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Effect of cyclosporin A and its vehicle on cardiac and skeletal muscle mitochondria: relationship to efficacy of the respiratory chain

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1 Although cyclosporin (CsA) is considered to be the best immunosuppressive molecule in transplantation, it has been suspected to alter mitochondrial respiration of various tissues.

2 We evaluated the acute effect of CsA and its vehicle on maximal oxidative capacity (V_{max}) of cardiac, soleus and gastrocnemius muscles of rats by an oxygraphic method in saponin skinned muscle fibres. The effects of Sandimmun (a formulation of CsA), vehicle of Sandimmun (cremophor and ethanol (EtOH)), CsA in EtOH and EtOH alone were tested. Increasing concentrations (5–20–50–100 μM) of CsA (or vehicles) were used.

3 Sandimmun profoundly altered the V_{max} of all muscles. For example, at 20 μM, inhibition reached 18 ± 3, 23 ± 5, 45 ± 5%, for heart, soleus and gastrocnemius respectively. There were only minor effects of CsA diluted in EtOH and EtOH alone on V_{max} of cardiac muscle. Because the effects of vehicle on V_{max} were similar or higher than those of Sandimmun, the inhibition of oxidative capacity could be entirely attributed to the vehicle for all muscles.

4 Next, we investigated the potential sites of action of the vehicle on the different complexes of the mitochondrial respiratory chain by using specific substrates and inhibitors. The vehicle affected mitochondrial respiration mainly at the level of complex I (≈ -85% in skeletal muscles, and -32% in heart), but also at complex IV (≈ -26% for all muscles).

5 The mechanism of action of the vehicle on the mitochondrial membrane and the implications for the clinical use of immunosuppressive drugs are discussed.

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Abbreviations: ACR, acceptor control ratio; COX, cytochrome oxidase; CsA, cyclosporin A; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; O_2, oxygen; OXPHOS, mitochondrial oxidative phosphorylation; PCr, creatine phosphate; Pi, inorganic phosphate; TMPD, N,N,N′,N′-tetramethyl-*p*-phenylenediamine dihydrochloride; V_0, mitochondrial basal respiration rate; Vmax, mitochondrial maximal respiration rate; VO_2, peak oxygen uptake

Introduction

Cyclosporin (CsA) represents the most widely used immunosuppressive molecule in organ transplantation and autoimmune disorders. While the main therapeutic drawback of this drug is its capability to produce nephrotoxicity (Goldstein *et al*., 1997), cyclosporin can adversely damage other organs, *in vitro*, such as cardiac (Olbrich *et al*., 1993; Hucheson *et al*., 1995; Banijamali *et al*., 1998) and skeletal muscles (Fernandez-Sola *et al*., 1990; Biring *et al*., 1998). Other experimental findings on isolated mitochondria indicate that cyclosporin alters mitochondrial respiration in different tissues such as kidney (Lemmi *et al*., 1990), liver (Fournier *et al*., 1987) and skeletal muscles (Hokanson *et al*., 1995; Mercier *et al*., 1995). Furthermore, Lampert *et al*. (1996) have previously shown that the relationship between total mitochondrial volume density of skeletal muscle and peak oxygen uptake (VO_2) was lost in heart transplant recipients, suggesting that immunosuppressive therapy could be partly responsible for persistent bioenergetic deficiency following heart transplantation. Whereas the mechanism of CsA toxicity is unknown, the former clinical and experimental findings suggest that it could be in part related to a mitochondrial disorder.

In a previous study we have demonstrated that the inhibitory effect of a chronic treatment with the clinical form of CsA, Sandimmun (Novartis, Basel, Switzerland), on muscle oxidative capacity was due to its vehicle, while CsA slightly affected glycolytic muscle (Sanchez *et al*., 2000). The vehicle of CsA is mainly composed of a lipid emulsion of cremophor, which is used as a solubilizer for lipophilic drugs and vitamins. In this case, the strength of the vehicle is in part owed to cremophor. While the vehicle has been

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implicated in the nephrotoxicity (Sokol *et al*., 1990), cardiotoxicity (Tatou *et al*., 1996), and hepatotoxicity (Roman *et al*., 1989) of CsA treatments, few data are available concerning toxic effect of cremophor on mitochondrial function. Following our previous study, two hypotheses were raised concerning vehicle effect on mitochondrial respiration: (1) a decrease in mitochondrial enzyme content or (2) a direct poisoning of mitochondria (Sanchez *et al*., 2000). As the mitochondrial content of creatine kinase and citrate synthase was not modified by chronic treatment with vehicle (Bigard *et al*., 2000), this latter result suggested that the expression of mitochondrial proteins was not altered by cremophor. Taken together these results suggest that cremophor exerts a direct inhibition on mitochondrial function, although the possible site(s) of action remain to be elucidated.

The aim of this study was thus, (1) to evaluate *in situ* the acute effect of CsA and its vehicle on muscle oxidative capacity, (2) to determine the effects of the vehicle on the different respiratory chain complexes, and (3) to examine a possible tissue specificity of these effects by investigating muscles having different oxidative metabolic requirements and functions such as heart (highly oxidative), soleus (slow oxidative muscle), and the superficial part of the gastrocnemius (fast glycolytic muscle).

Methods

Animals

Experiments were performed on adult male Wistar rats (IFFA CREDO, France) weighing ≈320 g. Animals were anaesthetized with intraperitoneal injection of sodium pentobarbital 0.2 g/100 g body wt. Left ventricle, soleus, and the superficial part of the gastrocnemius were removed and dissected in ice-cold zero-Ca^{2+} Krebs solution containing 118 mM NaCl, 4.7 mM KCl, 25 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, equilibrated with 95% O_2 – 5% CO_2, pH 7.4. This investigation was carried out in accordance with the Helsinki Recommendations for Humane Treatment of Animals during Experimentation.

Functional properties of mitochondria

Respiratory parameters of the total mitochondrial population were studied *in situ* in saponin-skinned fibres (Veksler *et al*., 1987; Saks *et al*., 1998). Briefly, thin fibre bundles (100–200 μm in diameter) were excised from ventricular and skeletal muscles under microscope in solution S (see below). Fibres were incubated with intense shaking for 30 min in the same solution containing 50 μg ml^{-1} saponin to selectively permeabilize the sarcolemma. They were then transferred into solution R (see below) for 10 min to wash out adenine nucleotides and creatine phosphate (PCr). All procedures were carried out at 4°C. Fibres were bathed in solutions mimicking the composition of the intracellular medium. Solutions S and R contained: 10 mM EGTA-CaEGTA buffer (free Ca^{2+} concentration 100 nM), 1 mM free Mg^{2+}, 20 mM taurine, 0.5 mM dithiothreitol and 20 mM imidazole (pH 7.1). Ionic strength was adjusted to 160 mM by addition of potassium methanesulphonate. Solution S also contained: 5 mM MgATP and 15 mM PCr, while solution R contained 5 mM glutamate, 2 mM malate, 3 mM phosphate and 2 mg ml^{-1} fatty acid free bovine serum albumin.

Respiratory rates were determined using a Clark electrode in oxygraph chambers (Strathkelvin Instruments, U.K.) containing ≈0.4–0.7 mg dry weight (dw) fibres in 3 ml solution R (at 22°C with continuous stirring). The solubility of oxygen (O_2) was taken as 267 nmol of O_2 ml^{-1}. Under these conditions, ADP content is the only limiting factor of respiration, while substrates (glutamate, malate), inorganic phosphate (Pi) and O_2 are present at saturating concentrations in solution R. Several parameters could be measured such as basal oxygen consumption without ADP (V_0) or maximal respiration rate (V_{max}) with a saturating concentration of ADP. The Acceptor Control Ratio (ACR), the ratio between V_{max} and V_0, which represents the degree of coupling between oxidation and phosphorylation, could be easily calculated. After measurements, the fibres were carefully removed, dried and weighed. Care was taken that a similar amount of fibres was used in each assay, according to the oxidative capacity of each tissue. Respiration was expressed as μmol O_2 min^{-1} g^{-1} dry weight for each experiment.

Protocols

The clinical form of CsA, Sandimmun (Novartis, Basel, Switzerland) was used in this study. This drug contains CsA diluted in a vehicle, which is composed of 2/3 cremophor and 1/3 ethanol (v v^{-1}). To determine the specific effect of CsA and vehicle, different combinations were tested: (1) Sandimmun, (2) the complete vehicle, (3) CsA diluted in ethanol and (4) ethanol alone. The full dissolution of CsA in ethanol was confirmed by the clarity of the solution.

Mitochondrial respiratory parameters V_{max} was first determined in the presence of 2 mM ADP, then increasing concentrations of a tested substance were examined. The range of concentrations for Sandimmun and cyclosporin diluted in ethanol was: 5–20–50–100 μM of CsA. Then, for vehicle and ethanol, which did not contain CsA, we used doses corresponding to a given CsA concentration in Sandimmun (i.e. equivalent doses of CsA).

For calculation of the ACR, V_0 was recorded with 100 μM final concentration of a substance; 2 mM ADP were then added for maximal activation of respiration.

Respiratory chain complexes The effects of the vehicle on respiratory chain complexes were investigated at maximal respiration rates (ADP 2 mM) and for 100 μM equivalent doses of CsA.

The specific substrates and inhibitors used to study the effects of vehicle on the respiratory chain complexes are summarized in Figure 3A. While the uncoupled respiratory chain, complex IV and complex II were studied by a polarographic method, complex I was studied by a spectrophotometric method (Letellier *et al*., 1992).

Uncoupled respiratory chain To uncouple respiration from phosphorylation we used the uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) in the absence of ADP. The amount of FCCP needed to maximally uncouple

respiration was determined for each muscle by stepwise increasing FCCP concentration. An optimal concentration of 1 μM was determined for all muscles.

Complex IV (cytochrome c *oxidase, COX)* The electron flow was blocked at complex III ($CoQH_2$-c reductase) with antimycin A (20 μg/ml) then, N,N,N′,N′-tetramethyl-*p*-phenylenediamine dihydrochloride (TMPD, 0.5 mM) and ascorbate (0.5 mM) were added as artificial electron donor to cytochrome *c* and COX. In these conditions, COX was studied as an isolated step of the respiratory chain.

Complex II (succinate-CoQ reductase) The electron flow was blocked at complex I with rotenone (38 μM), and succinate (25 μM) which enters the respiratory chain at complex II was added. In these conditions, mitochondrial respiration was performed by complexes II, III and IV (Figure 3A).

Complex I (NADH-CoQ reductase) Complex I activity was determined by a spectrophotometric method. Forty mg of each muscle was homogenized in 1 ml of 41 mM KH_2PO_4 pH 7.2, using both a Potter tissue homogenizer for 10 min and sonication to disrupt mitochondria. Then, the tissue preparation was centrifuged at $650 \times g$. Oxidation of NADH by complex I was recorded using the ubiquinone analogue UQ_1 as electron acceptor. The assay medium (35 mM KH_2PO_4, 5 mM $MgCl_2$, and 0.2 mM NaN_3, pH 7.2) was supplemented with fatty acid free BSA (2.5 mg ml^{-1}), antimycin A (2 μg ml^{-1}), 0.1 mM UQ_1 and 0.1 mM NADH in a final volume of 1 ml. Enzyme activity was measured at 30°C, and the reaction was started with 50 μl of muscle homogenate. Decrease in absorption due to NADH oxidation was measured at 340 nm, both in the absence and in the presence of rotenone (10 μg ml^{-1}). Experiments were performed both in the absence (control) and presence of 100 μM vehicle. As for COX, this complex was studied as an isolated step of the respiratory chain.

All enzymes and coenzymes were obtained from SIGMA ALDRICH CHIMIE (L'Isle D'Abeau Chesnes, France). Chemicals were purchased from PROLABO (Fontenay-sous-Bois, France) and were of the highest purity.

Statistical analysis

All data are presented as mean values ± standard errors of the means. A two-way analysis of variance (ANOVA) was used to determine the global effects of both substance and concentration on V_{max}. A one-way ANOVA was performed to check the global effect of a substance on ACR, or to verify the global effect of vehicle on the respiratory chain complexes. When appropriate, comparisons between groups were made using a Newman-Keuls test. Values of $P<0.05$ were considered significant.

Results

Effects of Sandimmun on maximal respiration rate

We first examined the effects of the immunosuppressive drug Sandimmun on maximal respiration rates (V_{max} in the presence of 2 mM ADP) of skinned preparations from muscles varying by their oxidative capacities. Since in permeabilized fibres the whole mitochondrial population participates in the measured oxygen consumption, when normalized to fibre dry weight, V_{max} represents the oxidative capacity of the different tissues. The effects of increased concentrations of Sandimmun (from 5 to 100 μM) on V_{max} are presented in Figure 1. As expected, the oxidative capacity was high in heart and low in gastrocnemius muscle, with intermediate values in soleus muscle. V_{max} was reduced by Sandimmun, and this reduction was concentration-dependent for all muscle types. This diminution became significant from 5 μM in heart ($P<0.05$) and gastrocnemius muscle ($P<0.001$) but only from 50 μM in soleus muscle ($P<0.001$).

Specific effects of the components of Sandimmun on maximal respiration rate

Next we investigated which of the drug components was responsible for the decreased respiration rates. For this, we compared the effects of ethanol alone, or complete vehicle, or CsA diluted in ethanol, on maximal respiration rates. The absolute decrease in maximal respiration are presented in Figure 2 for the different CsA concentrations tested in cardiac (Figure 2A), soleus (Figure 2B) or gastrocnemius muscles (Figure 2C). Ethanol alone significantly decreased V_{max} in heart preparations only, while having no significant effect on skeletal muscles. Because no difference was observed between CsA + ethanol and ethanol alone, it is likely that CsA *per se* had no specific effect on mitochondrial respiration. For all muscles, and beginning with the lowest concentration, complete vehicle dramatically decreased respiration rates. Moreover, it appeared that V_{max} was often more affected by the vehicle alone than by Sandimmun, the difference being significant for 100 μM in all muscles, starting from 50 μM in heart and soleus and even from 20 μM in heart.

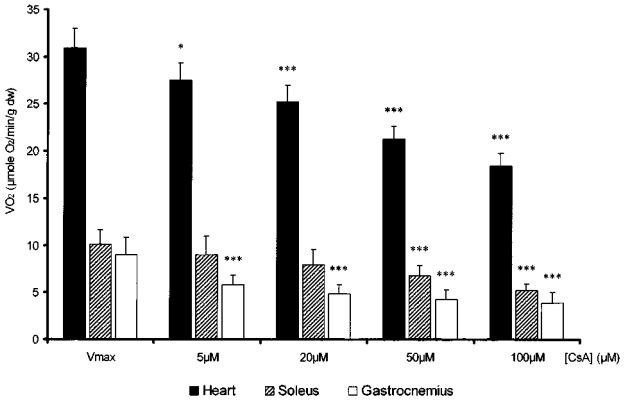

Figure 1 Effect of increasing concentrations of Sandimmun on maximal respiration rates of *in situ* mitochondria from cardiac, soleus, and white gastrocnemius muscles of rats. Oxygen consumption was measured in saponin permeabilized muscle fibres with glutamate and malate as substrates and with 2 mM ADP (V_{max}) in an oxygraph cell and with a Clark electrode. Values are expressed as μmoles O_2 consumed per min and per g of fibre dry weight. Values are means ± s.e.mean of 5–6 experiments on each muscle type. *, ***, values statistically different from V_{max} for all muscles with $P<0.05$ or $P<0.001$ respectively.

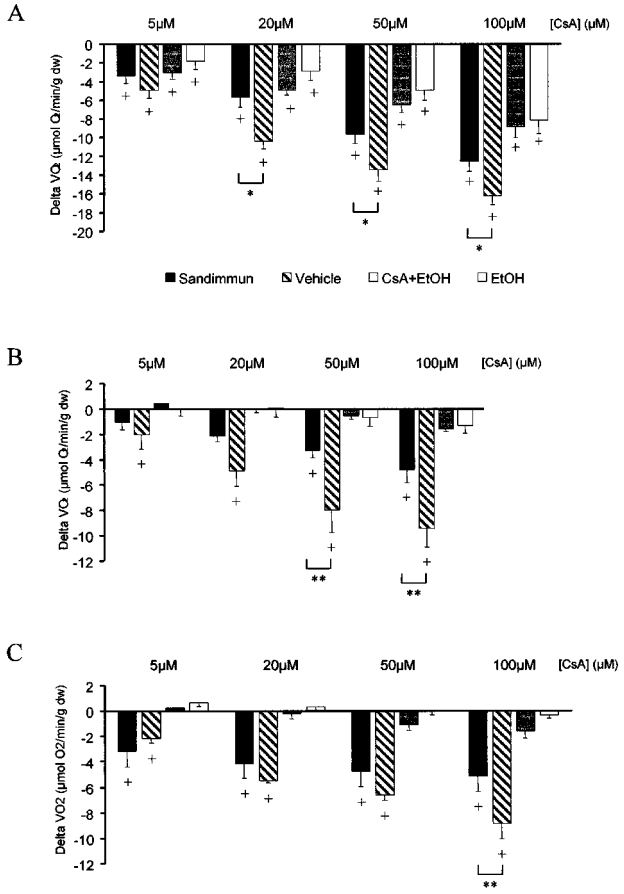

Figure 2 Effect of increasing concentrations of Sandimmun, complete vehicle, CsA diluted in ethanol, and ethanol on maximal respiration rates of *in situ* mitochondria from cardiac (A), soleus (B), and white gastrocnemius (C) muscles of rats. Same conditions as in Figure 1. Values are means ± s.e.mean of 5–6 experiments on each muscle type, and correspond to the difference between the respiration rate recorded for one concentration and V_{max}. They are expressed as μmoles O_2 consumed per min and per g of fibre dry weight. For simplicity, + represents values significantly different from V_{max} with $0.5 < P \leq 0.001$; *, **, values statistically different from Sandimmun, with $P<0.05$ or $P<0.01$ respectively.

Effects of Sandimmun and its components on ACR

We further studied whether either 100 μM Sandimmun or its constituents affected the coupling between oxidation and phosphorylation. Table 1 summarizes the effects on V_0, V_{max} and ACR. V_0 was either unchanged or slightly decreased, showing that none of the drugs increased uncoupled respiration. In control groups, ACR values were higher than 4, showing that mitochondria were functionally intact in these preparations. In all muscles, ACR tended to be decreased by vehicle and Sandimmun: ACR was significantly lower than control in heart ($P<0.05$) and soleus ($P<0.001$) in the presence of vehicle, and in soleus muscle in the presence of Sandimmun ($P<0.01$). As V_{max} was reduced without noticeable modification in V_0, this decrease in ACR mainly resulted from the decrease in V_{max}.

Effects of vehicle on the respiratory chain complexes

We demonstrated that Sandimmun affected the maximal respiration rate in cardiac and skeletal muscles and that this effect could be mainly attributed to the vehicle.

In order to get insight into the mechanisms of action of the vehicle, we next investigated the possible sites of action of the vehicle on the different complexes of the mitochondrial respiratory chain. Indeed, this effect could take place at any step of the oxidative phosphorylation from complex I of the respiratory chain (glutamate and malate as substrates) to ADP/ATP translocator (translocase) and F_0-F_1 ATP synthase. For this, specific substrates and inhibitors of the respiratory chain (Figure 3A), and a saturating concentration of vehicle was used (100 μM equivalent CsA).

In order to examine whether vehicle affected phosphorylation steps or the respiratory chain directly, we studied the effects of vehicle on respiration uncoupled by FCCP in the absence of ADP (Figure 3B). The results show that FCCP increased absolute respiration for the three muscles, and that uncoupled respiration was significantly decreased ($P<0.001$) for each muscle by a single addition of vehicle. Inhibition was 57 ± 5, 65 ± 5, and $71\pm7\%$ for heart, soleus and white gastrocnemius respectively, reaching values close to the effects of vehicle on V_{max}. This suggests that vehicle primarily affected the respiratory chain.

We next investigated the potential inhibition at the level of complex IV of the respiratory chain, cytochrome oxidase

Table 1 Effect of 100 μM of Sandimmun, complete vehicle, CsA diluted in ethanol and ethanol on mitochondrial parameters from heart and skeletal muscles

		Control	*Sandimmun*	*Vehicle*	*CsA + EtOH*	*EtOH*
Heart	V_{max}	27.3±1	18.4±1.3*	8.6±0.8***	19.1±1.8*	23.7±2.5
	V_0	6.2±0.2	6.8±0.7	5.0±0.4	3.8±0.3*	6.0±0.8
	ACR	4.9±0.3	3.8±0.5	2.1±0.1*	3.7±0.5	4.10±0.17
Soleus	V_{max}	9.9±0.6	5.2±0.6*	4.3±0.5**	6.9±1	7.6±0.5
	V_0	2.5±0.1	2.2±0.3	2.4±0.3	1.8±0.2	1.6±0.3
	ACR	4.1±0.3	2.3±0.1**	1.5±0.2***	3.7±0.2	3.51±0.35
Gastrocnemius	V_{max}	10.8±0.6	3.9±1.1***	4.4±0.6***	7.8±0.8	9.7±0.6
	V_0	2.7±0.2	1.7±0.4	1.7±0.3	1.3±0.1	1.4±0.4
	ACR	4.0±0.4	3.2±1.0	2.8±0.5	3.2±0.9	3.21±0.56

Mitochondrial respiration was measured in saponin-permeabilized muscle fibres in the absence of ADP (V_0), or with glutamate and malate as substrates and with 2 mM ADP (V_{max}). Respiration was expressed as μmol O_2 min g^{-1} dry weight. ACR was calculated as the ratio between V_{max} and V_0. *, **, *** values statistically different from control with $P<0.05$, or $P<0.01$, or $P<0.001$, respectively.

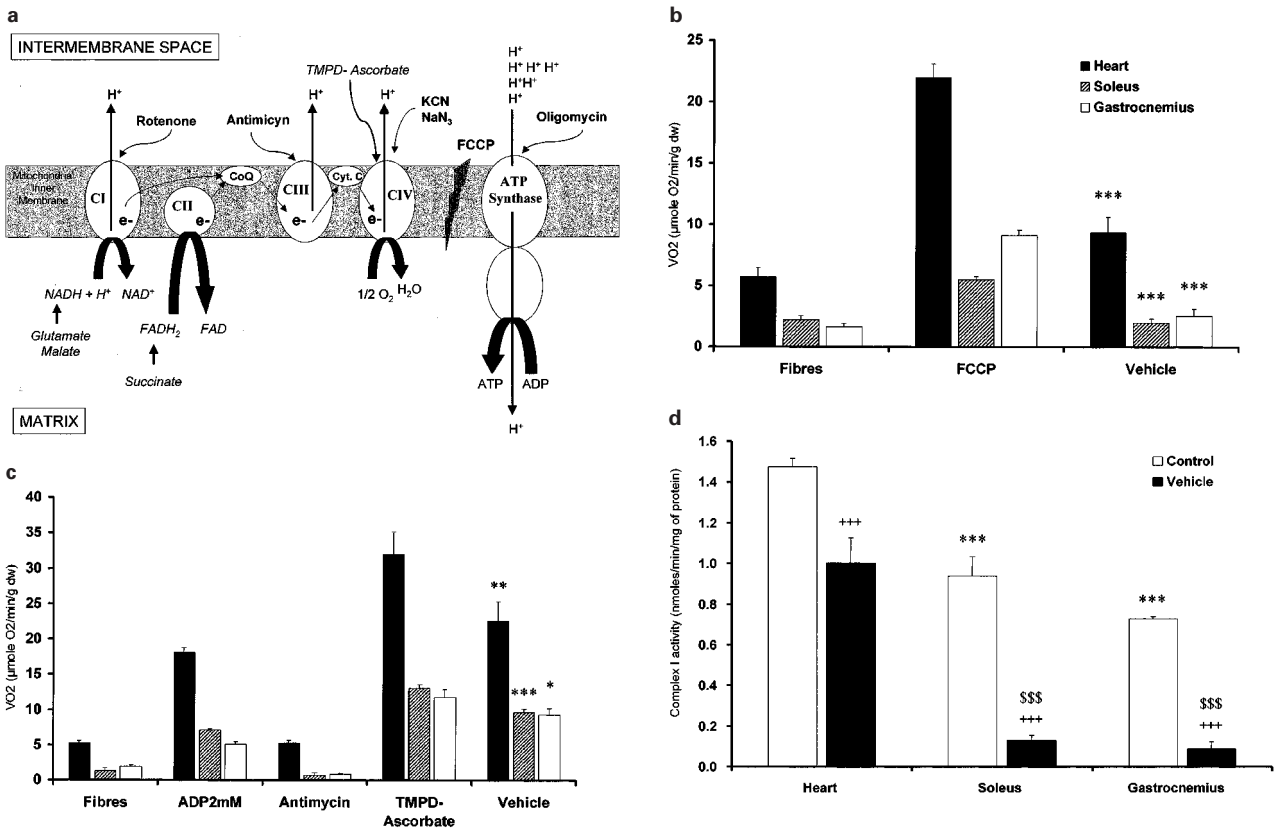

Figure 3 Effects of the vehicle on the respiratory chain complexes. (A) Schematic representation of the respiratory chain with specific substrates and inhibitors. CI: complex I (NADH-CoQ reductase), CII: complex II (succinate-CoQ reductase), CIII: complex III ($CoQH_2$-c reductase), CIV: complex IV (cytochrome *c* oxidase, COX), CoQ: coenzyme Q, Cyt.c: cytochrome *c*, TMPD: N,N,N′,N′-tetramethyl-p-phenylenediamine dihydrochloride, FCCP: carbonyl cyanide p-trifluoromethoxyphenylhydrazone, KCN: potassium chloride, NaN_3: sodium azide. (B) Effect of 100 μM vehicle on uncoupled respiratory chain from cardiac, soleus, and white gastrocnemius muscles of rats. Oxygen consumption was measured in saponin permeabilized muscle fibres with glutamate and malate as substrates. Basal oxygen consumption without ADP was measured (Fibres), then to uncouple respiration from phosphorylations, 1 μM FCCP was added, finally 100 μM of vehicle were added. Values are expressed as μmoles O_2 consumed per min and per g of fibre dry weight. Values are means$\pm$s.e.mean of 5–6 experiments on each muscle type. ***, values statistically different from FCCP respiration for all muscles with $P<0.001$. (C) Effect of 100 μM vehicle on Cytochrome *c* Oxydase (COX) respiration from cardiac, soleus, and white gastrocnemius muscles of rats. Same conditions as B. Basal oxygen consumption without ADP (Fibres) and maximal respiration rate (ADP 2 mM) were measured, then the electron flow was blocked at complex III with antimycin, then TMPD and ascorbate were added as artificial electron donor to COX, finally 100 μM of vehicle were added. *, ** and ***, values statistically different from TMPD-Ascorbate respiration with $P<0.05$, $P<0.01$ and $P<0.001$ respectively, for all muscles. (D) Effect of 100 μM vehicle on specific activity of complex I from cardiac, soleus, and white gastrocnemius muscles of rats. All spectrophotometric measurements were performed at 30°C with an UVIKON spectrophotometer. They were realised in the absence (control) and in the presence of 100 μM vehicle. Values represent the rotenone-sensitive complex I activity. +++, values statistically different from control for all muscles with $P<0.001$. ***, values statistically different from heart control with $P<0.001$. \$\$\$, values statistically different from heart vehicle with $P<0.001$.

(COX). After addition of 2 mM ADP, respiration was inhibited at the level of complex III by antimycin A; subsequently electron donors were added as TMPD/ascorbate and respiration rate was recorded for complex IV (Figure 3C). Under these conditions, respiration rates were much higher for all muscles than the maximal ADP stimulated respiration with glutamate/malate as substrate. Values were $+76\pm16$, $+84\pm5$ and $+135\pm26\%$ in heart, soleus and gastrocnemius, muscles, respectively. Vehicle significantly decreased complex IV respiration of heart ($P<0.01$), soleus ($P<0.001$) and white gastrocnemius ($P<0.05$) muscles. However, the corresponding inhibitions of -30 ± 2, -27 ± 2, and $-22\pm3\%$ respectively for heart, soleus and white gastrocnemius, were lower than the effects of vehicle on respiration from complex I, suggesting that other steps could be inhibited as well.

We further investigated respiration rates from complex II with succinate as substrate after inhibiting complex I with rotenone. Under these conditions, addition of vehicle had no significant effect on respiration rates, showing that complex II was not affected by the vehicle (results not shown).

Finally, we checked whether complex I activity by itself could be involved in the inhibiting effect of the vehicle. Enzymatic activity was determined with a spectrophotometric method by measuring the NADH oxidation, both in the absence and in the presence of rotenone. Experiments were realized both in the absence (control) and in the presence of 100 μM vehicle (Figure 3D). As expected, complex I activity

was high in heart and low in white gastrocnemius muscle with intermediate values in soleus muscle. This activity was dramatically decreased ($P<0.001$) by vehicle, and this effect was more pronounced for soleus ($-86\pm3\%$) and white gastrocnemius ($-89\pm6\%$) muscles than for heart ($-32\pm7\%$).

Discussion

The results of the present study can be summarized as follows: (1) Immunosuppressive drug Sandimmun induces a dose-dependent decrease in maximal respiration for all muscle type studied. (2) This effect is entirely attributable to the vehicle of CsA (ethanol and cremophor), with no specific effect of the immunosuppressant itself. (3) The vehicle affects respiration mainly at the level of complex I, but also at complex IV of the respiratory chain.

Effects of immunosuppressant on mitochondrial respiration

One of the main results of this study was that, whatever the muscle type, the immunosuppressant molecule CsA had no effect on mitochondrial respiration whereas the vehicle was mainly responsible for the drop of maximal oxidative capacity observed with Sandimmun. This confirmed our earlier results showing that a long term treatment of rats with the immunosuppressive drug induced skeletal and cardiac muscle mitochondrial respiration alterations which we attributed to deleterious effects of vehicle (Sanchez *et al*., 2000). Previous studies already described alterations of oxidative capacity or mitochondrial function but the effects of the immunosuppressive molecule on one hand and the effects of vehicle on the other were not clearly elucidated (Hokanson *et al*., 1995; Mercier *et al*., 1995; Nässberger, 1990).

The vehicle of Sandimmun is an emulsifier widely used in drug delivery to dissolve lipophilic components in a safe and tolerable matrix. It is composed of ethanol and an amphiphilic molecule Cremophor EL, which is a polyoxyethylated derivative of castor oil. As ethanol exerted only a minor effect on mitochondrial respiration in our study, the toxic effect of vehicle on the mitochondrial respiration could be mainly attributed to Cremophor EL. There is an abundant literature on the toxicity of this compound including hypertensive effects (Mehring *et al*., 1992), haemodynamic effects (Bowers *et al*., 1991; Abraham *et al*., 1991; Tatou *et al*., 1996), neurotoxicity (Windebank *et al*., 1994), hepatotoxicity (Roman *et al*., 1989) and nephrotoxicity (Sokol *et al*., 1990). However, few data concern the mitochondrial effects of this compound and the site of action of the molecule on mitochondrial oxidative phosphorylation (OXPHOS) is still unknown.

Effects of vehicle on mitochondrial respiration

The main purpose of this study was to investigate which step(s) of the OXPHOS pathway could be inhibited by the vehicle. The respiratory chain is composed of redox proteins and proton pumps (complexes I–IV), which are coupled to the ATP synthase. As the respiration uncoupled from phosphorylation by FCCP was greatly affected by a single concentration of vehicle for all muscle types, it could be concluded that the ATP synthase is not an essential target of the vehicle, and that the respiratory chain complexes might be preferentially involved. On the other hand, the fact that basal respiration was hardly changed, even for the highest concentration of the drug, allows the exclusion of the possibility that the drug acts through uncoupling of respiration.

When mitochondrial respiration was initiated at complex IV of the respiratory chain, the vehicle inhibited respiration showing that this step was sensitive to the drug. However, this effect could not account for the entire inhibiting effect of the drug for several reasons. Indeed, the activity of COX was largely above the value needed for maximal ADP stimulated respiration for all muscles. This excess capacity of cytochrome *c* oxidase (Gnaiger *et al*., 1995; 1998) was inhibited by vehicle in a range of 20–32%, depending on the muscle, but the corresponding mitochondrial respiration remained higher than V_{max} determined with glutamate/malate.

Other investigators have determined the biochemical threshold on isolated mitochondria of various tissues for each oxidative phosphorylation complex (Mazat *et al*., 1997; Letellier *et al*., 1998; Rossignol *et al*., 1999). This parameter corresponds to the maximal inhibition tolerated by a complex without any consequences for the global respiratory rate. These authors reported a threshold value for COX close to 67% for heart and skeletal muscles indicating that this complex was not a main controller step of respiration. In our study, for all muscle types, the vehicle-induced inhibition of COX was always lower than this critical value, adding support to the notion that inhibition of complex IV could not explain alteration of maximal respiration by vehicle.

This is further supported by the absence of effects of vehicle when respiration was induced from the complex II of the respiratory chain with succinate as substrate. Two conclusions can be drawn, first, complex II is not implicated in the inhibition process and second, COX inhibition hardly affects respiration, when complex I is not involved.

Conversely, this suggests a major involvement of complex I. Indeed, vehicle dramatically decreased the specific activity of complex I of the respiratory chain. This effect ranged from 37% in heart and reached 87% in skeletal muscle, showing a possible tissue specific effect of the drug at this step of the respiratory chain. As the biochemical threshold of complex I was 64 and 74% respectively in heart and skeletal muscles (Rossignol *et al*., 1999), the strong inhibition of complex I in soleus and gastrocnemius would lead to a low oxidative capacity whereas in heart muscle this inhibition would have moderate effect on the global respiratory rate.

However, it should be kept in mind that in our experimental conditions oxygen level was largely higher than in the intracellular microenvironment of tissues, where it is as low as 1–2% of standard atmospheric oxygen pressure (Gnaiger *et al*., 1995). Under these *in vivo* conditions, there is a regulatory control of oxygen on the mitochondrial respiration in particular at the COX level (Gnaiger *et al*., 1998), and inhibition of complex I and IV may both participate in the worsening of mitochondrial function *in vivo*.

Mechanism of action of the vehicle on the mitochondrial membrane

As ethanol *per se* had moderate effects on respiration, it could be inferred that the effect of vehicle was due to cremophor. The membrane fluidity is altered by this component (Dudeja *et al*., 1995), which is capable of integrating into a membrane due to its amphiphilic properties. The mitochondrial phospholipid composition is known to influence the non-phosphorylating respiratory rate (Brand *et al*., 1991), and removal of less than 1% phospholipids from inner mitochondrial membrane induces noticeable effects on mitochondrial function (Spencer *et al*., 1976). Thus, a modification of the lipid environment of respiratory chain complexes could influence the efficacy of the respiratory chain. Complex I was the most sensitive to vehicle in our study. This complex is also the target of other lipophilic molecules affecting the mitochondrial bioenergetics such as local anaesthetics, whose potency depends on their partition coefficient (Sztark *et al*., 2000). Thus, accumulation of cremophor in the mitochondrial inner membrane could cause alterations of the lipid environment of the complex I and IV, decreased membrane fluidity, worsening of electron transfer and inhibition of respiration. Additionally, lipid peroxydation of the membrane by reactive oxygen species may also be involved in the toxic effects of this substance (Tatou *et al*., 1996).

In this study, vehicle alone appeared more toxic than the lipid-emulsion formulation which contains vehicle and CsA. As this beneficial effect of CsA was not observed when CsA was dissolved in EtOH, this suggests a specific interaction between CsA and cremophor. One possibility would be that in the galenic formulation of CsA, as molecules of cremophor make droplets with CsA molecules inside, this decreases the amount of free lipophilic molecules able to interact with the mitochondrial membrane. Alternatively, a direct positive effect of CsA on the deleterious effect of cremophor on inner mitochondrial should also be considered. Clearly, more work is needed to understand the nature of interaction between these two compounds.

Relevance to clinical use of immunosuppressive drugs

The range of concentrations used in this study (5–100 μM) to determine the maximal effect of the substances was much higher than the tissue concentrations of CsA measured in heart and skeletal muscles (2–3 μM; data from Novartis). However, the fact that lower concentrations of these substances used in chronic treatment in animals (10 to 25 mg of CsA/kg/day) cause an important drop in mitochondrial respiration (Mercier *et al*., 1995; Sanchez *et al*., 2000), and that this deleterious effect persists despite fibre isolation and permeabilization of the sarcolemma by saponin, suggests that the molecules of cremophor can accumulate with time in the inner mitochondrial membrane.

In clinical conditions, the immunosuppressive treatment with CsA formulations were adapted to the transplanted patients with low concentrations, but an accumulation of cremophor molecules could occur. Considering that immunosuppressive treatments last for many years, the amount of cremophor accumulating in mitochondrial membranes may reach inhibitory levels. Thus, it can be concluded that part of immunosuppressive treatment toxicity may arise from alterations in mitochondrial respiratory chain by vehicle. Although only demonstrated in this study for heart and skeletal muscle, it is highly conceivable that such an effect may also alter the energetics of other organs like brain, kidney and liver, having high oxidative demands.

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