Biochemical Pharmacology, Vol. 46, No. 5, pp. 829–832, 1993.
Printed in Great Britain.

0006-2952/93 $6.00 + 0.00

COMPARISON OF THE EFFECTS OF THE IMMUNOSUPPRESSIVE AGENTS FK 506 AND CYCLOSPORIN A ON RAT KIDNEY MITOCHONDRIA

WOLFGANG HENKE* and KLAUS JUNG
Research Division of the Urological Clinic, Medical Faculty (Charité), Humboldt-University Berlin, Schumannstraße 20/21, 10098 Berlin, Germany

(*Received* 13 *January* 1993; *accepted* 4 *June* 1993)

Abstract— Interactions of FK 506 with renal cortical mitochondria have been investigated by measuring respiration, ATP net uptake and Ca^{2+}/P_i-induced swelling. Both FK 506 and cyclosporin A (CsA) inhibit the succinate-supported state 3 and uncoupled respiration and are without effect on the glutamate/malate-supported state 3 and uncoupled respiration. FK 506, like CsA, inhibits net uptake of ATP, but, in contrast to CsA, is without effect on Ca^{2+}/P_i-induced swelling.

The macrolide FK 506 is a recently introduced immunosuppressive compound with more potent effects than cyclosporin A (CsA†) [1]. Both CsA and FK 506 block activation of T cells by inhibiting the transcription of interleukin-2. It has been suggested that the binding proteins (immunophilins) for CsA and FK 506 called cyclophilin and macrophilin, respectively, when bound by the drugs form inactive complexes with the Ca^{2+}-dependent protein phosphatase calcineurin and calmodulin and that calcineurin is involved in regulating the transcription of interleukin-2 [2, 3].

One of the major side effects of CsA is its renal toxicity [4]. Several studies have also been reported on nephrotoxicity induced by FK 506 [5–9]. However, the nephrotoxic effects of FK 506 have not been completely elucidated [1]. Whereas CsA effects on renal mitochondria, e.g. inhibited respiration [10], ATP net transport [11] and Ca^{2+}/P_i-induced swelling and Ca^{2+} release [12], have been suggested to be decisive for the nephrotoxic property of this drug, there are no reports dealing with the interaction of FK 506 and renal mitochondria. The purpose of this study was to compare FK 506 and CsA effects on rat renal mitochondria as possible targets of nephrotoxic action. FK 506 induces respiratory dysfunction and inhibits net uptake of ATP in mitochondria, and is without effect on Ca^{2+}/P_i-induced swelling.

MATERIALS AND METHODS

Materials. FK 506 and CsA were generous gifts from Fujisawa Pharmaceutical Co., Ltd, (Japan) and Sandoz AG (Basel, Switzerland), respectively.

* Corresponding author: Dr W. Henke, Forschungsabteilung der Urologischen Klinik, Universitätsklinikum Charité, Medizinische Fakultät der Humboldt-Universität zu Berlin, Schumannstraße 20/21, 10098 Berlin, Germany. Tel. (49) 30/2802-4073; FAX (49) 30/2802-1402.

† Abbreviations: RCR, respiratory control ratio; CsA, cyclosporin A.

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.) as described [11] and finally 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 [11].

Measurement of respiratory rates. Respiratory rates were determined with a Clark-type oxygen electrode at 25° using a medium of 210 mM sucrose, 10 mM KCl, 10 mM KH_2PO_4, 0.5 mM EDTA, 60 mM Tris–HCl and pH 7.4. Succinate (10 mM) and 10 mM glutamate/malate were applied as substrates and 0.05 mM 2,4-dinitrophenol as uncoupler. FK 506 and CsA were dissolved in dimethyl sulfoxide. An equimolar volume of the solvent was added to the control.

ATP net uptake studies. Mitochondria (3 mg/mL) were incubated at 30° in a medium consisting of 4 mM ATP, 5 mM $MgCl_2$, 2 mM K_2HPO_4, 0.5 mM EDTA, 90 mM sucrose, 75 mM Tris–HCl and pH 7.4 as published [11].

Determination of adenine nucleotides. Adenine nucleotides were determined by an ion-pair microbore HPLC method [11].

Measurement of swelling. Mitochondrial swelling was determined by monitoring the decrease in absorbance at 546 nm. Mitochondria (1 mg/mL) were preincubated with 0.1 mM $CaCl_2$, 230 mM mannitol, 70 mM sucrose, 10 mM Tris–HCl and 10 mM succinate at pH 7.4 and 25° for 1 min. Mitochondrial swelling was induced with 2 mM K_2HPO_4.

RESULTS

FK 506 inhibits *in vitro* like CsA, succinate-supported state 3 and uncoupled respiration (Fig. 1). With 100 μM FK 506, the highest concentration used, the rate of state 3 and uncoupled respiration is decreased by 24% ($P < 0.01$) and 35% ($P < 0.001$),

Table 1. Influence of FK 506 and cyclosporin on RCR and ADP/O ratio calculated from respiration of kidney cortex mitochondria

	Succinate		Glutamate/malate	
	RCR	ADP/O	RCR	ADP/O
Control	5.69 ± 0.44	1.52 ± 0.15	8.1 ± 1.33	2.21 ± 0.2
100 μM FK 506	3.78 ± 0.23†	1.38 ± 0.14	4.19 ± 0.63*	2.03 ± 0.16
100 μM CsA	5.79 ± 0.13	1.55 ± 0.18	7.12 ± 0.71	2.14 ± 0.16

Data are given as arithmetic means ± SD; N = 5. Differences to the controls were estimated with the Student's *t*-test of paired data.
* $P < 0.01$ compared with controls.
† $P < 0.001$ compared with controls.

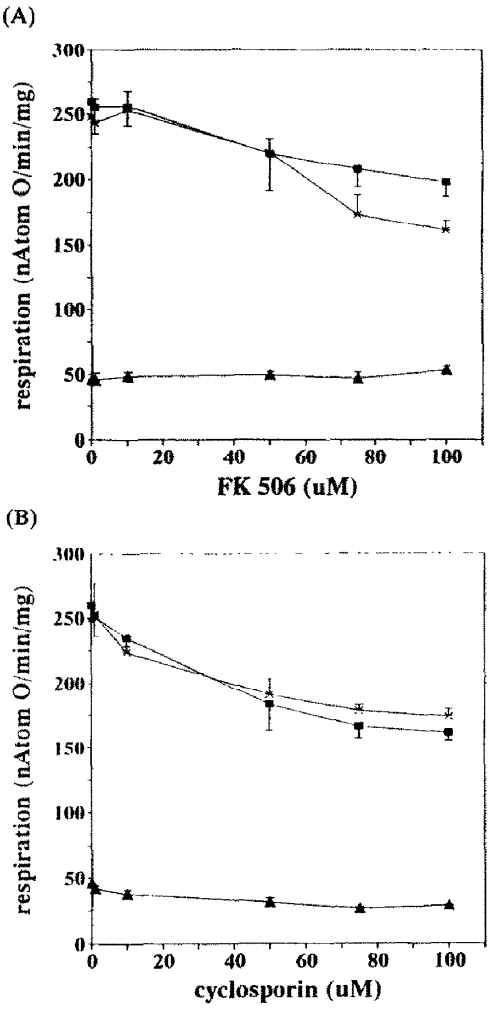

Fig. 1. Parameters of succinate-supported respiration as a function of FK 506 (A) or cyclosporin concentration (B). For conditions see Materials and Methods. (■) State 3, (▲) state 4 and (×) uncoupler-stimulated respiration; N = 5.

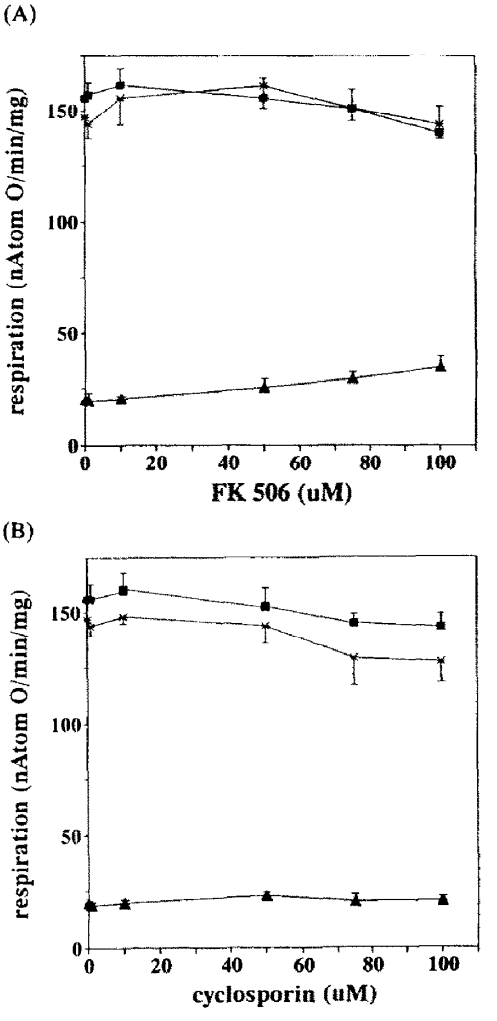

Fig. 2. Parameters of glutamate/malate-supported respiration as a function of FK 506 (A) or cyclosporin concentration (B). For conditions see Materials and Methods. (■) State 3, (▲) state 4 and (×) uncoupler-stimulated respiration; N = 5.

respectively. At this concentration, the state 4 respiratory rate is raised by 15% ($P < 0.05$). The respiratory control ratio (RCR) is diminished by 33% ($P < 0.001$) and the ADP/O ratio is unchanged.

Using glutamate/malate as respiratory substrates, FK 506 is without influence on the state 3 and uncoupled respiratory rate as well as on the ADP/O ratio (Fig. 2). The state 4 respiration rate is increased

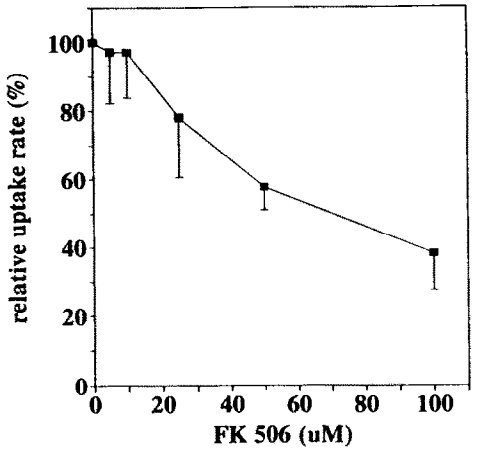

Fig. 3. Inhibition of the ATP uptake rate by FK 506. ATP uptake rates were determined at indicated FK 506 concentrations by incubating for 5 min as described in Materials and Methods; N = 3.

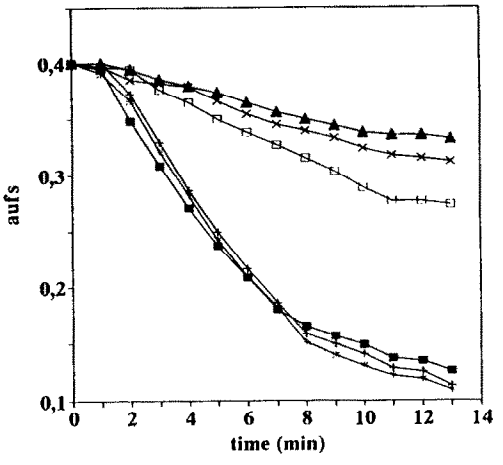

Fig. 4. Influence of FK 506 or cyclosporin on Ca^{2+}/P_i-induced swelling of renal mitochondria. Mitochondrial swelling was induced with 2 mM P_i after preincubation with 100 nmol Ca^{2+}/mg for 1 min. (■) Control; FK 506, (*) 550 pmol/mg, (+) 1100 pmol/mg; cyclosporin, (□) 275 pmol/mg, (×) 550 pmol/mg and (▲) 1100 pmol/mg.

and the RCR is decreased by 75% ($P < 0.01$) and 48% ($P < 0.01$), respectively. The efficacy of CsA on state 3 and uncoupled respiration is also considerably lower as with succinate (Fig. 2).

Furthermore, FK 506 inhibits the net uptake of ATP measured as increase of adenine nucleotide content (AMP + ADP + ATP). About 70 μM FK 506 inhibits the ATP uptake rate by 50% (Fig. 3).

In the presence of 2 mM P_i, 100 nmol Ca^{2+}/mg protein induce swelling of mitochondria. This swelling is prevented by CsA, but FK 506 is without such inhibitory effects (Fig. 4).

DISCUSSION

It has been suggested that the interaction between CsA and mitochondria, especially by inhibiting respiration, net transport of ATP and Ca^{2+}/P_i-induced swelling and Ca^{2+} release, may contribute to the drug nephrotoxicity [10–16]. FK 506 inhibits, like CsA, the succinate-supported state 3 respiration as well as the uncoupled respiration of kidney cortex mitochondria. Both drugs have no significant effect on the NAD-linked state 3 respiration. The results obtained with CsA are in agreement with published data [12, 17] but in contrast with those of our group [10]. In our previous experiments we used CsA dissolved in polyoxyethylated castor oil and ethanol [10]. An effect of this vehicle might be the reason for the observed differences.

The mitochondrial adenine nucleotide content, which varies with changes in physiological conditions, is involved in regulating mitochondrial functions [18]. Net transport of ATP, discussed as one of the mechanisms accounting for changes of adenine nucleotide content [19], is well-established for liver [18] and recently described in kidney mitochondria [20]. Inhibition of 50% of the uptake rate is obtained with about 70 μM FK 506 or 15 μM CsA. The CsA experiments were performed with 1 mM ATP [11]. This might contribute to the affinity difference.

Mitochondrial swelling, Ca^{2+} release and hydrolysis of matrix pyridine nucleotides, attributed to a large permeability transition pore [12] or a specific Ca^{2+} release pathway [15], respectively, may be provoked by Ca^{2+} loading and an inducing agent. CsA inhibits these processes and the mitochondrial peptidyl-prolyl *cis-trans* isomerase activity [12, 14, 15, 21]. This CsA action might be of special importance, because, concentrations necessary for these effects are about three orders of magnitude lower than those required for inhibiting oxidative phosphorylation [10, 12, 17] or net uptake of ATP [11]. FK 506, applied in concentrations which are effective with CsA, is without effect on mitochondrial swelling of renal mitochondria. These results agree with recently published data obtained with liver and heart mitochondria [22]. Cyclophilin and macrophilin have the same enzymatic activities but discriminate in binding their corresponding effectors [23, 24]. Obviously, only cyclophilin interacts with the mitochondrial pore mechanism or Ca^{2+} release pathway. So far as the high-affinity CsA effect on mitochondrial swelling and Ca^{2+} flux dominates the nephrotoxic action of this drug then the FK 506 toxicity caused by interacting with mitochondria should be of minor importance.

FK 506 is immunologically effective at doses of about two orders of magnitude lower than CsA [1]. This raises the question about the relevance of the described FK 506 effects on mitochondria. Both CsA [13] and FK 506 [6] induce similar morphological changes in renal mitochondria. Nephrotoxic FK 506 effects have been detected in experimental studies [5–8] and in the course of clinical application [1]. FK 506 is metabolized and excreted by the liver. A

dysfunction of the transplanted liver may be connected, as has been demonstrated recently [9, 25, 26], with raising FK 506 plasma level and nephrotoxic side effects. Nevertheless, it left as an open question if the FK 506 concentration is raised to a level which might cause, at least partially, nephrotoxicity by interacting with mitochondrial respiration and net uptake of ATP.

Acknowledgements—This work was supported by the Deutsche Forschungsgemeinschaft, SFB 330-Organprotektion, Göttingen, F.R.G. We gratefully thank Sabine Becker, Silke Klotzek and Erika Nickel for technical assistance.

REFERENCES

1. Fung JJ, Abu Elmagd K, Todo S, Shapiro R, Tzakis A, Jordan M, Armitage J, Jain A, Alessiani M, Martin M *et al.*, Overview of FK 506 in transplantation. *Clin Transplant* **6**: 115–121, 1990.
2. Liu J, Farmer JDJ, Lane WS, Friedman J, Weissman I and Schreiber SL, Calcineurin is a common target of cyclophilin–cyclosporin A and FKBP-FK 506 complexes. *Cell* **66**: 807–815, 1991.
3. Clipstone NA and Crabtree GR, Identification of calcineurin as a key signalling enzyme in T-lymphocyte activation. *Nature* **357**: 695–697, 1992.
4. Kopp JB and Klotman PE, Cellular and molecular mechanisms of cyclosporin nephrotoxicity. *J Am Soc Nephrol* **1**: 162–179, 1990.
5. Ochiai T, Nakajima K, Nagata M, Hori S, Asano T and Isono K, Studies of the induction and maintenance of long-term graft acceptance by treatment with FK 506 in heterotopic cardiac allotransplantation in rats. *Transplantation* **44**: 734–738, 1987.
6. Nalesnik MA, Lai HS, Murase N, Todo S and Starzl TE, The effect of FK 506 and CyA on the Lewis rat renal ischemia model. *Transplant Proc* **22**: 87–89, 1990.
7. Kumano K, Wang G, Endo T and Kuwao S, FK 506-induced nephrotoxicity in rats. *Transplant Proc* **23**: 512–515, 1991.
8. Moutabarrik A, Ishibashi M, Kameoka H, Ogata A, Kawaguchi N, Takano Y, Kokado Y, Takahara S, Jiang H, Onishi S, Sonoda T and Okuyama A, *In vitro* FK 506 kidney tubular cell toxicity. *Transplant Proc* **23**: 3137–3140, 1991.
9. Winkler M, Jost U, Ringe B, Gubernatis G, Wonigeit K and Pichlmayr R, Association of elevated FK 506 plasma levels with nephrotoxicity in liver-grafted patients. *Transplant Proc* **23**: 3153–3155, 1991.
10. Jung K and Pergande M, Influence of cyclosporin A on the respiration of isolated rat kidney mitochondria. *FEBS Lett* **183**: 167–169, 1985.
11. Henke W, Nickel E and Jung K, Cyclosporine A inhibits ATP net uptake of rat kidney mitochondria. *Biochem Pharmacol* **43**: 1021–1024, 1992.
12. Strzelecki T, Kumar S, Khauli R and Menon M, Impairment by cyclosporine of membrane-mediated functions in kidney mitochondria. *Kidney Int* **34**: 234–240, 1988.
13. Mihatsch MJ, Oliviere W, Marbet U, Thiel G, Hrder F and Zollinger HU, Giant mitochondria in renal tubular cells and cyclosporin A. *Lancet* **1**: 1162–1163, 1981.
14. Halestrap AP and Davidson AM, Inhibition of Ca^{2+}-induced large-amplitude swelling of liver and heart mitochondria by cyclosporin is probably caused by the inhibitor binding to mitochondrial-matrix peptidyl-prolyl *cis-trans* isomerase and preventing it interacting with the adenine nucleotide translocase. *Biochem J* **268**: 153–160, 1990.
15. Richter C, Theus M and Schlegel J, Cyclosporine A inhibits mitochondrial pyridine nucleotide hydrolysis and calcium release. *Biochem Pharmacol* **40**: 779–782, 1990.
16. Schlegel J, Meier P, Kass GE and Richter C, Inhibition by cyclosporine A of the prooxidant-induced but not of the sodium-induced calcium release from rat kidney mitochondria. *Biochem Pharmacol* **42**: 2193–2197, 1991.
17. Jackson NM, O'Connor RP and Humes HD, Interactions of cyclosporine with renal proximal tubule cells and cellular membranes. *Transplantation* **46**: 109–114, 1988.
18. Aprille JR, Regulation of the mitochondrial adenine nucleotide pool size in liver: mechanism and metabolic role. *FASEB J* **2**: 2547–2556, 1988.
19. Henke W, Ziegler M, Dubiel W and Jung K, Mechanisms accounting for changes of adenine nucleotide content in mitochondria at ischemia. *Adv Exp Med Biol* **309A**: 309–312, 1991.
20. Henke W and Nickel E, The contribution of adenine nucleotide loss to the ischemia-induced impairment of rat kidney cortex mitochondria. *Biochim Biophys Acta* **1098**:P 233–239, 1992.
21. McGuinness O, Yafei N, Costi A and Crompton M, The presence of two classes of high-affinity cyclosporin A binding sites in mitochondria. Evidence that the minor component is involved in the opening of an inner-membrane Ca^{2+}-dependent pore. *Eur J Biochem* **194**: 671–679, 1990.
22. Griffiths EJ and Halestrap AP, Further evidence that cyclosporin A protects mitochondria from calcium overload by inhibiting a matrix peptidyl-prolyl cis-trans isomerase. Implications for the immunosuppressive and toxic effects of cyclosporin. *Biochem J* **274**: 611–614, 1991.
23. Harding MW, Galat A, Uehling DE and Schreiber SL, A receptor for the immunosuppressant FK 506 is a *cis-trans* peptidyl-prolyl isomerase. *Nature* **341**: 758–760, 1989.
24. Tropschug M, Wachter E, Mayer S, Schonbrunner ER and Schmid FX, Isolation and sequence of an FK 506-binding protein from *N. crassa* which catalyses protein folding. *Nature* **346**: 674–677, 1990.
25. Abu Elmagd K, Fung JJ, Alessiani M, Jain A, Venkataramanan R, Warty VS, Takaya S, Todo S, Shannon WD and Starzl TE, The effect of graft function on FK 506 plasma levels, dosages, and renal function, with particular reference to the liver. *Transplantation* **52**: 71–77, 1991.
26. Hebert MF, Ascher NL, Lake JR and Roberts JP, Efficacy and toxicity of FK 506 for the treatment of resistant rejection in liver transplant patients. *Transplant Proc* **23**: 3109–3110, 1991.