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ORIGINAL ARTICLE

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Preventive effects of diltiazem on cyclosporin A-induced vascular smooth muscle dysfunction

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Abstract Cyclosporin A may cause vascular smooth muscle dysfunction due to calcium overload as a consequence of chronically augmented calcium influx. In the present study, the responsiveness to vasoconstrictors was investigated in rats after chronic treatment for 6 weeks with placebo, cyclosporin A (30 mg/kg per day), diltiazem (60 mg/kg per day), or cyclosporin A plus diltiazem. Twenty-four hours after the last oral treatment the animals were sacrified and rings of the thoracic aorta were suspended in organ chambers under isometric conditions in the absence of cyclosporin A or diltiazem. Chronic treatment with cyclosporin A significantly augmented contractions to angiotensin II (10^{-9}–10^{-5} M). This effect was prevented by cotreatment with diltiazem. Diltiazem did not affect the cyclosporin A-induced reduction in the response to potassium chloride (10–80 mM). The contractions to phenylephrine (10^{-9}–10^{-6} M) and endothelin-1 (10^{-9}–10^{-7} M) were not significantly different in the four groups. The preventive effect of diltiazem against the cyclosporin A-induced hypersensitivity to angiotensin II supports the hypothesis of increased calcium influx during cyclosporin A therapy. The results may provide an additional rationale for the use of calcium antagonists in the treatment of the vascular side effects of cyclosporin A.

Key words Diltiazem, rat, cyclosporin A. Cyclosporin A, diltiazem, rat · Angiotensin II, diltiazem

Introduction

Cyclosporin A is an immunosuppressive drug frequently used in patients after organ transplantation and in the treatment of autoimmune diseases [5, 7, 13, 34, 37]. Chronic treatment with this drug disturbs vascular smooth muscle function [3, 12, 16, 22, 29, 30]. The altered reactivity of the vascular smooth muscle may contribute to the side effects frequently observed with clinical use of the drug, such as the development of hypertension and renal dysfunction [2, 24, 32]. The mechanisms mediating the chronic effects of cyclosporin A on the arterial wall are not completely understood, nor do recommendations exist for the treatment of the cardiovascular side effects in clinical use. An increased influx of calcium was proposed as a possible mechanism leading to the cyclosporin A-induced dysfunction of vascular smooth muscle cells. Calcium influx during cyclosporin A exposure may cause direct vasoconstrictor effects [41]; chronic exposure may lead to an overload of intracellular calcium stores. The latter may explain the augmented effect of those vasoconstrictors, which mobilize calcium from intracellular stores [11, 18–20, 26, 27]. Evaluating the effect of chronic calcium entry blockade in the prevention of cyclosporin A-induced vascular dysfunction may, therefore, be interesting. Previous studies have suggested a beneficial effect of calcium antagonists in the prevention of renal failure in cyclosporin A-treated patients [8, 14, 39]. The effect of chronic calcium entry blockade on the cyclosporin A-induced alterations of vascular reactivity has not yet been investigated.

Thus, the objective of the present study was to determine the effect of cotreatment with the calcium antagonist diltiazem on vascular smooth muscle reactivity occurring during chronic treatment with cyclosporin A.

Materials and methods

Animals

Forty male Wistar rats were randomly assigned to four groups: controls (CO), a cyclosporin A-treated group (CyA), a diltiazem-treated group (DIL), and a cyclosporin A plus diltiazem-treated group (CyA/DIL). The mean weight at the beginning of the treatment period was 328 ± 5 g, 316 ± 7 g, 317 ± 10 g, and 324 ± 7 g, respectively. In addition to their standard diet (Altromin, Lange, FRG), the rats received 1 ml of olive oil (CO), 30 mg/kg per day of cyclosporin A dissolved in 1 ml of olive oil (CyA), 60 mg/kg per day of diltiazem dissolved in 1 ml of distilled water (DIL), or 30 mg/kg per day cyclosporin A plus 60 mg/kg per day diltiazem (CyA/DIL) daily, administered through an oral gastric tube over a period of 6 weeks. In order to test the preventive effect of diltiazem, the duration and dosage of treatment with cyclosporin A was chosen according to previous experimental studies showing altered reactivity of the rat aorta [3, 29]. Two rats in the CyA group and in the CyA/DIL group were lost because of aspiration while receiving the treatment.

Experimental protocol

Twenty-four hours after the last administration of the treatment the rats were anesthetized with ether. Systolic blood pressure was measured under anesthesia (VSM-Physio Control, Redmont, Wash., USA) through a cannula that was inserted into the abdominal aorta. Approximately 5 ml of blood was withdrawn for laboratory tests. Whole blood levels of cyclosporin A were measured by high pressure liquid chromatography. Laboratory tests for renal and liver function in the plasma were performed with an automatic analyzer (BM/Hitachi System 717, Boehringer, Mannheim, Germany). After the animals had been exsanguinated, the aorta was rapidly excised. The blood vessel was immediately flushed with and placed into modified Krebs-Henseleit bicarbonate solution of the following composition (mM): NaCl (118), KCl (4.7), $CaCl_2$ (2.5), $MgSO_4$ (1.2), KH_2PO_4 (1.2), $NaHCO_3$ (25.0), edetate calcium disodium (0.026), glucose (11.1; control solution).

The thoracic part of the aorta was cut into four rings each 4 mm in length. The rings were suspended in organ chambers between a clip and a force transducer by two stainless steel wires inserted into the lumen of the vessels. The organ chambers were filled with 10 ml control solution, kept at 37 °C, and aerated with a 95 % O_2 and 5 % CO_2 gas mixture. Changes in isometric force were measured. The preparations were set individually at the optimal point of their length-tension relationship as determined by repeated exposure to potassium chloride (20 mM) that was comparable among the four groups (approximately 5 g).

After at least 1 h of equilibration, in which all of the rings returned to their basal tension, cumulative concentration response curves to angiotensin II, potassium chloride, endothelin-1, and phenylephrine were registered. To control for possible angiotensin II tachyphylaxis, pilot experiments were performed, that did not reveal any difference in angiotensin II-induced contractions in response to a single dose or cumulative application of angiotensin II [1]. Because angiotensin II causes transient contractions, higher concentrations were applied at exactly defined time intervals. Indomethacin (10^{-5} M) was present throughout the experiments to prevent the generation of vasoactive prostaglandins.

Drugs

Angiotensin II and endothelin-1 were obtained from Novabiochem (Sandhausen, Germany). Indomethacin and phenylephrine were purchased from Sigma Chemicals (8024 Deisenhofen, Germany). Cyclosporin A was obtained from Sandoz (Nürnberg, Germany). Diltiazem was obtained from Gödecke-AG (Berlin, Germany). The drugs were prepared daily in distilled water except for indomethacin, which was dissolved by sonication in Na_2CO_3 (10^{-5} M). The drugs were added to the organ bath in small volumes (100 µl) in a cumulative manner, except for the higher concentrations of potassium chloride (40, 60, and 80 mM). Those were prepared with a control solution containing a reduced sodium concentration in order to maintain the osmolarity of the buffer. All concentrations are expressed as final molar (M) bath concentrations. Since the study was designed to determine the chronic effects of cyclosporin A or diltiazem, these drugs were not added to the incubation medium during the in vitro tests.

Calculations and statistical analysis

Increases in isometric force in response to contracting agents are expressed in grams (g). Results are given as means ± SEM, with n referring to the number of rats the vessels were taken from. Statistical comparisons between the four groups were performed by one-way analyses of variance (ANOVA). When P was less than 0.05, the means were considered statistically different.

Results

Vascular smooth muscle function

Angiotensin II

Cumulative addition of angiotensin II (10^{-9}–10^{-5} M) caused transient contractions in the rat aorta reaching a maximum at 10^{-7} M in the CO, DIL, and CyA/DIL groups and a maximum at 10^{-6} M in the CyA group. Higher concentrations did not cause a further increase in tension. Yet, the contractions to angiotensin II (10^{-7} and 10^{-5} M) were significantly higher in the CyA-treated group than the response in the CO group. In the CyA/DIL group, however, angiotensin II elicited similar contractions to those observed in the CO group (Fig. 1).

Potassium chloride

Contractions to potassium chloride (10–80 mM) were significantly reduced in CyA or CyA/DIL-treated rats at concentrations of 60 and 80 mM potassium chloride. Chronic treatment with diltiazem, either alone or in com-

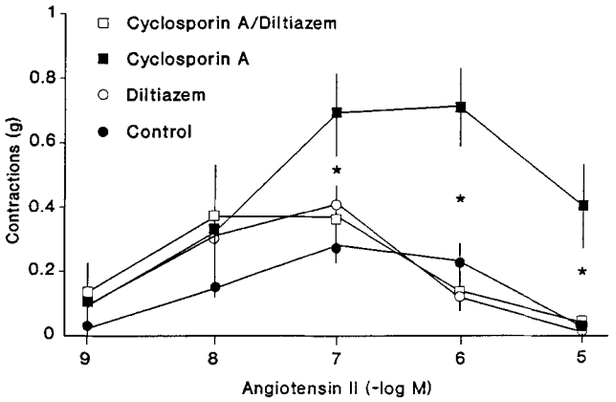

Fig. 1 Contractions to angiotensin II (10^{-9}–10^{-5} M) in the rat aorta, expressed as gram *(g)* increase in isometric force. Results are shown as means ± SEM of *n* experiments (● rings from control rats, ■ rings from rats in CyA group, ○ rings from rats in DIL group, □ rings from rats in CyA/DIL group). *$P < 0.05$

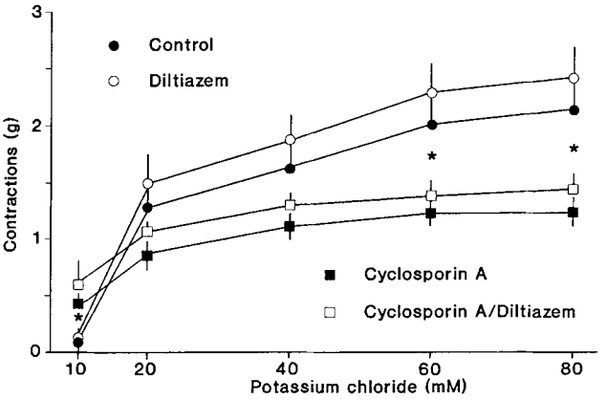

Fig. 2 Contractions to potassium chloride (10–80 mM) in the rat aorta, expressed as gram *(g)* increase in isometric force. Results are shown as means ± SEM of *n* experiments (● rings from control rats, ■ rings from rats in CyA group, ○ rings from rats in DIL group, □ rings from rats in CyA/DIL group). *$P < 0.05$

Table 1 Vascular smooth muscle contractions to phenylephrine and endothelin-1 in the aorta of rats treated for 6 weeks with either vehicle (1 ml of olive oil = control), or cyclosporin A (30 mg/kg per day), or diltiazem (60 mg/kg per day), or cyclosporin A (30 mg/kg per day) plus diltiazem (60 mg/kg per day). Contractions are expressed as gram *(g)* increase in isometric force. Results are shown as means ± SEM of *n* experiments

	Control ($n = 10$)	Cyclosporin A ($n = 8$)	Diltiazem ($n = 10$)	Cyclosporin A plus diltiazem ($n = 8$)
(a) Phenylephrine				
10^{-9} M	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.1
10^{-8} M	0.6 ± 0.2	1.0 ± 0.2	0.8 ± 0.2	0.9 ± 0.2
10^{-7} M	1.8 ± 0.2	1.5 ± 0.2	1.9 ± 0.2	1.6 ± 0.1
10^{-6} M	2.3 ± 0.3	1.8 ± 0.1	2.2 ± 0.2	1.9 ± 0.1
(b) Endothelin-1				
10^{-9} M	0.1 ± 0.0	0.1 ± 0.1	0.0 ± 0.0	0.1 ± 0.0
10^{-8} M	0.8 ± 0.2	0.6 ± 0.1	1.0 ± 0.2	0.6 ± 0.1
10^{-7} M	2.5 ± 0.1	2.0 ± 0.2	2.7 ± 0.1	2.2 ± 0.2

bination with cyclosporin A, did not affect the response to potassium chloride (Fig. 2).

Phenylephrine and endothelin-1

Contractions to the α-adrenergic agonist phenylephrine (10^{-9}–10^{-6} M) were not significantly altered by any of the treatments (Table 1 a).

The contractile response to endothelin-1 (10^{-9}–10^{-7} M) did not significantly differ in the four groups (Table 1 b).

Blood pressure weight and laboratory tests

Mean arterial blood pressure measured intra-aortically under anesthesia was not different between the four groups (CO 81 ± 4 mm Hg, CyA 81 ± 4 mm Hg, DIL 80 ± 5 mm Hg, CyA/DIL 77 ± 5 mm Hg).

Cyclosporin A treatment resulted in comparable blood levels of 5313 ± 913 μg/l (CyA) and 5615 ± 888 μg/l (CyA/DIL) of cyclosporin A.

Plasma nitrogen urea was significantly elevated in the CyA and CyA/DIL-treated groups (CO 23 ± 1 mg/100 ml, CyA 38 ± 1* mg/100 ml, DIL 22 ± 1 mg/100 ml, CyA/DIL 40 ± 2* mg/100 ml; * $P < 0.05$). Plasma creatinine levels were comparable between the four groups.

Treatment with cyclosporin A caused significant increases in levels of gamma glutamyltransferase (CO 0.2 ± 0.1 U/l, CyA 3.0 ± 1.3* U/l, * $P < 0.05$), aspartate aminotransferase (CO 15 ± 1 U/l, CyA 80 ± 34* U/l, * P

< 0.05), alanine aminotransferase (CO 28 ± 1 U/l; CyA 100 ± 38* U/l, * $P < 0.05$), and bilirubin (CO 0.09 ± 0.01 mg/100 ml, CyA 0.48 ± 0.13* mg/100 ml, * $P < 0.05$).

Under cotreatment with diltiazem some animals showed more pronounced hyperbilirubinemia (CyA/DIL 1.18 ± 0.78 mg/100 ml) and increases in serum transaminases. Mean values, however, were not significant in the CyA-treated group (CyA/DIL aspartate aminotransferase 531 ± 428 U/l, alanine aminotransferase 425 ± 226 U/l).

In both groups that were treated with cyclosporin A, the weight gain during the treatment period was significantly reduced compared to the rats that did not receive cyclosporin A (CO $23 \pm 1\%$, CyA $-3 \pm 2\%$*, DIL $22 \pm 1\%$, CyA/DIL $0 \pm 1\%$*; * $P < 0.05$).

Discussion

This study demonstrates that cotreatment with the calcium antagonist diltiazem partially prevents the vascular smooth muscle dysfunction that occurs during chronic treatment with cyclosporin A.

The chronic treatment with cyclosporin A or diltiazem was withdrawn 24 h before the in vitro testing in order to avoid acute effects of the drugs. The effective washout of diltiazem was proven by the unaffected response to potassium chloride (as well as all of the other responses) in the group treated with diltiazem alone, since diltiazem remaining in the tissue would have reduced contractions to potassium chloride. Thus, the observed effect of cotreatment with diltiazem can most likely be assigned to the long-term effect of the drug.

One of the most striking effects of chronic exposure to cyclosporin A on vascular reactivity is the augmented responsiveness to vasoconstrictor peptides such as angiotensin II. The combination of cyclosporin A with diltiazem during chronic in vivo treatment effectively prevents the hyperreactivity of the tissue to angiotensin II. This protective effect indicates a role for chronic calcium influx through diltiazem-sensitive calcium channels in the pathogenesis of cyclosporin A-induced vascular smooth muscle dysfunction.

Angiotensin II mobilizes calcium from intracellular stores in vascular smooth muscle cells [6, 9, 25, 35]. In accordance with our observations, previous studies described an increased rise in intracellular calcium provoked by angiotensin II and vasopressin in cultured vascular smooth muscle or mesangial cells after exposure to cyclosporin A [18–20, 26, 27]. Different results were obtained with in vivo application of angiotensin II after 1 week of treatment with cyclosporin A [36]. The reason for this discrepancy might be the rather short-term treatment with cyclosporin in the latter experiments or the difference in the experimental conditions, since the response to norepinephrine was also reduced in the in vivo study.

The dysfunction of the vascular smooth muscle depends on the vasoconstrictor applied. In contrast to angiotensin II contraction responses to potassium chloride are significantly decreased after treatment with cyclosporin A. This difference may reflect the involvement of complementary second messenger pathways mediating these contractions. In the rat aorta the contraction response to potassium chloride is dependent on the influx of calcium from extracellular sources [10]. Diltiazem does not affect the reduced reactivity to potassium chloride, which suggests that additional effects of cyclosporin A on vascular smooth muscle function may occur that are not prevented by calcium entry blockade. The minor effects of cyclosporin A treatment on the responsiveness to α-adrenergic stimulation and endothelin-1 may reflect the involvement of both sources of calcium in the contractions to these drugs. An augmented response mediated by the mobilization of intracellular calcium may be masked by a reduced contraction in response to the influx of extracellular calcium.

The vascular alterations observed in this study mainly seem to be due to the direct effect of cyclosporin A on the vascular wall, since treatment with cyclosporin A did not cause a significant increase in blood pressure, which may affect the function of the vascular wall by itself [38]. A lack of increases in blood pressure during treatment with cyclosporin A has been observed in other studies employing normotensive rats as well, whereas in spontaneously hypertensive rats the development of hypertension is accelerated [12, 17, 23, 31, 36]. However, in the latter model it might be difficult to attribute the changes in vascular function to either the direct effect of the drug or the augmented blood pressure or genetic variations.

Chronic treatment with cyclosporin A in the concentration used causes toxic effects, as judged from the reduced weight gain and the impaired renal and liver function. These effects are similar to those observed in animal studies and during clinical use [17, 21, 31, 40]. Diltiazem alone does not cause any toxic effects at all. Cotreatment with diltiazem and cyclosporin A, however, affects serum transaminases, bilirubin, and alkaline phosphatase more than treatment with cyclosporin A alone, indicating increased liver toxicity of the combination. Although the toxic effects are, statistically, not significantly different between the cyclosporin A and the cotreatment group in the present study, the possible potentiation of liver toxicity has to be carefully monitored by further investigations into the combination of cyclosporin A with calcium antagonists. The effect cannot be explained by the pharmacokinetic interaction between cyclosporin A and diltiazem described in humans [4, 15, 28], since whole blood levels of cyclosporin A are comparable in these animals. Although in humans an increase in cyclosporin A levels during cotherapy with diltiazem due to competition in the metabolic pathway has been described [4, 15, 28], this was not observed in the rat model in the present study. The lack of this effect may be explained by species differences.

The present study demonstrates the protective effects of calcium antagonism in the model of cyclosporin A-induced vasculopathy in the rat. Recently, the prevention of coronary artery narrowing in heart transplant recipients by cotreatment with diltiazem has been reported [33]. Although the mechanisms involved in this particular form of coronary artery disease are extremely complex and not well understood, the effectiveness of a calcium antagonist and the results of our study encourage further evaluation of calcium-dependent mechanisms and potential vasoprotective effects of calcium antagonists in transplant patients.

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