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Impact on energy metabolism of quantitative and functional cyclosporine-induced damage of kidney mitochondria

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In this study we have measured, under experimental conditions which maintained efficient coupling, respiratory intensity, respiratory control, oxidative phosphorylation capacity and protonmotive force. Succinate cytochrome-*c* reductase and cytochrome-*c* oxidase activities were also studied. These investigations were carried out using kidney mitochondria from cyclosporine-treated rats (in vivo studies) and from untreated rats in the presence of cyclosporine (in vitro studies). Inhibition of respiratory intensity by cyclosporine did not exceed 21.1% in vitro and 15.9% in vivo. Since there was no in vitro inhibition of succinate cytochrome-*c* reductase and cytochrome-*c* oxidase activities, the slowing of electron flow observed can be interpreted as a consequence of an effect produced by cyclosporine between cytochromes *b* and c_1. Cyclosporine had no effect on respiratory control either in vitro or in vivo. Statistically significant inhibition of the oxidative phosphorylation was observed both in vitro (6.6%) and in vivo (12.1%). Moreover, cyclosporine did not induce any change of membrane potential either in vivo or in vitro. Our findings show that cyclosporine is neither a protonophore, nor a potassium ionophore. In cyclosporine-treated rats we noticed a decrease of protein in subcellular fraction, including the mitochondrial fraction. The role of the inhibition respiratory characteristics by cyclosporine in nephrotoxicity in vivo must take account of these two parameters: inhibition of the respiratory characteristics measured in vitro and diminution of mitochondrial protein in cyclosporine-treated rats.

Introduction

Cyclosporine is a natural cyclic peptide used clinically as an immunosuppressant to prevent rejection after transplantation. Cyclosporine is not myelotoxic at therapeutic dose levels [1]. It initiates a new category of immunosuppressant which has revolutionized the prognosis of organ transplants. Unfortunately, the clinical utility of the agent is limited, mainly by severe nephrotoxicity [2–5]. Cyclosporine induces morphological alterations of the kidney tissue. At the ultrastructural level an increase in empty vacuoles and in lysosome numbers is observed together with focal alteration of mitochondria [6–8]. The structural changes affecting mitochondria gave rise to the notion of a possible effect on one of the main functions of these organelles: energy metabolism.

Abbreviations: RI, respiratory intensity; RC, respiratory control; *P/O*, oxidative phosphorylation capacity; $\Delta\Psi$, potential difference; ΔpH, pH difference; BSA, bovine serum albumin.

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The findings concerning this possibility can be summarized as follows: All authors agree that cyclosporine induces inhibition of the respiratory intensity [9–14]. However, differing views exist with regard to the action of cyclosporine on respiratory control and the P/O ratio: Jung et al. [12–14] and Pfaller et al. [15] report inhibition by cyclosporine of both respiratory control and of P/O ratio, whereas Backman et al. [16] conclude that "the respiratory control was completely intact and the level of oxidative phosphorylation was unchanged". These discrepancies can be explained on the basis of differing experimental conditions (composition of the medium, difficulty in dissolving cyclosporine in aqueous buffers). However, it is the respiratory control and phosphorylation capacity which provide a true indication of the efficacy of oxidative phosphorylation rather than respiratory intensity. This why the present authors thought it important:

– to tackle the question under experimental conditions providing both efficient coupling (presence of magnesium and of inorganic phosphate in the absence of EDTA: which is not always the case) and high level of reproducibility (standardized method, use of succinate yielding easily determined respiratory intensity values and a high level of cyclosporine solubility in the medium);

– to investigate the origins of the impairment of phosphorylating oxidation firstly observed in direct investigation of the effect of cyclosporine on electron transfer (determination of the enzyme activities of succinate cytochrome-*c* reductase and of cytochrome-*c* oxidase) and on the energy conservation mechanism (determination of membrane pH difference and potential difference).

Two types of study were performed:

in vitro: addition of cyclosporine to rat kidney mitochondria: and

'in vivo': study of the biochemical characteristics of cyclosporine-treated rat kidney mitochondria.

Materials and Methods

Doses of cyclosporine used. For the in vitro studies, cyclosporine was dissolved in ethanol due to its hydrophobic properties, and the highest concentration which could be used in aqueous buffers was 10^{-5} M. In vivo, the doses administered were 30 and 45 mg/kg per day. Higher doses could not be used due to high mortality.

Animals. Three groups of five male Wistar rats weighing 300–35 g were treated with 0, 30 and 45 mg/kg per day cyclosporine dissolved in olive oil. The drug was administered by gastric intubation. Animals had free access to tap water and a standard laboratory feed. All animals were killed on day 14 without anesthesia.

Preparation of mitochondrial fraction. Rats were killed in the laboratory between 9 a.m. and 10 a.m. and the kidneys were removed and placed in an ice-bath within 15 min of death. The kidneys were then washed with a buffer (250 mM sucrose/30 mM Tris/1 mM EDTA/0.2% BSA (pH 7.4)) and homogenized in the same buffer (10 ml/g of tissue) using a coaxial glass homogenizer. The mitochondria were prepared according to the method of Hogeboom [17]. After centrifugation ($750 \times g$, 10 min) the pellet (unbroken cells and nuclei) was discarded and the supernatant recentrifuged ($1000 \times g$, 10 min). The pellet (mitochondria) was washed using the method of Harel et al. [18] and resuspended in 'respiratory buffer' (250 mM sucrose/5 mM $MgCl_2$/0.5 mM EGTA/10 mM Tris/10 mM KH_2PO_4/0.075% BSA (pH 7.0)). Protein concentration was determined by the method of Lowry et al. [19]. The purity of the mitochondrial preparation was checked using enzyme markers as following: glucose-6-phosphate dehydrogenase [20], antimycin A-sensitive NADPH cytochrome-*c* reductase [21]. Cytochrome-*c* oxidase [22], succinate cytochrome-*c* reductase [23], malate dehydrogenase [24] and ATPase [25]. These methods were also used to investigate the effect of cyclosporine on mitochondrial succinate cytochrome-*c* reductase and cytochrome-*c* oxidase activities.

Determination of respiratory parameters. The respiratory characteristics of the mitochondrial fraction and the action of cyclosporine on these characteristics were determined polarographically with a Clark oxygen electrode at 40 °C in 2 ml of 'respiratory buffer'. For all studies in vitro, cyclosporine was diluted in ethanol. For all determinations of respiratory characteristics (respiratory intensity, respiratory control and P/O ratio) we used the methods described by Chance [26] and by Chance and Williams [27]. Results are

expressed in comparison with an ethanol reference.

Determination of the protonmotive force. The protonmotive force (Δp) is composed of a membrane potential component ($\Delta\Psi$) and a pH difference (ΔpH). ΔpH was estimated from the accumulation of [1-^{14}C]acetate (10 μM final concentration, 1.97 MBq/mmol) in the presence of tritiated water (0.11 MBq/ml) as described by Rottemberg [28]. 1-ml samples of mitochondria (1 mg protein/ml) were incubated for 5 min in the presence of the radioactive probes, succinate (15 mM final concentration), catalase (5000 U/ml) and 10 μl of 0.2 M H_2O_2 to prevent anaerobiosis during centrifugation. The 1-ml samples were then centrifuged for 2 min on a bench microfuge. The pellet and aliquot (100 μl) of the supernatant were counted for radioactivity. The membrane potential ($\Delta\ \Psi$) was estimated from the accumulation of [^{14}C]tetraphenylphosphonium (10 μM, spec. act. 2.17 GBq/mmol), using the method described by Rottemberg [28]. The procedure was the same as for the pH difference (ΔpH) determination, except that no tritiated water was present. The [^{14}C]tetraphenylphosphonium uptake was corrected for unspecific binding by substracting a 'blank' obtained under identical conditions except that the mitochondria were pretreated with the protonophore carbonyl cyanide chlorophenylhydrazone at 20 μM. For ΔpH and for $\Delta\Psi$ all measurements were performed in triplicate. In all calculations a matrix volume of 1 μl/mg protein was assumed.

Statistical analysis. Statistical significance was determined with Student's *t*-test. Due to the slight changes induced by cyclosporine, we were forced to increase the number of determinations considerably (from three to nine) as required for each study in order to permit reliable statistical analysis.

Reagents. Cyclosporine was obtained from Sandoz, France, EDTA, EGTA were purchased from Merck, catalase and ADP from Boehringer, carbonyl cyanide chlorophenylhydrazone from Sigma. Other reagents were commercial products of analytical grade. Tritiated water and [1-^{14}C]acetate were obtained from CEA and [^{14}C]tetraphenylphosphonium from Amersham.

Results

Characteristics of the mitochondrial fraction

The purity of the mitochondrial fraction was satisfactory. The mitochondria-associated enzymes were all enriched in the mitochondrial fraction, whereas the microsomal marker antimycin-A insensitive NADPH cytochrome-*c* reductase was reduced and the cytosolic enzyme glucose-6-phosphate dehydrogenase was absent. The protein content of the mitochondrial fraction of treated rats was found to be reduced (Table I) and this corresponded to a reduced body weight: -13% ($P < 0.01$, $n = 7$) for the 30 mg/kg per day dose of cyclosporine and -15% ($P < 0.02$, $n = 7$) for the 45 mg/kg per day dose.

Effect of cyclosporine on mitochondrial respiratory characteristics

In vivo. The maximum inhibition of the respiratory intensity of the kidney mitochondria recorded for both doses administered (30 and 45 mg/kg per day) was -15.9% (Table II). Under these experimental conditions, a slight, but statistically significant, reduction of the oxidative phosphorylation capacity was observed (-12.1%) in the absence of any inhibition of respiratory control (2.80 in the

TABLE I

CYCLOSPORINE REDUCES THE PROTEIN CONTENT OF THE HOMOGENATE, CYTOPLASM AND MITOCHONDRIA IN KIDNEYS OF TREATED RATS

The rats were treated with 45 mg/kg per day for 14 days, before being killed. The protein content was expressed in mg/g of renal tissue. Each value represents the mean ± S.D. of seven determinations carried out from a homogenate of ten rat adrenals. The *P* values compare the test values with those of untreated animals.

Subcellular fraction	CsA (mg/kg per day)	Protein content	% decrease	*P*
Homogenate	0	68.5 ± 3.7		
	45	58.0 ± 6.1	15.3	< 0.02
Mitochondria	0	8.0 ± 0.9		
	45	6.2 ± 0.3	22.5	< 0.001
Cytoplasm	0	60.7 ± 3.0		
	45	49.0 ± 4.8	19.20	< 0.02

TABLE II

CYCLOSPORINE INHIBITS RESPIRATORY INTENSITY (RI) OF TREATED RAT KIDNEY MITOCHONDRIA

The rats were treated with 30 or 45 mg of cyclosporine/kg per day for 14 days before being killed. Each value represents the mean ± S.D. of five determinations. The values of *P* compare the test values with those obtained using mitochondria from the kidneys of untreated rats. General conditions of incubation are the following: 1 mg of mitochondrial protein was incubated at 37 °C in a final volume of 2 ml of 'respiratory buffer' (pH 7.00). Each determination was performed in the presence of 15 mM succinate.

Cyclosporine (mg/kg per day)	RI (nmol O_2/ min per mg P)	% inhibition	*P*
0	51.0 ± 3.1		
30	42.9 ± 6.1	15.9	< 0.01
45	44.0 ± 4.7	13.7	< 0.01

presence of cyclosporine, 2.80 in its absence; Table III).

In vitro. It should be noted that the inhibition range from 0 to 80 s of preincubation in the presence of cyclosporine (Table IV) and that this may result in variable results. This is why we performed a 40 s preincubation in a subsequent determinations. The inhibition of rat kidney mitochondrial respiratory intensity by 10^{-5} M cyclosporine did not exceed 21%. The inhibition

TABLE III

IN VIVO STUDY COMPARISON OF RESPIRATORY CONTROL (RC) AND *P/O* OF KIDNEY MITOCHONDRIA FROM CYCLOSPORINE-TREATED AND UNTREATED RATS

The rats were treated with 30 or 45 mg of cyclosporine/kg per day for 14 days. Each value represents the mean ± S.D. of five determinations. Each determination was carried out in the presence of 15 mM succinate and 0.3 mM ADP. For general conditions of experimentation see Table II. The *P* values compare the test values with those obtained using mitochondria from the kidneys of untreated rats. RI is expressed in nmol O_2/min per mg protein.

Cyclosporine (mg/kg per day)	RC	*P/O*	*P*
0	2.8 ± 0.1	1.15 ± 0.02	
30	2.8 ± 0.1	1.02 ± 0.01	< 0.001
45	2.8 ± 0.1	1.01 ± 0.08	< 0.001

TABLE IV

DETERMINATION OF THE MINIMUM CONTACT TIME TO STUDY, IN VITRO, THE INHIBITION BY CYCLOSPORINE OF RI OF RAT KIDNEY MITOCHONDRIA

Each value represents the mean ± S.D. of nine different determinations. The values of *P* compare the values for the test with the cyclosporine-free control. The determinations in the absence of cyclosporine were carried out using an equivalent volume of ethanol. In the course of this investigation 1 mg of mitochondrial protein was incubated for various times at 37 °C in our experimental buffer in the presence of 10^{-5} M cyclosporine before adding 15 mM succinate.

Contact time (s)	Cyclosporine (M)	RI (nmol O_2/ min per mg P)	% inhibition	*P*
0	0	50.0 ± 3.0		
	10^{-5}	44.5 ± 4.3	11.0	< 0.01
20	0	53.4 ± 4.9		
	10^{-5}	44.6 ± 8.8	16.4	< 0.01
40	0	40.5 ± 7.0		
	10^{-5}	32 ± 6.4	21.0	< 0.001
80	0	42.5 ± 6.2		
	10^{-5}	34.4 ± 6.6	19.0	< 0.001

produced is dose-dependent (Table V). 10^{-5} M cyclosporine induces slight, but statistically significant, inhibition of the oxidative phosphorylation capacity (6.6%) but no inhibition of respiratory control (2.50 in the presence of cyclosporine and 2.50 in its absence; Table VI).

TABLE V

IN VITRO, CYCLOSPORINE PRODUCES DOSE-DEPENDENT INHIBITION OF RI OF RAT KIDNEY MITOCHONDRIA

For general conditions of incubation see Table II. Each value represents the mean ± S.D. of five or seven determinations for 10^{-5} and $5 \cdot 10^{-6}$ M cyclosporine respectively. The time of incubation in the presence of cyclosporine was 40 s. The determinations in the absence of cyclosporine were carried out using an equivalent volume of ethanol.

Cyclosporine (M)	RI (nmol O_2/ min per mg P)	% inhibition	*P* value
0	51.7 ± 4.8		
$2 \cdot 10^{-6}$	52.0 ± 5.1	0	
$5 \cdot 10^{-6}$	45 ± 7.0	12.9	< 0.001
10^{-5}	40.8 ± 6.6	21.1	< 0.01

TABLE VI

ACTION OF CYCLOSPORINE IN VITRO ON RC AND *P/O* RATIO OF RAT KIDNEY MITOCHONDRIA

Each value represents the mean ± S.D. value of five determinations. For general conditions of experimentation see Table II. In this experimentation ADP concentration was 0.3 mM and succinate 15 mM. The *P* values compare the values for the test with the cyclosporine free control. The determinations in the absence of cyclosporine were carried out using an equivalent volume of ethanol.

Cyclosporine (M)	RC	*P/O*	*P*
0	2.5 ± 0.2	1.06 ± 0.07	
10^{-5}	2.5 ± 0.2	0.99 ± 0.05	< 0.02

Absence of any direct effect of cyclosporine on succinate cytochrome-c reductase and on cytochrome-c oxidase

The 15.9% in vivo and 21.1% in vitro decrease of respiratory intensity induced by cyclosporine led us to test in vitro the possibility of direct inhibitory action of cyclosporine on the two multi-step enzyme activities involved in succinate oxidation: succinate cytochrome-*c* reductase and cytochrome-*c* oxidase. Data in Table VII show that 10^{-5} M cyclosporine does not exert an inhibitory action on either succinate cytochrome-*c* reductase or cytochrome-*c* oxidase, as compared to the vehicle, ethanol.

Action of cyclosporine on the protonmotive force

As shown in Table VIII, cyclosporine (10^{-5} M) had nearly no effect on the protonmotive force. In these experiments the matrix volume was not measured and was assumed to be 1 µl/mg protein. Nevertheless, a putative variation of the matrix volume, induced by cyclosporine would not alter the results significantly, since a variation of a factor 2 in the matrix volume results in a variation of 18 mV for $\Delta\Psi$. When the external potassium concentration was raised to 50 mM, cyclosporine had no further effect on the membrane potential, ruling out the possibility that cyclosporine could act as a potassium ionophore of the valinomycin type. Moreover, when the protonmotive force of kidney mitochondria, isolated from rats treated for 14 days with 30 or 45 mg/kg per day cyclosporine, was measured, no significant difference from the control was observed (Table VIII).

TABLE VII

ACTION OF CYCLOSPORINE ON DIFFERENT ENZYME ACTIVITIES OF THE RAT KIDNEY MITOCHONDRIA

Each value represents the mean ± S.D. of six different experiments. Specific activities are expressed in nmol/mg protein per min.

Enzyme	Cyclosporine 10^{-5} M	EtOH 25 µl
Succinate cytochrome-*c* reductase	140 ± 19	127 ± 14
Cytochrome-*c* oxidase	241 ± 27	235 ± 16

TABLE VIII

ACTION OF CYCLOSPORINE ON PROTONMOTIVE FORCE

Each value represents the mean of three determinations. 1 mg of mitochondrial proteins was incubated at 25 °C in a final volume of 1 ml. Each determination was carried out in the presence of 15 mM succinate and of potassium chloride at concentrations shown in this Table. Results are expressed in mV. $Z = 2.3\ RT/F$ with $Z = 59$ mV at 25 °C. $\Delta P = \Delta\Psi - Z\Delta pH$.

	In vitro		In vivo		
	ethanol 25 µl	cyclosporine (10^{-5} M)	control	cyclosporine (mg/kg per day	
				30	45
$\Delta\Psi$ (10 mM K^+)	193	185	190	195	199
$-Z\Delta pH$	42	50	18	30	30
ΔP	235	235	208	225	229
$\Delta\Psi$ (50 mM K^+)	188	184	–	–	–

Discussion

The results obtained in vitro by the action of cyclosporine on mitochondria isolated from untreated rats kidney and from kidney mitochondria of cyclosporine-treated rats were similar, with a slight accentuation of the inhibition of the oxidative phosphorylation capacity in the treated animals. Our results were reproducible under experimental conditions providing effective coupling (absence of any agent producing membrane permeabilization or changing the general ultrastruc-

ture of the mitochondria, presence of Mg^{2+} ions available for coupling and therefore the absence of EDTA) and good contact between cyclosporine and the mitochondrion (cyclosporine dissolved in ethanol and a sufficiently long preincubation with cyclosporine). Changes in these experimental conditions may result in modification of the results. Our results can be summarized as follows. Cyclosporine: (i) produced slight, but statistically significant, inhibition of respiratory intensity; (ii) induced significant inhibition of P/O ratio; (iii) had no effect on the membrane potential; (iv) produced no inhibition of either succinate cytochrome-*c* reductase or cytochrome-*c* oxidase or of membrane potential; (v) produced in vivo reduction of the quantity of mitochondrial proteins.

These results led us to the following conclusions.

Cyclosporine does not act as a dinitrophenol-like uncoupler. The inhibition of respiratory intensity by cyclosporine rules out this possibility, which is characterized by stimulated oxygen consumption.

Cyclosporine does not induce any major, nonspecific change of the mitochondrial membrane. Any major, nonspecific, detergent-like change would result in collapse of the membrane potential.

Cyclosporine does not produce any major modification of the mitochondrial membrane which is specific to the energy metabolism. There was no protonophoric effect and no potassium ionophoric effect. Clinically observed abnormalities of blood potassium [29,30] could suggest possible disturbance of transmembrane potassium exchanges. The absence of any effect of cyclosporine on the membrane potential in the presence of potassium rules out this mode of action at mitochondrial level.

The in vitro inhibition of respiratory intensity and of oxidative phosphorylation capacity may be explained by slowed electron transfer between cytochromes b and c_1. The absence of any effect of cyclosporine on the activity of succinate cytochrome-*c* reductase showed that there is no slowing of the oxidation of succinate to form fumarate or of the transfer of the electrons taken from the succinate to the cytochrome *b*. This finding would appear to indicate that cyclosporine does not alter the accessibility of succinate to succinate dehydrogenase.

Similarly, the lack of any inhibitory effect of cyclosporine on cytochrome-*c* oxidase demonstrates that there is also no slowing of the transfer of electrons between cytochromes *c* and a_3. However, under conditions which do not produce any change in mitochondrial structure, an overall reduction of electron transfer within the respiratory chain as a whole was noted, since respiratory intensity was reduced in the presence of cyclosporine. We are forced to conclude that the slowing of electron transfer must occur between cytochromes *b* and c_1. This confirms the finding of Humes et al. [11], which were obtained by demonstrating cyclosporine-induced spectral changes in cytochromes *b* and c_1. We can suggest the following explanation: the lipophilic nature of cyclosporine makes it possible that it may be inserted into the lipid portion of the internal membrane of mitochondria and this insertion would reduce the mobility of the lipids in their environment and could hamper the transfer of electrons between cytochromes *b* and c_1. This could explain the reduction of both the respiratory intensity and of the oxidative phosphorylation capacity. As a matter of fact, Le Grue et al. [31] have demonstrated that cyclosporine is bound to phospholipid vesicles, and Mathyus et al. [32] have shown that it alters "the apparent motional freedom of membrane lipids in vitro". In conclusion, our results show that hypotheses suggesting a protonophoric or potassium ionophoric nature can be ruled out. The inhibition of the respiratory intensity and of the oxidative phosporylation capacity observed is slight and cannot alone account fully for the nephrotoxicity. However, a recent study by Sumpio et al. [33] has shown that treatment with ATP-$MgCl_2$ reduces cyclosporine nephrotoxicity by improving glomerular filtration rate, tubular absorption and renal perfusate flow. This shows that the changes of respiratory characteristics induced by cyclosporine, even though they are slight, do contribute to its nephrotoxicity. However, the indirect consequences of the changed respiratory characteristics induced by cyclosporine probably account for the nephrotoxicity of the drug, as Verpooten et al. [8] have suggested. As a matter of fact, in order to estimate the role played by energy metabolism in nephrotoxicity the following fact must also be taken into account: we observed not

only a reduction of the body weight of the animals treated (which agrees with the findings of Jackson et al. [34]) but also reduction of the quantity of protein in the different subcellular fractions including mitochondria (which supports the findings of Backman et al. [16]). The combination of these two factors: the inhibition of some respiratory parameters and the reduction of mitochondrial protein levels, lead us to reconsider the part played by mitochondrial damage in nephrotoxicity, which could at first glance appear to be limited.

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