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Tacrolimus and sirolimus decrease oxidative phosphorylation of isolated rat kidney mitochondria

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1 Tacrolimus and sirolimus are potent immunosuppressors used in transplantation. Tacrolimus has been suspected to alter mitochondrial respiration of different tissues but sirolimus has not been evaluated.

2 We evaluated the *in vitro* effect of tacrolimus and sirolimus on oxidative phosphorylation of isolated rat kidney mitochondria.

3 Oxygen consumption was measured with a Clark-type electrode. Tacrolimus and sirolimus increased the resting rate (state 4) and had no significant effect on ADP-stimulated respiration (state 3). The decrease of respiratory control ratio was concentration-dependent with a biphasic curve for tacrolimus. The EC_{50}s were 3.4×10^{-11} M and 2.3×10^{-8} M for tacrolimus and 4.4×10^{-10} M for sirolimus. The maximal inhibition was 20 and 14% for tacrolimus and sirolimus, respectively.

4 Tacrolimus and sirolimus had an uncoupling effect on oxidative phosphorylation related to a decrease of the inner membrane fluidity. At the opposite of cyclosporin A, no effect on swelling or Ca^{2+} fluxes was observed.

5 All events occurred at therapeutic concentrations and then could appear during long-term treatment. Cellular consequences such as chronic nephrotoxicity with tacrolimus are suggested. The risk of cyclosporin A nephrotoxicity potentiation by sirolimus is discussed.
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Abbreviations: CCCP, carbonyl cyanide m-chlorophenylhydrazone; DPH, 1,6-diphenyl-1,3,5- hexatriene; DUQ, decylubiquinone; HP, haematoporphyrin; P/O, ratio of ADP used by atom of Oxygen; RCR, respiratory control ratio; TMPD, N,N,N′,N′- tetramethyl-p-phenylenediamine

Introduction

Immunosuppression therapy currently used cyclosporin A and tacrolimus. Recently sirolimus has received approval from the U.S. Food and Drug Administration for marketing as an agent for the prevention of acute rejection in renal transplant recipients. Sirolimus shared with tacrolimus a similar molecular structure and a binding to the cytosolic immunophilin FK Binding Protein 12 (FKBP12) (Saunders *et al*., 2001). Cyclosporin A and tacrolimus act by inhibiting the calcineurin phosphatase. By contrast, sirolimus-FKBP12 complex has no effect on calcineurin phosphatase but its molecular targets include RAFT1/FRAP proteins in mammalian cells (Gummert *et al*., 1999). Thus, despite a similar molecular structure, tacrolimus and sirolimus have different mechanisms of action. Several trials are still ongoing which will help to define the best place of sirolimus in the immunosuppression therapy.

If the use of calcineurin phosphatase inhibitors for preventing transplant rejection has been a major improvement, they also lead to acute and chronic nephrotoxicity (de Mattos *et al*., 2000). The mechanism of acute nephrotoxicity seems to be linked with a direct glomerular vasoconstriction (de Mattos *et al*., 2000), whereas chronic nephrotoxic mechanism is less clear. Several studies have tried to elucidate this pejorative effect and interestingly they observed different properties of cyclosporin A and tacrolimus. Among them, cyclosporin A has been shown to act on mitochondria by inhibiting the permeability transition (Broekemeier & Pfeiffer, 1995). This effect leads to an accumulation of calcium into the matrix and a subsequent decrease of energy supply (Simon *et al*., 1997). In this study, we proposed that after long term cyclosporin A therapy, the sustained decrease of ATP synthesis could lead to chronic toxicity. To the same extent, tacrolimus was shown to inhibit the oxidative respiration of mitochondria (Henke & Jung, 1993) but the investigations always focused on one particular function and never involved sirolimus. In this study we determined the effects of tacrolimus and sirolimus on various mitochondrial tests such as oxidative phosphorylation, Ca^{2+} fluxes, swelling, cytochrome *c* release, and membrane fluidity. The relationship between the *in vitro* activities and side-effects are discussed.

Methods

Materials

Sucrose, EGTA, D-mannitol, rotenone, malonate, antimycin A, oligomycin, succinate, malate, pyruvate, ADP, carbonyl

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cyanide m-chlorophenylhydrazone (CCCP), sodium ascorbate, N,N,N′,N′-tetramethyl-p-phenylenediamine (TMPD), decylubiquinone (DUQ), 1,6-diphenyl-1,3,5-hexatriene (DPH); haematoporphyrin (HP) and bovine serum albumin (BSA) were purchased from Sigma (Saint Quentin Fallavier, France). KCl and $MgCl_2$ were obtained from Prolabo (Paris, France). KH_2PO_4 was purchased from Merck (Paris, France).

Tacrolimus was a gift of Fujisawa Laboratories (Deerfield, IL, U.S.A.). Sirolimus was purchased from Sigma (Saint Quentin Fallavier, France). They were solubilized in dimethylformamide (DMF) and distilled water (v v^{-1}) in order to obtain a stock solution at 10^{-3} M. All the controls were carried out using the same solvent mixture. The final solution contained no more than 0.05% DMF.

Decylubiquinol ($DUQH_2$) was immediately prepared from decylubiquinone (DUQ) as described by Veitch *et al.* (1992). The solution was evaporated to dryness under nitrogen gas, dissolved in 1 ml of DMF and stored at −70°C until used.

Isolation of kidney mitochondria

Mitochondria were extracted from a homogenate of rat kidney by differential centrifugations according to Simon *et al.* (1997). Rats (male, Wistar, weighing 280–300 g) were killed by decapitation and the kidneys removed and placed quickly in an ice-bath. The kidneys were then minced in ice-cold isolation medium (Tris 20 mM, sucrose 250 mM, KCl 40 mM, EGTA 2 mM and bovine serum albumin 1 mg ml^{-1}; pH 7.2) and homogenized (6 ml g^{-1} of tissue) using a Potter-Elvejhem homogenizer. Mitochondria isolation was performed at 4°C using differential centrifugation: the homogenate was centrifuged at $2000 \times g$ for 8 min to remove cell debris and nuclei, mitochondria were separated from the supernatant by centrifugation at $12{,}000 \times g$ for 10 min. The pellet (mitochondria) was washed and resuspended in a medium containing sucrose 250 mM, KH_2PO_4 5 mM for the Ca^{2+} fluxes and induced swelling experiments, or in a respiratory buffer (Mannitol 300 mM, KH_2PO_4 10 mM, KCl 10 mM, $MgCl_2$ 5 mM, pH 7.2) for measuring respiratory activity. Protein concentrations of the mitochondrial suspension were determined by the method of Lowry. All assays were done on freshly isolated mitochondria. This protocol meets the guidelines of the French agency regarding animal experimentation (authorization No00748 delivered to Pr. J.P. Tillement).

Assay of mitochondrial oxygen consumption

Oxygen uptake was determined with a Clark-type microelectrode (Hansatech, U.K.). Each experiment was carried out as follows: 45 μl of mitochondria suspension (0.4 mg ml^{-1}, except for evaluation of complexes I to V where 1.2 mg ml^{-1} was used) were preincubated during 15 min at 4°C with (or without) the tested drug, then incubated 1 min at 37°C in 500 μl of the respiratory buffer without or with the inhibitors, then the substrates (listed in the following paragraph) were added and oxygen consumption was checked (State 2). To initiate state 3 respiratory activity, 200 μM ADP was added to the cuvette. When all ADP was converted to ATP the state 4 was measured. The following parameters were determined: the respiratory rates calculated as nM O_2 min^{-1} mg^{-1} mitochondrial protein, the respiratory control ratio (RCR) expressed as the ratio of state 3/state 4 oxygen consumption and the ratio of ADP used by atom of oxygen (P/O).

The rates of oxygen consumption by the different complexes were determined according to Rustin *et al.* (1994). Briefly, rotenone (2 μM), malonate (10 mM), antimycin (1 μM), and oligomycin (10 μM) were used to inhibit complexes I, II, III and V, respectively and carbonyl cyanide-m-chlorophenylhydrazone (CCCP) was added as uncoupling agent (10 μM). Malate (10 mM) plus pyruvate (10 mM), succinate (10 mM), decylubiquinone (150 μM), ascorbate (5 mM) plus TMPD (1 mM) were used as subtrates for complexes I, II, III and IV respectively.

Ca^{2+} fluxes

A specific Ca^{2+} electrode (Orion 9320) fitted to a Hansatech recorder *via* a 720A Orion ionometer was used to record Ca^{2+} movements in extramitochondrial medium in a thermostat-controlled reaction chamber (3.8 ml) at 37°C containing sucrose 250 mM, KH_2PO_4 5 mM, plus succinate 6 mM (Simon *et al.*, 1998). The study was performed in presence of 25 μM of Ca^{2+}. Tacrolimus and sirolimus effects were measured at 1 μM and were added prior to Ca^{2+} and mitochondria to ensure that they did not modify the electrode response. The Ca^{2+} concentration in extramitochondrial medium decreased rapidly when mitochondria (1 mg ml^{-1} protein) were added, due to Ca^{2+} uptake into mitochondria. After all of the oxygen in the medium was consumed, anaerobiosis led to Ca^{2+} release.

Measurement of swelling of energized mitochondria

The method of Halestrap & Davidson (1990) was used with some modifications. Mitochondria (1 mg ml^{-1} of protein) were added to 1.8 ml of a medium containing sucrose 250 mM, KH_2PO_4 5 mM and rotenone 3 μM at 37°C. After mixing, swelling was initiated by addition of Ca^{2+} 25 μM plus succinate 6 mM to the sample cuvette. Swelling of energized mitochondria was determined by measuring the decrease of optical density at 520 nm, with a Hitachi spectrophotometer (U3000) (Simon *et al.*, 1998).

Enzyme activity determination

Mitochondrial complex III assay (ubiquinol-cytochrome c *oxidoreductase)* Mitochondrial complex III activity was measured as the rate of cytochrome *c* reduction at 550 nm and 37°C triggered by decylubiquinol ($DUQH_2$) in Hitachi U-3000 spectrophotometer (Zini *et al.*, 1998). The reaction mixture (1 ml) contained: Tris-HCl 10 mM pH 7.2, ADP 0.2 mM, rotenone 3 μM, KCN 0.3 mM, succinate 10 mM and 40 μg of mitochondria. Antimycin A (1 μM) was added to the reference cuvette to inhibit complex III activity. The reaction was initiated by the addition of 40 μM cytochrome *c* in the assay cuvette. After 1 min, 10 mM malonate (complex II inhibitor) was added and inhibited rate measured for a further 1 min, then 50 μM $DUQH_2$ (substrate) was added and the rate of cytochrome *c* reduction (product) measured for a further 1 min.

Mitochondrial complex V assay (ATPase) ATPase activity was measured as the hydrolysis rate of ATP to ADP + Pi

(Morin *et al*., 2000). Mitochondria (25 μg) were incubated in ATPase buffer (500 μl; Tris 50 mM, $MgCl_2$ 5 mM; pH 7.2) at 37°C with 5 mM of ATP for 10 min. Then the reaction was stopped by the addition of trichloracetic acid 10% (500 μl). Each assay was centrifuged at $3000 \times g$ for 20 min and 500 μl of supernatant were mixed with 500 μl of water. A control was performed in same conditions in order to obtain the non enzymatic hydrolysis of ATP. Pi production was measured using the method of Fiske & Subbarow (1925).

Cytochrome c *release*

The amount of cytochrome *c* was measured using a Quantikine® M Rat/Mouse Cytochrome *c* immunoassay (R&D Systems, U.K.) after addition of drugs in energized mitochondrial (3 min) or after Ca^{2+}-induced mitochondrial swelling (3 min). The suspension was taken and centrifuged in order to eliminate mitochondria. Then the supernatant was diluted 1/50 and the cytochrome *c* release was measured.

Membrane fluidity

The membrane fluidity was measured by the changes in fluorescence anisotropy (*r*) of mitochondrial membranes with two fluorescent probes: 1,6- diphenyl-1,3,5-hexatriene (DPH) and haematoporphyrin (HP). DPH is typically used to probe highly hydrophobic lipid phases of membranes, while HP mostly interacts with very polar, solvent-accessible regions of the lipid bilayer (Ricchelli *et al*., 1991) and with protein sites in biological membranes (Ricchelli *et al*., 1995; 1999a). HP preferentially accumulates in protein regions of the inner membrane of mitochondria (Ricchelli *et al*., 1999b). Briefly, DPH (5 mM) diluted in tetrahydrofuran was added into mitochondrial suspension to give a final phospholipid/probe molar ratio of about 200 (Mecocci *et al*., 1997). The labelling was achieved after a 2 h-incubation at 4°C. HP (3 μM) was added into stirred mitochondria suspension (1 mg ml^{-1}) and the mixture incubated for 2 min before measuring anisotropy. Then DPH was excited at 340 nm, its fluorescence detected at 460 nm; HP was excited at 520 nm and its fluorescence detected at 626 nm with a Perkin-Elmer LS 50B spectrophotofluorimeter. The anisotropy values (*r*) were obtained by measuring the polarized fluorescence intensities, parallel (I_{vv}) and perpendicular (I_{vh}) to the vertical plane of polarization of the excitation beam. These values are defined by the following equation:

$$r = (I_{vv} - G\,I_{vh})/(I_{vv} + 2G\,I_{vh})$$

where G equals I_{hv}/I_{hh}, I_{hv} is the intensity with the polarizers in horizontal and vertical positions (excitation and emission) and I_{hh} is the intensity with the polarizers in horizontal and horizontal positions (excitation and emission) and represents the correction factor for instrumental artefacts. A significant increase of the fluorescence anisotropy reflects a decrease of membrane fluidity (Ricchelli *et al*., 1999a).

Statistical analysis

For each experiment, mean values of RCR or percentages were compared with a one-way analysis of variance. When appropriate, comparisons between groups were made using a Scheffe's test. Values of $P < 0.05$ were considered significant. EC_{50} was calculated by non-linear regression of effect-concentration curve to the equation:

$$E = E_0 + (E_{max} \times EC_{50})/(C + EC_{50}),$$

where E_0, E_{max} and EC_{50} are the residual effect, the maximal effect and the concentration producing 50% of maximal effect respectively, using a commercially available software (Micropharm) (Urien, 1995).

Results

Oxygen consumption

When the respiratory chain was activated by malate/pyruvate and ADP (complexes I to V), the RCR was significantly decreased by 10 or 11% for tacrolimus and sirolimus, respectively (Table 1). Significant increase of state 4 was observed whereas the state 3 was not modified. So the decrease of RCR value was due to an increase of state 4 even if a slight increase of state 3 occurred. The P/O ratio was also significantly decreased by tacrolimus and sirolimus.

When the respiratory chain was activated by succinate and complex I inhibited by rotenone, tacrolimus and sirolimus decreased significantly RCR (Figure 1). This effect was concentration-dependent for the two compounds, with a biphasic curve for tacrolimus. The EC_{50} values were 3.4×10^{-11} M and 2.3×10^{-8} M for tacrolimus; 4.4×10^{-10} M for sirolimus. The maximal inhibitory effect was about 20% for tacrolimus and 14% for sirolimus. These results show that tacrolimus and sirolimus decrease oxidative phosphorylation without inhibiting complex I.

When complexes III and IV were activated alone, tacrolimus and sirolimus (1 μM) significantly reduced oxygen consumption by 25 and 17%, respectively (Table 1). Finally, when complex IV operated alone, none of the tested compounds modified the oxygen consumption (Table 1).

Ca^{2+} fluxes through mitochondria

Ca^{2+} overloading experiments were carried out to confirm the Ca^{2+} dependency of the mitochondrial effects of tacrolimus and sirolimus. None of them (1 μM) modified Ca^{2+} fluxes (Figure 2).

Swelling of energized mitochondria

Tacrolimus and sirolimus (1 μM) did not modify the swelling of energized mitochondria induced by 25 μM Ca^{2+} plus inorganic phosphate (KH_2PO_4 5 mM) (Figure 3). The rates of swelling were 104 and 102% versus control for tacrolimus and sirolimus.

Enzyme activity determination

Both tacrolimus and sirolimus decreased the complex III activity. This inhibition was about 30 and 24% for tacrolimus and sirolimus respectively (Table 2). When the enzyme activity of complex V was investigated, the decrease was about 8% for both compounds (Table 2).

Table 1 Effects of tacrolimus and sirolimus on oxidative respiration of isolated rat kidney mitochondria

		Control	*Tacrolimus*	*Sirolimus*
Complexes I→V				
S3	(nmol O_2 min^{-1} mg^{-1})	50.4 (3.05)	51.8 (3.61)	53.1 (1.83)
S4	(nmol O_2 min^{-1} mg^{-1})	14.7 (1.18)	16.8 (1.01)*	17.5 (1.04)*
RCR		3.43 (0.08)	3.08 (0.07)†	3.05 (0.10)†
P/O	(mol ADP/atom O)	2.41 (0.08)	2.14 (0.19)*	2.32 (0.09)*
Complexes II→V				
S3	(nmol O_2 min^{-1} mg^{-1})	76.5 (9.50)	79.4 (32.2)	75.0 (8.16)
S4	(nmol O_2 min^{-1} mg^{-1})	25.6 (3.45)	30.3 (12.9)	27.1 (0.51)
RCR		3.00 (0.25)	2.64 (0.13)‡	2.77 (0.34)†
Complexes III→IV				
	(nmol O_2 min^{-1} mg^{-1})	41.4 (7.34)	31.0 (6.67)‡	34.4 (3.19)†
Complex IV				
	(nmol O_2 min^{-1} mg^{-1})	215 (37.0)	232 (48.8)	220 (31.1)

*$P<0.05$; †$P<0.01$; ‡$P<0.001$ compared to control. Each value represents the mean (±s.d.) of six determinations. The duration time for compounds incubation (1 μM) was 15 min. The controls were carried out using an equivalent volume of dimethyl formamide (0.05%). For evaluation of complexes I to V, the oxygen consumption was measured with malate (10 mM) plus pyruvate (10 mM) as substrates and ADP (0.2 mM) for a mitochondrial concentration of 1.2 mg ml^{-1}. For evaluation of complexes II to V, the oxygen consumption was measured with rotenone (2 μM), succinate (10 mM) and ADP (0.2 mM) for a mitochondrial concentration of 0.4 mg ml^{-1}. To investigate complexes II and IV, the oxygen consumption was measured with rotenone (2 μM), malonate (10 μM) and oligomycin (10 μM) in order to inhibit complexes, I, II and V, and decylubiquinol (150 μM) plus carbonyl cyanide m-chlorophenylhydrazone (CCCP; 10 μM). Then complex III was inhibited by antimycin (1 μM) and complex IV was stimulated by sodium ascorbate (5 mM) and N,N,N′,N′-tetramethyl-p-phenylenediamine (TMPD, 1 mM). The mitochondrial concentration for these last experiments was 0.4 mg ml^{-1}.

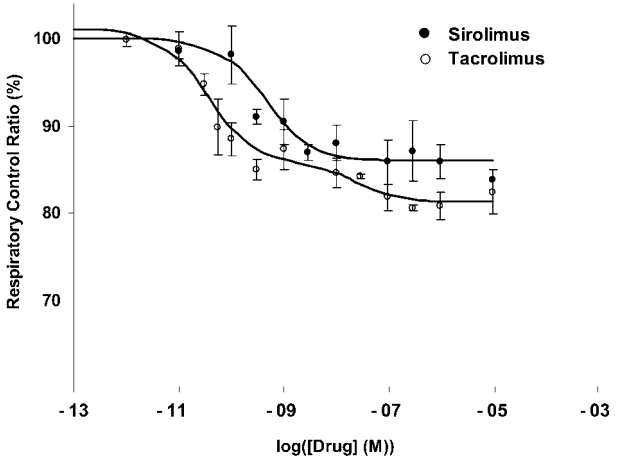

Figure 1 Effect of tacrolimus and sirolimus on respiratory control ratio (RCR) of rat kidney mitochondria. Concentration-response curves were expressed as the percentages of RCR, tacrolimus (open circles) and sirolimus (closed circles). All experiments were carried out at 37°C with succinate (10 mM), rotenone (2 μM), ADP (0.2 mM) and 0.4 mg ml^{-1} of mitochondrial protein. Each point is the mean±s.d. of eight determinations.

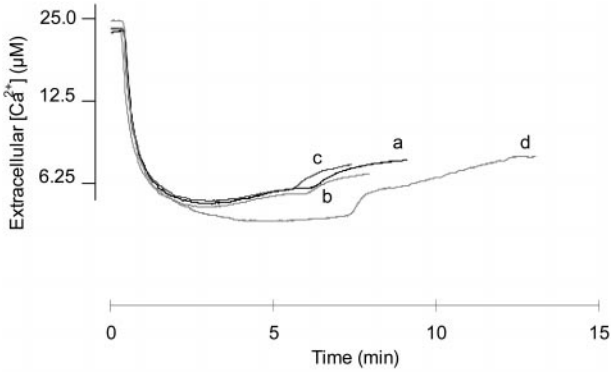

Figure 2 Representative plot of tacrolimus and sirolimus effect on Ca^{2+} fluxes on rat kidney mitochondria. At the beginning of the experiment, Ca^{2+} medium concentration was 25 μM. Then mitochondria (1 mg ml^{-1}) were added in the cuvette (arrow). The change of Ca^{2+} in medium was recorded with a Ca^{2+}-sensitive electrode system. A downward deflection corresponded to a decrease of Ca^{2+} concentration in the medium, i.e. an increase of mitochondrial Ca^{2+}. The medium contained succinate 6 mM and either control (a), tacrolimus 1 μM (b), sirolimus 1 μM (c) or cyclosporin A (d).

Cytochrome c *release*

Tacrolimus and sirolimus did not induce a release of cytochrome *c* by kidney mitochondria (Figure 4). The Ca^{2+}-induced (25 μM) swelling released 29% of the whole mitochondrial cytochrome *c* concentration (1021 ng cytochrome *c* mg^{-1} protein). Tacrolimus or sirolimus did not influence the release.

Membrane fluidity

The dynamic properties of mitochondrial membranes were investigated by following the changes in the steady state fluorescence anisotropy (*r*) of mitochondriabound HP or DPH. Tacrolimus and sirolimus increased significantly the anisotropy of HP-labelled mitochondria (Figure 5). The *r* value which theoretically ranges from 0 to 0.4 was increased by 0.01 and 0.005 units for tacrolimus and sirolimus respectively. In contrast with HP, no change in anisotropy was observed for DPH-labelled mitochondria (data not shown).

Discussion

Mitochondria are essential for the viability of eukaryotic cells and among their properties they are the main source of

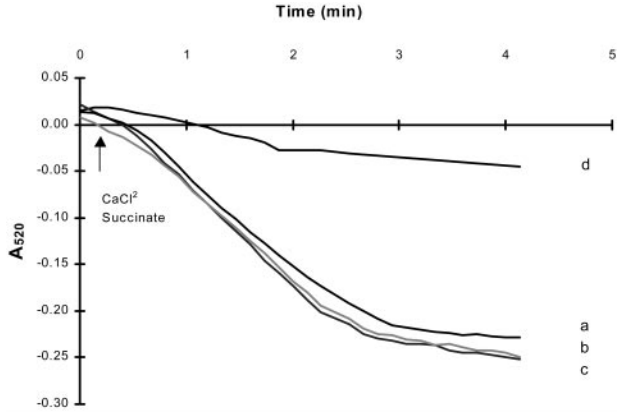

Figure 3 Representative plot of tacrolimus and sirolimus effect on mitochondrial swelling. The swelling was induced by adding Ca^{2+} 25 μM and succinate 6 mM to a suspension of mitochondria (1 mg ml^{-1}) containing 250 mM sucrose; 5 mM KH_2PO_4 and either blank control (a), tacrolimus 1 μM (b), sirolimus 1 μM (c) or cyclosporin A 1 μM (d). In each experiment, the absorbance at 520 nm was measured.

Table 2 Effects of tacrolimus and sirolimus on enzyme activity of complex III and V

	Complex III (nmol cytochrome *c* reduced min^{-1} mg^{-1})	*Complex V* (nmol inorganic phosphate min^{-1} mg^{-1})
Control	1285 (120)	149.0 (5.1)
Tacrolimus	903 (55)†	137.5 (2.4)*
Sirolimus	976 (111)†	137.9 (2.7)*

*$P<0.01$; †$P<0.001$ compared to control. Each value represents the mean (±s.d.) of six determinations. The duration time for compounds incubation (1 μM) was 15 min. The substrates for the mitochondrial complex III assay (ubiquinol-cytochrome *c* oxidoreductase) were 0.2 mM ADP, 3 μM rotenone, 0.3 mM KCN, 10 mM succinate and 40 μM cytochrome *c*. After 1 min, 10 mM malonate was added and inhibited rate measured for a further 1 min; then 50 μM $DUQH_2$ was added and the rate measured for a further 1 min. ATPase activity was measured with 5 mM of ATP. The mitochondrial concentration for these experiments was 0.4 mg ml^{-1}.

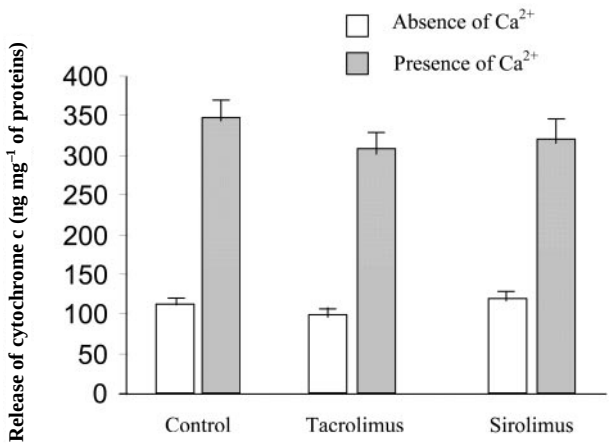

Figure 4 Effect of tacrolimus and sirolimus on cytochrome *c* release The release of cytochrome *c* was measured after 5 min of incubation of kidney mitochondria (2 mg ml^{-1}) in a buffer containing 250 mM sucrose, 5 mM KH_2PO_4, 6 mM succinate, pH 7.2 at 37°C. The presence of 25 μM of Ca^{2+} (swelling conditions) induced an increase of the cytochrome *c* release compared to controls without Ca^{2+}. Tacrolimus (1 μM) and sirolimus (1 μM) did not modify the release of cytochrome *c* in control or swelling conditions. Each value represents the mean±s.d. of six determinations.

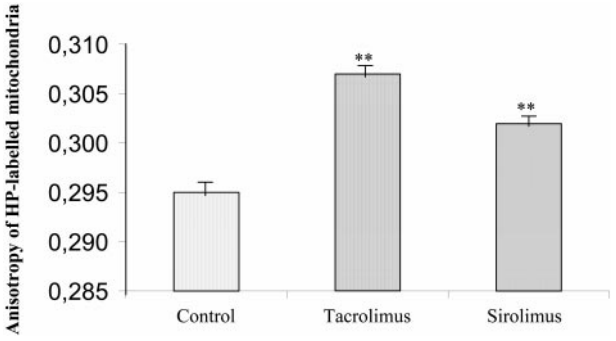

Figure 5 Effect of tacrolimus and sirolimus on anisotropy of haematoporphyrin-labelled kidney mitochondria. The membrane fluidity was evaluated by measuring the changes in fluorescence anisotropy (*r*) of mitochondrial membranes with haematoporphyrin (HP) as fluorescent probe. HP (3 μM) was injected into stirred mitochondria suspension (1 mg ml^{-1}) and the mixture incubated for 2 min before measuring anisotropy in presence of either tacrolimus 1 μM or sirolimus 1 μM. HP was excited at 520 nm and its fluorescence detected at 626 nm. The data were the mean±s.e.mean (bars) of four experiments in triplicate. **$P<0.01$ versus control.

respiratory energy, i.e. energy produced by the oxidative phosphorylation. Currently an efficient way to investigate the ability of mitochondria to produce ATP is to monitor the oxygen consumption in different conditions. Without any substrates no oxygen consumption is observed, but as soon as succinate (or other substrates) is added a decrease in oxygen concentration was observed corresponding to the consumption by mitochondria. This oxygen consumption called state 4, corresponds to basal conditions required to maintain the membrane potential due to the gradient of protons. When ADP is added, the oxygen consumption is significantly increased (state 3). The sudden ADP increase is related to an ATP depletion which stimulates ATP synthesis by the complex V (ATP synthase). The ability of mitochondria to respond to the 'ATP depletion' is measured by the respiratory control ratio (RCR) which is the state 3/state 4 ratio. Another parameter defining the ability of mitochondria to face with an ATP depletion is the ratio of ADP used (equivalent as mole of ATP synthesized) to consumed oxygen atom (P/O). A decrease of RCR and P/O indicates a loss of the coordination between the activity of the oxidative chain (complex I to IV) and the ATPase (complex V).

Polarographic experiments showed that sirolimus and tacrolimus are both inhibitors of the respiratory control ratio and these effects were concentration-dependent (Figure 1). For tacrolimus, this is in agreement with published data (Henke & Jung, 1993; Zini *et al*., 1998). However, if several authors found that the respiratory control ratio is decreased, little is known on the mechanism. The decrease of the respiratory control ratio can be explained by an increase of the state 4, a decrease of the state 3 or both. Henke & Jung (1993) described different effects on state 3

of the oxygen consumption depending on the substrate used. The state 3 respiration was decreased with succinate while other substrates such as glutamate/malate had no effect. Using mitochondria from hepatocytes, Pelekanou *et al*. (1991) did not observe an effect of tacrolimus on the state 3 respiration. In our experiments, both state 3 and state 4 were increased but only the effect on state 4 was significant (Table 1). Because the increase is more pronounced for state 4 than for state 3, we observed a decrease in the respiratory control ratio. Furthermore this effect was associated with a decrease in the P/O ratio which highly suggests an uncoupling effect of tacrolimus and sirolimus on kidney mitochondria. When activation of the oxidative chain was performed from complex III, we observed a decrease in oxygen consumption (Table 1). At the opposite, no effect was observed when only complex IV was activated. These results were confirmed by determination of enzymatic activity, tacrolimus and sirolimus decreased the activity of complex III by 32% and 25% respectively and had no effect on complex V (Table 2). In fact, it is necessary to inhibit more than 80% of the complex III activity to observe a decrease of the respiratory rate (Rossignol *et al*., 1999). Thus, the specific inhibition of the complex III activity by sirolimus and tacrolimus seems to be insufficient to explain their action on oxygen consumption.

Fluidity changes of mitochondrial membranes were measured with two probes, DPH and HP which monitor hydrophobic and hydrophilic regions of lipidic membrane. Incubation with tacrolimus or sirolimus increased the fluorescence anisotropy (r) corresponding to a decrease in membrane fluidity. This effect was only observed with HP which means that the decrease in fluidity was confined to rich protein areas (Figure 5). These results confirmed the uncoupling effects of tacrolimus and sirolimus. Indeed, uncoupling agents such as the protonophore CCCP, also increase the fluorescence anisotropy (Ricchelli *et al*., 1999b). The decrease in membrane fluidity, especially in protein region, could decrease the ability of mitochondria to arrange membrane components such as respiratory complexes. This finally leads to a higher consumption of oxygen to produce an equivalent amount of ATP, i.e. an uncoupling effect.

Experiments on Ca^{2+} fluxes or Ca^{2+}-induced swelling did not show any effect of tacrolimus and sirolimus. This result is on agreement with previous studies where tacrolimus was compared to cyclosporin A (Pelekanou *et al*., 1991; Prasad *et al*., 1991; Henke & Jung, 1993; Friberg *et al*., 1998). Furthermore, neither tacrolimus nor sirolimus had anti-oxidant properties, as investigated by lipid peroxydation assay (data not shown).

In vitro experiments agree that tacrolimus, sirolimus and cyclosporin A produced inhibitory effects on respiratory control ratio. However, if the cyclosporin A mechanism of action is related to Ca^{2+} fluxes and Ca^{2+}-induced swelling, for tacrolimus and sirolimus another mechanism occurred. This study showed that tacrolimus and sirolimus had an uncoupling effect on oxidative phosphorylation which could not be explained by the low alteration of complex III activity but by a decrease in the membrane fluidity. These substances probably act indirectly by disarranging membrane structure and thus eliciting an increase of proton conductance of the membrane. Whether or not these two effects are related requires further explanations.

The therapeutic concentrations of tacrolimus (Spencer *et al*., 1997) and sirolimus (Meier-Kriesche & Kaplan, 2000) are around 10 ng ml^{-1}, which correspond to the IC_{50} we found in our experiments. Thus, the levels reached in the tissues could produce mitochondrial effects. However, because tacrolimus had a lower IC_{50} and a higher maximal inhibition on respiratory control ratio, we can expect more cellular consequences than with sirolimus.

Previous studies have shown that the mitochondrial effects of tacrolimus could have consequences on intestinal cells, especially in increasing the intestinal permeability (Madsen *et al*., 1995). These results suggest a link between an *in vitro* effect on mitochondria and cellular consequences. Indeed, tacrolimus has been found to uncouple mitochondrial oxidative phosphorylation and increase intestinal permeability in experimental animals. The authors confirmed in humans that inhibition of cellular energy production was associated with an increased intestinal permeability, endotoxemia and an impaired intestinal absorptive capacity (Gabe *et al*., 1998). Even if the consequences of increased intestinal permeability remain a subject of speculation, they could account for adverse effects observed in patients receiving tacrolimus for immunosuppression such as diarrhoea with malabsorption. Also, the mechanism of tacrolimus chronic nephrotoxicity is poorly understood. Interestingly, it has been described a modulation of energy status associated with cytotoxicity on a renal epithelial cell line incubated with tacrolimus (Massicot *et al*., 1997). The decrease in cell viability was observed with tacrolimus and cyclosporin A and was related to the ATP depletion. As mentioned above, inhibition of oxidative phosphorylation and/or uncoupling of mitochondria by tacrolimus could have cellular consequences. Under particular circumstances, such as long-term exposure of tacrolimus, a deleterious effect on kidney cells could be evoked to explain chronic nephrotoxicity. Further studies are required to define the cause-effect relationships between this inhibition and the nephrotoxicity.

Sirolimus used alone does not seem to induce a clear nephrotoxicity. However in humans, sirolimus can potentiate the nephrotoxic effect of cyclosporin A (Morales *et al*., 2001). This potentiation could be explained by a pharmacokinetic and/or a pharmacodynamic interaction. A pharmacokinetic interaction between cyclosporin A and sirolimus has been described in animals which led to higher concentrations of cyclosporin A and thus could explain the increase of cyclosporin A side-effects (Podder *et al*., 2001). There is a controversy on the ability of sirolimus to produce a toxic effect on kidney when used alone. In a salt-depleted rat model of cyclosporin A nephrotoxicity, the combination with sirolimus produced a functional and morphological deterioration (Andoh *et al*., 1996). Because no measurement of sirolimus was performed, it is difficult to ascertain whether this was a pharmacodynamic or a pharmacokinetic potentiation. More recently, Serkova *et al*., 2001 showed that following a 6-day administration of sirolimus in rat, a significant reduction of nucleoside triphosphate was observed as well as an increase of nucleoside diphosphate. The rat metabolism was investigated in brain using magnetic resonance spectroscopy. Interestingly, the combination of cyclosporin A with sirolimus leads to a synergic effect on mitochondrial metabolism. These results are in agreement with ours. Indeed, we showed that sirolimus had an

uncoupling effect which led to a decrease in the respiratory control ratio and thus a lower ability to synthetize ATP. One could argue that this effect is insufficient to trigger a clear nephrotoxic effect. Indeed, because sirolimus required a higher concentration and has a lower magnitude of effect on mitochondria than tacrolimus, it could never reach the necessary threshold to produce a cellular effect. The biochemical mechanisms involved in the sirolimus effects are concentration dependent. Thus either the sirolimus concentrations in patients are not sufficient to produce an effect or the effect produced is too low to have cellular consequences. However, sirolimus could enhance mitochondrial disorder of cyclosporin A by inhibiting a compensatory mechanism. Then the combination of two drugs with an inhibitory effect on oxidative phosphorylation, cyclosporin A and sirolimus, could finally turn to a more frequent cell injury.

It is concluded that tacrolimus and sirolimus could lead to an ATP depletion in kidney by an uncoupling effect on oxidative phosphorylation linked to a modification of the mitochondrial membrane structure. This mitochondrial effect could explain a toxic effect of tacrolimus on kidney and could enhance the cyclosporin A effect on mitochondria when combined with sirolimus.

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