low concentrations of CCB restore the V_3 and P/O values decreased by Cys A (Table I). At these concentrations, the CCB alone do not significantly modify V_3 and P/O values (13). Therefore their increase observed here corresponds to a counteraction of the Cys A effect and not to the expression of the action of calcium channel blockers themselves on mitochondria. Classically a decrease in V_3 and P/O represents an inhibition of mitochondrial ATP synthesis. Since CCB restore these values, the drugs counteract the Cys A inhibitory effect on ATP synthesis in vitro. The lipophilicity of the drugs does not appear to be involved in this action. Indeed nifedipine and verapamil, which have different lipophilicities (14), appear to be the most potent in our model. On the contrary diltiazem and verapamil have the same lipophilicity but the former has a lower activity than the latter on ATP synthesis. Classically diltiazem interferes with Cys A metabolism in vivo. The inhibition of cytochrome P450 might be involved in this interaction (15). This finding may be related to the particular behavior exhibited by diltiazem in our experimental conditions (Table I).

The inhibition of ATP synthesis by Cys A is Ca^{2+} mediated since EGTA totally suppresses it (8). It was therefore

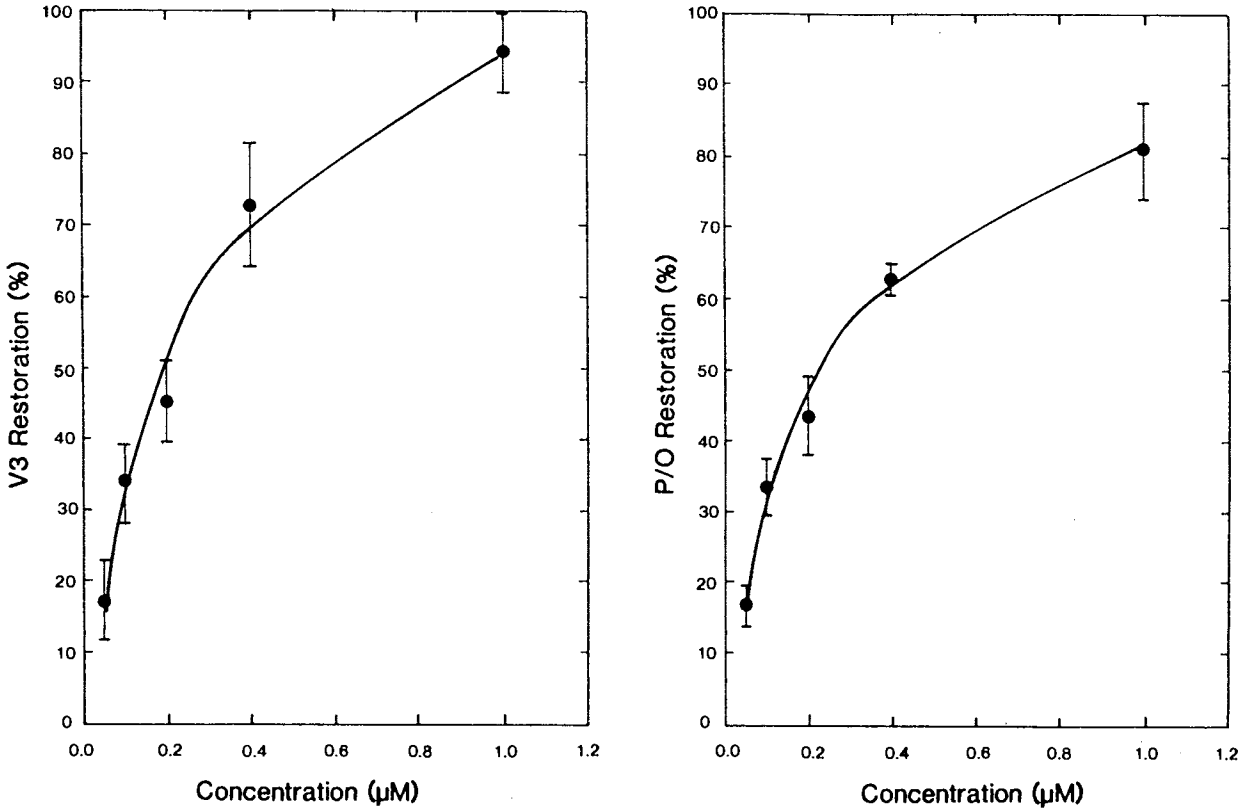

Fig. 2. Restoration by verapamil of V_3 and P/O altered by Cys A (1.0 μM). Results as expressed as percentage of restoration. Each point represents mean ± SD of six experiments.

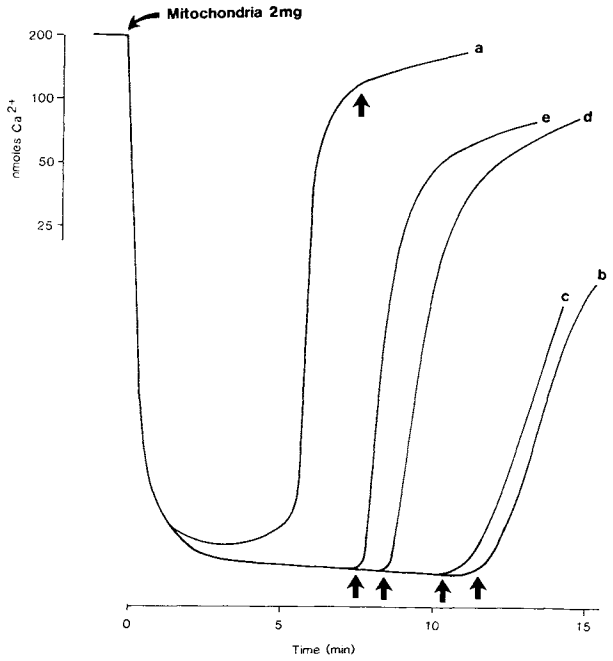

Fig. 3. Counteraction by bepridil of the Cys A effect on calcium fluxes through mitochondrial membrane. Cys A = 0.2 μM. 100 nmoles of Ca^{2+} per mg of mitochondrial protein was added. Arrows indicate the anaerobiosis. a, Ca^{2+} alone; b, Ca^{2+} + Cys A; c, d and e, Ca^{2+} + Cys A + bepridil (1 μM, 5 μM, 10 μM, respectively).

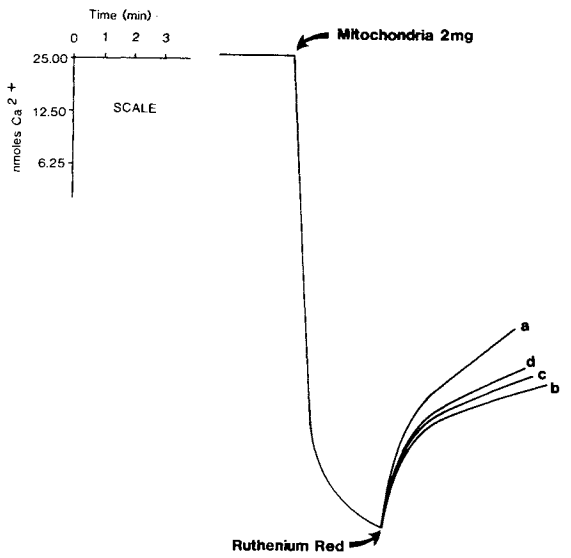

Fig. 4. Counteraction by diltiazem of the Cys A effect on RR-induced Ca^{2+} efflux. RR = 1 nmole per mg of mitochondrial protein. 12.5 nmoles of Ca^{2+} per mg of mitochondrial protein was added. Cys A = 1.0 μM. a, RR alone; b, RR + Cys A; c and d, RR + Cys A + diltiazem 0.1 μM and 0.5 μM, respectively.

of interest to study the action of CCB on calcium fluxes through mitochondrial membrane in presence of Cys A. As said above, steady state (see Method) corresponds to a dynamic equilibrium between Ca^{2+} entry and release. Since Cys A blocks the Ca^{2+} efflux from mitochondria (3,4,5,6) it increases the SSD. The corrective effect of CCB (Fig. 3, Table II) may occur during either Ca^{2+} uptake or release. But in our experimental conditions the drugs did not appear to modify the Ca^{2+} influx (not shown). Therefore the corrective effect observed may be related to an action on the Ca^{2+} efflux mechanisms. Experiments with RR supported this hypothesis. Since RR is a poison of Ca^{2+} carrier, it induces a Ca^{2+} release when it is added during the steady state because it disrupts the dynamic equilibrium. Since Cys A inhibits mitochondrial Ca^{2+} release, it totally or partially suppresses the RR effect according to the concentration

Table II. Restoration by Calcium Channel Blockers of SSD Increased by Cys A (0.2 μM)

Concentration, μM	SSD Diminution, %			
	Verapamil (n = 3)	Nifedipine (n = 3)	Bepridil (n = 3)	Diltiazem (n = 3)
0.25	17 ± 2	6 ± 2	NS[a]	NS[a]
0.50	25 ± 3	20 ± 4	13 ± 2	NS[a]
1.00	34 ± 5	30 ± 3	21 ± 5	18 ± 2
5.00	40 ± 7	44 ± 7	45 ± 6	29 ± 3
10.00	42 ± 6	61 ± 6	61 ± 4	22 ± 3

Results are expressed as percentages of SSD decrease. Each value represents the mean ± SD.
[a] Not significant.

(Fig. 4). Therefore the restoration of Ca^{2+} efflux induced by CCB is consistent with a hindering of the Cys A effect at the level of the Ca^{2+} release mechanism, thus leading to a decrease in Cys A-induced Ca^{2+} storage. This finding may explain the in vitro corrective effect of CCB on Cys A-impaired ATP synthesis since calcium storage classically inhibits oxidative phosphorylation (7).

The inhibition of ATP synthesis by Cys A is apparently in contradiction with previous results showing that the drug is able to maintain the cellular ATP level during an experimental oxydative stress such as that observed during acute ischemic reperfusion injury (16). In fact this apparent discrepancy may be easily explained. During oxidative stress, a deleterious mitochondrial Ca^{2+} release occurs. Therefore the ability of Cys A to block the mitochondrial Ca^{2+} efflux will be beneficial during this acute situation. In contrast our mitochondrial model corresponds to a chronic administration of Cys A without any acute injury. In these conditions Cys A proves toxic because of the permanent increase in mitochondrial calcium storage.

Our results obtained with liver mitochondria might occur with other mitochondria. Ca^{2+} release from liver mitochondria is essentially driven by a Na^{+}-independent pathway as in mitochondria from kidney, lung and smooth muscle (17). Moreover our hypothesis linking Cys A nephrotoxicity with the inhibition of Ca^{2+} release from liver mitochondria (8) is in good accordance with that of Khauli, for whom the toxic effect of Cys A is related to kidney mitochondrial calcium overload (18).

Now the question is whether the effect we observed with isolated mitochondria occurs in the cell. This seems possible since CCB enter the cell (19) and mitochondrial binding sites have been described for them (20). Furthermore the concentrations of CCB we used were in the range of those measured in the cell.

Finally, verapamil and nifedipine appear to be the most

potent in our mitochondrial model both for restoration of ATP synthesis and correction of SSD. These results obtained with concentrations in the pharmacological range (≤ 1 μM) are consistent with the fact that these two drugs also appear the most efficient in clinical use for the correction of Cys A toxicity.

Therefore our in vitro findings may intervene in vivo. If so, the results indicate that through a decrease in mitochondrial Ca^{2+} storage, CCB restore the intracellular ATP level diminished by Cys A. The dysfunctioning of the cellular extrusion Ca^{2+} pump would thereby be prevented and the toxic vasoconstrictive effect of Cys A would be corrected. This hypothesis derived from our in vitro model is consistent with the well documented clinical efficacy of CCB in preventing and correcting cyclosporine A toxicity.

ACKNOWLEDGMENTS

We thank Mrs. Vidalin and Mr. Bouteille for their skillful technical assistance.

REFERENCES

1. R. G. Luke. Mechanism of Cyclosporine induced hypertension. *Am J Hypert* 4, 468-471 (1991).
2. J. S. Abraham, F. R. Bentley, R. N. Garrison, H. M. Cryer. Cyclosporine A directly constricts intrarenal arterioles. *Transplant Proc* 23, 356-359 (1991).
3. N. Fournier, G. Ducet, and A. Crevat. Action of Cyclosporine A on mitochondrial calcium fluxes. *J. Bioenenerg Biomembrane* 19, 297-303 (1987).
4. C. Richter, M. Theus, J. Schlegel. Cyclosporine A inhibits mitochondrial pyridine nucleotide hydrolysis and calcium release. *Biochem Pharmacol* 4, 779-782 (1990).
5. KM. Broekemeier, ME. Dempsey, DR. Pfeiffer. Cyclosporine A is a potent inhibitor of the inner membrane permeability transition in liver mitochondria. *J Biol Chem* 264, 7826-7830 (1989).
6. P. Bernardini, P. Veronese, V. Petronilli. Modulation of the mitochondrial cyclosporine A sensitive permeability transition pore. *J Biol Chem* 268, 1005-1010 (1993).
7. M. Tuena De Gomes-Puyou, MN. Gavilanes, A. Gomes-Puyou, C. Ernster. Control of activity states of heart mitochondrial ATPase. Role of the proton-motive force and Ca^{2+}. *Biochim Biophys Acta* 592, 336-405 (1980).
8. M. D. Salducci, A. M. Chauvet-Monges, Y. Berland, B. Dussol, R. Elsen, A. Crevat. The restoration of ATP synthesis may explain the protective effect of calcium channel blocker against Cyclosporine A nephrotoxicity. *Life Sci* 50, 2053-2058 (1992).
9. H. Meyer-Lehnert, U. Friedrichs, D. Bokemeyer, S. Drechsler, H. J. Kramer. Cyclosporin A-induced calcium accumulation in glomerular mesangial cells. Inhibition by atrial natriuretic peptide (ANP) (abstract). *J Amer Soc Nephrol* 4, 756, (1993).
10. I. Dawidson, P. Rooth, W. R. Fry et al. Prevention of acute cyclosporine-induced renal blood flow inhibition and improved immunosuppression with verapamil. *Transplantation* 48, 575-580 (1989).
11. J. E. Scoble, J. C. M. Senior, P. Chan, Z. Varghese, P. Swery, J. F. Moorhead. In vitro Cyclosporine toxicity. *Transplantation* 47, 647-650 (1989).
12. G. Johnson, H. Lardy. Isolation of liver or kidney mitochondria. In P. W. Estabrook and M. Pullman, *Methods in Enzymology*, Academic Press New York, 1967, p. 94-96.
13. G. Ubeaud, A. M. Chauvet-Monges, J. P. Monti, J. P. Tillement, and A. Crevat. Calcium antiionophoretic effect of some calcium channel blockers in rat liver mitochondria. *J Pharm Sci* 83, 165-168 (1994).
14. M. Spedding. Calcium antagonist subgroups. *Trends Pharmacol Sci* 3, 109-114 (1985).
15. J. Brockmöller, H. H. Neumayer, K. Wagner, W. Weber, G. Heinemeyer, H. Kewitz, and I. Roots. Pharmacokinetic interaction between cyclosporin and diltiazem. *Eur J Clin Pharmacol* 38, 237-242 (1990).
16. G. E. N. Kass, M. J. Juedes, S. Orrenius. Cyclosporine A protects hepatocytes against prooxidant-induced cell killing. *Biochem Pharmacol* 44, 1995-2003 (1992).
17. C. Richter, J. Schlegel. Mitochondrial calcium release induced by prooxidants. *Toxicol Lett* 67, 119-127 (1993).
18. R. B. Khauli, T. Strzelecki, S. Kumar, M. Fink, J. Stoff, M. Menon. Mitochondrial alterations after Cyclosporine and ischemia, Insights on the pathophysiology of nephrotoxity. *Transplant Proc* 19, 1395-1397 (1987).
19. D. C. Pang, N. Sperelakin. Nifedipine, Diltiazem, Bepridil and Verapamil uptake into cardiac and smooth muscles. *Eur J Pharmacol* 87, 199-207 (1983).
20. G. Zernig, I. Graziadei, T. Moshammer, C. Zech, N. Reider, H. Glossmann. Mitochondrial Ca^{2+} antagonists binding sites are associated with an inner mitochondrial membrane anion channel. *Mol Pharmacol* 38, 362-369 (1990).