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CYCLOSPORINE A INHIBITS ATP NET UPTAKE OF RAT KIDNEY MITOCHONDRIA

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Abstract—The adenine nucleotide content of mitochondria varies at several physiological and pathological situations. Both net transport and intramitochondrial catabolism of adenine nucleotides has been suggested to be responsible for these changes. Here, the influence of cyclosporine A on the ATP net uptake of isolated rat kidney mitochondria was examined. The ATP net uptake of mitochondria depleted of matrix adenine nucleotides by pyrophosphate treatment was inhibited by cyclosporine A showing a I_{50} value of about 4 nmol/mg mitochondrial protein. Because intramitochondrial adenine nucleotide content is important for several mitochondrial functions such as oxidative phosphorylation, Ca^{2+} homoeostasis and mitochondrial biogenesis, it is concluded that the inhibition of adenine nucleotide net transport and a decrease of adenine nucleotide content may be involved in the immunosuppressive and nephrotoxic effects of cyclosporine A.

The immunosuppressive oligopeptide cyclosporine A is extensively applied in organ transplantation and autoimmune disorders. Its therapeutic use is, however, often limited by side effects including nephrotoxicity and hepatic disorders [1]. Mechanisms of cyclosporine A-induced toxicity have been investigated in several models including mitochondria [2–11].

The pool size of mitochondrial adenine nucleotide (AdN†) varies during several physiological situations such as during postnatal development, glucagon treatment, ischemia and hibernation. Mechanisms suggested to account for these changes include a net transport by the Mg^{2+}ATP/phosphate carrier (see Ref. 12) and/or an intramitochondrial catabolism of AdN [13, 14]. The ATP net uptake is well-established with mitochondria of liver [15] and described recently with those of kidney cortex [16].

In the present paper, it is shown that ATP net uptake of kidney mitochondria is inhibited by cyclosporine A.

MATERIALS AND METHODS

Materials. Nucleotides, nucleosides and nucleobases used as standards for HPLC were from Sigma (Deisenhofen, F.R.G.). Oligomycin was purchased from Serva (Heidelberg, F.R.G.). Cyclosporine A was a generous gift from Sandoz.

Preparation of mitochondria. Kidney cortex mitochondria were prepared from fed male Wistar rats (200–250 g body wt; Biomodelle Berlin GmbH, Schönwalde, F.R.G.) by standard procedures [17]. The final pellet was resuspended in a medium of 250 mM mannitol, 70 mM sucrose and 1 mM EDTA, buffered with traces of Tris to pH 7.4 at a protein content of about 15 mg/mL. Protein content was measured by a biuret method as described in Ref. 17.

AdN depletion of mitochondria. Mitochondria were depleted from matrix AdN by incubating in the preparation medium with pyrophosphate at 30° for 5 min. At the end of incubation, aliquots were placed on ice for 3 min, then centrifuged at 10,000 *g* for 5 min and washed in 8 mL preparation medium.

ATP net uptake studies. Mitochondria (3 mg/mL) were incubated in a reloading medium consisting of 1 mM ATP, 5 mM $MgCl_2$, 10 mM K_2HPO_4, 0.75 mM phosphoenolpyruvate, 0.5 mM EDTA, 90 mM sucrose, 75 mM Tris–HCl, pH 7.4 and 0.012 μkat desalted pyruvate kinase/mL. If used, cyclosporine A was added to mitochondrial incubations from stocks dissolved in dimethyl sulfoxide. The controls for all experiments involving this compound contained an equimolar volume of the solvent. The mitochondria were reisolated by centrifugation at 10,000 *g* for 5 min, washed once and immediately quenched with perchloric acid for AdN determination as described in Ref. 18.

Determination of AdN. AdN were determined by an ion-pair microbore HPLC method published elsewhere [18]. A 1090 M HPLC system of Hewlett–Packard, Vienna, Austria and a vertex microbore column (3 μm Hypersil, Shandon, U.K.; 100 × 2 mm i.d.) including a precolumn (3 μm Hypersil, Shandon, U.K.; 7 × 2 mm i.d.) from Knauer (Bad Homburg, F.R.G.) were applied.

RESULTS

Pyrophosphate treatment of kidney mitochondria reduced the content of intramitochondrial AdN determined by the sum of AMP, ADP and ATP. Incubation with 1 mM pyrophosphate, the highest

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† Abbreviation: AdN, adenine nucleotide.

Table 1. *In vitro* effect of pyrophosphate on adenine nucleotide content in renal mitochondria

Pyrophosphate (mM)	Content of AMP+ADP+ATP (nmol/mg protein)
0.0	5.1 ± 0.8
0.1	3.1 ± 0.5
0.5	2.3 ± 0.4
1.0	1.7 ± 0.2

Mitochondria were incubated with pyrophosphate for 5 min as described in Materials and Methods.

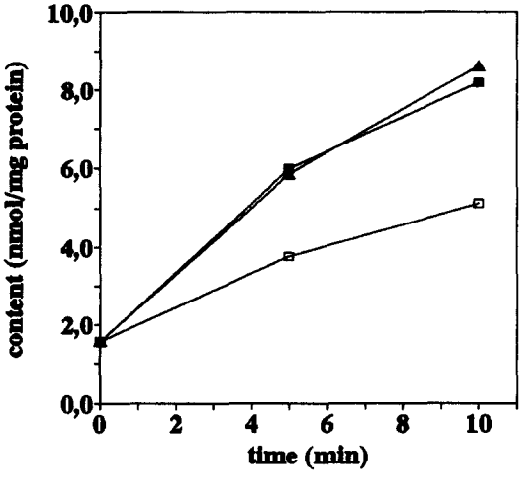

Fig. 1. ATP net uptake of kidney mitochondria. Mitochondria were depleted of matrix adenine nucleotide by treatment with 1 mM pyrophosphate for 5 min and then incubated with 1 mM ATP as described in Materials and Methods. (■) Control; (▲) 7.5 μM oligomycin; (□) 50 μM cyclosporine A. N = 3.

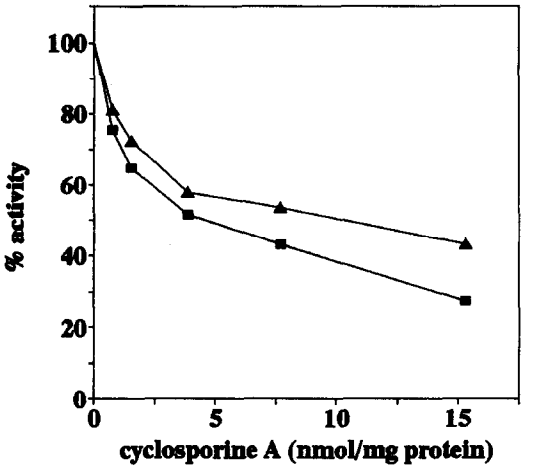

Fig. 2. Inhibition of the ATP net uptake rate by cyclosporine A. ATP net uptake rate was measured at different indicated cyclosporine A concentrations by incubating for 10 min as described in Fig. 1. The cyclosporine A inhibition was estimated as a percentage of the uptake rate measured without cyclosporine A. (■) Without and (▲) with 7.5 μM oligomycin. N = 3.

concentration applied, for 5 min decreased the matrix AdN content to 33% (Table 1).

Mitochondria depleted in this way from AdN were used to study the ATP net uptake. The matrix AdN content increased during the incubation with 1 mM ATP, 5 mM Mg^{2+} and 10 mM phosphate. Oligomycin had no effect and cylosporine A inhibited this ATP net uptake (Fig. 1).

Figure 2 demonstrates the decrease of ATP net uptake rates measured in dependence on the cyclosporine A concentration. About 4 nmol cyclosporine A/mg mitochondrial protein were required to inhibit the ATP net uptake rate by 50%. A similar inhibition pattern was observed in the presence of oligomycin. Oligomycin prevents an increase of matrix ATP/ADP ratio [15]. Therefore, it can be ruled out that the cyclosporine A inhibition is caused secondarily by an increase of the matrix ATP/ADP ratio.

DISCUSSION

Rat kidney mitochondria perform an ATP net uptake [16]. As shown here, cyclosporine A is an inhibitor of the ATP net uptake by kidney mitochondria. Two action modes of cyclosporine A on mitochondrial functions—a high affinity and a low affinity—have been described. The inhibition of Ca^{2+} efflux from mitochondria or of mitochondrial swelling provoked by Ca^{2+} accumulation or by adding an inducing agent, e.g. phosphate, *t*-butylhydroperoxide or ruthenium red, occurs with high affinity [4–7, 9–11, 19, 20]. The inhibitor constant and the number of cyclosporine A binding sites have been estimated to be about 5 nM and about 50–100 pmol/mg of mitochondrial protein, respectively [7, 10, 11, 20]. Three hypotheses are published to explain the inhibitory cyclosporine A effects: firstly, binding to a putative pore being responsible for the permeability transition linked with Ca^{2+} efflux and swelling of mitochondria [7, 19]; secondly, binding to a peptidyl-prolyl *cis–trans* isomerase, recently identified as the cyclosporine A binding protein cyclophilin [21, 22] and now also detected in mitochondria of *Neurospora crassa* [23], rat liver and heart [10, 11], and removing it from a complex formed with the Ca^{2+}-liganded AdN translocator which functions as a non-specific pore [10, 11]; and thirdly, inhibition of the initial reaction during protein ADP-ribosylation, i.e. pyridine nucleotide hydrolysis, suggested to regulate the mitochondrial Ca^{2+} release [9].

In vitro, cyclosporine A inhibits oxidative phosphorylation [2–4] and displaces [^{3}H]Ro5, a tight-binding benzodiazepine derivative, from its mitochondrial receptor [8]. The corresponding I_{50} values of cyclosporine A inhibition are in the same order of magnitude as the I_{50} value measured for the inhibition of ATP net uptake and about three orders

of magnitude higher than those of the provoked mitochondrial Ca^{2+} efflux and swelling [7, 10, 11, 20].

In the kidneys of cyclosporine A-treated animals, histological alterations were most obvious in the proximal tubules and included varying degrees of cytosolic vacuolization, formation of myeloid bodies and evidence of autophagocytosis of mitochondria [24–26]. Mitochondria isolated from rats treated with cyclosporine A are characterized by a decreased respiratory capacity [6, 27, 28]. In these experiments, 25–50 mg cyclosporine A/kg body weight were applied. Multiple doses of 10 mg/kg to rats cause a cyclosporine A content of about 30 μg/g in renal tissue [29]. This value corresponds to the concentration range used in this study.

Matrix AdN content is important for several mitochondrial functions such as oxidative phosphorylation [16, 30], gluconeogenesis [31], ureogenesis [32], retainment of Ca^{2+} [33], pyridine nucleotide degradation [34], protein synthesis [35] and mitochondrial biogenesis [12]. We therefore suggest that the inhibition of ATP net uptake by cyclosporine A and a decrease of AdN content may be involved in the immunosuppression and nephrotoxicity of the drug.

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