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VASCULAR CHANGES IN CYCLOSPORINE A-INDUCED HYPERTENSION AND NEPHROTOXICITY IN SPONTANEOUSLY HYPERTENSIVE RATS ON HIGH-SODIUM DIET

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Functional and morphological changes of blood vessels in cyclosporine A (CsA)-induced hypertension and nephrotoxicity were studied in spontaneously hypertensive rats (SHR). The role of the L-arginine–nitric oxide (NO) pathway and the importance of oxidative stress in CsA toxicity were also assessed. SHR (7–8 week old) on a high-sodium diet were treated with CsA (5 mg kg⁻¹d⁻¹ s.c.) for 6 weeks. A proportion of the rats were treated concomitantly with the NO precursor L-arginine (1.7 g kg⁻¹d⁻¹ p.o.). CsA elevated blood pressure and caused renal dysfunction and morphological nephrotoxicity. CsA also impaired mesenteric and renal arterial function and caused structural damage to intrarenal and extrarenal small arteries and arterioles. Medial atrophy of the mesenteric resistance vessels and decreased viability of smooth muscle cells of the thoracic aorta were observed. Renal and arterial damage was associated with the presence of inflammatory cells. CsA did not affect markers of the L-arginine–NO pathway (urinary cyclic GMP excretion or endothelial or inducible NO synthase expression in kidney, aorta or heart) or oxidative stress (urinary excretion of 8-isoprostaglandin F_{2α}, plasma urate concentration or total radical trapping capacity). Concomitant L-arginine treatment did not affect CsA-induced changes in blood pressure or histological findings but tended to alleviate the arterial dysfunction. The renal and cardiovascular toxicity of CsA was associated with arterial dysfunction and morphological changes in small arteries and arterioles in SHR on a high-sodium diet. The findings did not support the role of oxidative stress or a defect in the L-arginine–NO pathway.

Key words: *cyclosporine A, sodium, nitric oxide, L-arginine, oxidative stress, blood vessels, hypertension, nephrotoxicity*

INTRODUCTION

Cyclosporine A (CsA) is an immunosuppressive drug widely used to prevent the rejection of transplanted organs and to treat autoimmune diseases. Unfortunately, CsA treatment is often limited by undesirable effects such as

hypertension and nephrotoxicity (for review see 1 and 2). A harmful interaction between CsA and high intake of dietary sodium has been described in spontaneously hypertensive rats (SHR) (3—4). CsA caused more severe hypertension and renal dysfunction in SHR on a high-sodium diet than in those with a moderate sodium intake (3).

Hypertension and nephrotoxicity by CsA have been related to arterial dysfunction seen as impaired endothelium-dependent relaxation in experimental animals (5—8). On the other hand, CsA has been shown to constrict renal arterioles *in vitro* (9—10). Furthermore, CsA given acutely (10) or repeatedly (11) sensitised renal arteries to contractile responses.

Endothelial dysfunction is widely thought to be attributed to a decreased production of endothelium-derived nitric oxide (NO), which may be due to an altered activity of NO synthase (NOS) or to a deficiency in the availability of L-arginine, the precursor of NO (12). Increased NO synthesis and endothelial NOS (eNOS) expression due to transcriptional induction of the eNOS gene have been reported in bovine endothelial cells exposed to CsA (13—14). CsA also caused eNOS overexpression in monocytes of renal transplant patients (15) and in rat kidneys (16—17). However, increased eNOS expression by CsA has not been confirmed in all *in vivo* studies (18).

Contrary to the effects of CsA on eNOS, CsA seems to inhibit inducible NOS (iNOS). CsA inhibited transcription of iNOS in murine macrophages and vascular smooth muscle cells (19) and in rat aortic smooth muscle cells *in vitro* (20). Depressed renal iNOS expression after CsA treatment has also been reported *in vivo* in rats (17—18).

CsA-induced endothelial dysfunction (21) and up-regulation of vascular eNOS (22) have been linked to increased synthesis of reactive oxygen species. Various antioxidants, such as vitamin E, ascorbate, lazaroids, and superoxide dismutase/catalase, have been reported to diminish CsA-induced renal toxicity (23—26). However, the role or the mechanisms of oxidative stress in CsA toxicity are not understood (for review see 27).

Short-term L-arginine administration has been shown to antagonize the CsA-induced increase in blood pressure in rats (8) but the long-term effects of L-arginine are unknown. Likewise, CsA-induced vascular endothelial dysfunction (5—8), deterioration of renal function (25, 28) and morphological renal damage (28—29) have also been alleviated by L-arginine.

The aim of the present study was to assess the effects of long-term treatment with CsA on the L-arginine-NO pathway, oxidative stress and the arteries in SHR during a high intake of sodium, an experimental model concomitantly showing both hypertension and the renal toxicity of CsA.

MATERIAL AND METHODS

Experimental protocol

Thirty-four 7–8 week-old male spontaneously hypertensive rats (SHR) (186–254g, Harlan Sprague Dawley, Indianapolis, IN, USA) were used. The protocol of the study was approved by the Animal Experimentation Committee of the Institute of Biomedicine, University of Helsinki, Finland.

All the rats received a high-sodium diet (Na 2.6%, Mg 0.2%, K 0.8%, Ca 1.0%, P 0.75% of the dry weight of the chow; R36, Finnewos Aqua, Helsinki, Finland). At the beginning of the study, SHR matched for blood pressure and body weight were divided into 4 different drug regimens for 6 weeks ($n = 8$ –9 per group): Control (vehicle s.c.); CsA (5 mg kg⁻¹d⁻¹ s.c.); L-arginine (1.7 mg kg⁻¹d⁻¹ in drinking water) and CsA + L-arginine. The rats had free access to tap water and chow and were weighed daily during the experiment. The dose of L-arginine was chosen on the basis of a previous study to antagonise the renal effects of CsA in normotensive rats (28). The present dose of CsA has been shown to produce plasma concentrations similar to those measured in CsA-treated patients (3).

Measurement of systolic blood pressure and heart rate

Systolic blood pressure and heart rate were measured each week using a tail cuff blood pressure analyser (Apollo—2AB Blood Pressure Analyser, Model 179—2AB, IITC Life Science, Woodland Hills, CA, USA).

Collection of samples

After 3 weeks the rats were housed individually in metabolic cages for a 24-hour period. They had free access to tap water and food. The consumption of food and water was measured by weighing the chow and the water bottles, respectively. Twenty-four-hour urine volumes were measured and the samples were stored at -80°C for the biochemical determinations. At the end of the study, the rats were kept in metabolic cages for 48 hours. During the first 24 hours, urine was collected into a vial in which 1 ml of 6 M HCl was added in order to preserve cyclic GMP in the sample. Papaverine (The University Pharmacy, Helsinki, Finland) was used to inhibit phosphodiesterases during storage.

After the 6-week experimental period, unconsciousness was induced in the animals with CO₂/O₂ (AGA, Riihimäki, Finland) prior to decapitation 24 hours after the last CsA administration. Blood samples for urate and total antioxidant capacity (total radical-trapping antioxidant parameter, TRAP) were taken into chilled tubes containing EDTA (4.5 mM). Samples for serum creatinine determination were taken into glass tubes without an anticoagulant. The heart was excised, large vessels, atria and the free wall of the right ventricle were dissected, and the left ventricular mass was weighed. The right kidney was washed with ice-cold saline and weighed. The left ventricle and kidney weights were expressed as a ratio relative to body weight.

Arterial responses

A 3-mm-long section of the mesenteric artery was excised 5 mm distally from the mesenteric artery-aorta junction. Likewise, a 3-mm-long section of the right renal artery was excised. The rings were placed between stainless steel hooks and mounted in an organ bath chamber in Krebs-Ringer

buffer (pH 7.4) of the following composition (mM): NaCl 119.0, NaHCO₃ 25.0, glucose 11.1, CaCl₂ 1.6, KCl 4.7, KH₂PO₄ 1.2, MgSO₄ 1.2 and aerated with 95% O₂ and 5% CO₂. The rings were equilibrated at 37 °C for 30 min with a resting tension of 0.2 g for renal and 1.0 g for mesenteric arteries. The force of contraction was measured with an isometric force displacement transducer and registered with a polygraph (FTO3 transducer, Model 7P122E Polygraph; Grass Instrument Co, Quincy, MA, USA).

Cumulative concentration-response curves were determined for noradrenaline (1 nM—1 µM, renal artery; 1 nM—10 µM, mesenteric artery) and potassium chloride (20 mM—125 mM). Cumulative relaxation responses to acetylcholine (1 nM—100 nM, renal artery; 1 nM—1 µM, mesenteric artery) and sodium nitroprusside (100 pM—10 µM, renal artery; 10 pM—10 µM, mesenteric artery) were examined after precontraction with noradrenaline (1 µM).

Histology

A section of the mesenteric arterial trunk with the first and the second order branches and the arterial bed together with a section of the renal artery and a cross-section of the left kidney were fixed in 10% formalin for 24 to 48 hours. The samples were dehydrated and embedded in paraffin by using the standard protocol. The samples were deparaffinised, hydrated and stained with Masson's trichrome or with hematoxylin and eosin.

In renal samples, interstitial, tubular and glomerular changes were investigated. In renal and extrarenal vessels, periadventitial, adventitial, medial and intimal changes were registered.

Vessels from the mesenteric bed were studied by morphometric analysis with an image operating system (AnalySIS, 3.0 Soft Imaging System GmbH, Munich, Germany). The corrected area of the media was determined from its cross-sectional area by applying a correction for eccentricity of the sections (30). The corrected medial area was expressed as a ratio to the mean diameter of the whole vessel to exclude the effect of vessel size on the measured area. The mean axis was determined as a mean of the longest and the shortest axis seen in the picture. On the basis of vascular diameter, the arteries were divided into 2 categories:

1. less than 100 µm in diameter, representing resistance arteries and arterioles
2. 100–400 µm in diameter, the first and the second order branch of artery from the superior mesenteric artery representing large muscular vessels.

The slides of vessels and kidneys were blindly investigated by the authors.

Hormonal and biochemical determinations

One total protein concentration was determined by the method of Lowry *et al.* (31). Urine creatinine excretion and serum creatinine levels were analysed with the Jaffe method (32) (BM/Hitachi 917 analyzer, Boehringer Mannheim, Germany/Hitachi Ltd, Tokyo, Japan). Creatinine clearance as an index of glomerular filtration rate was calculated from the serum concentration of creatinine and the 24-hour urinary excretion of creatinine. Urine sodium and potassium excretions were determined by flame photometer (IL model 943, Instrumentarium Laboratory, Milan, Italy). Cyclic GMP (33) and 8-isoprostaglandin F_{2α} were analysed by radioimmunoassay. Unlabelled 8-isoprostaglandin F_{2α} was obtained from Cayman Chemical Co (Ann Arbor, MI, USA). 8-[¹²⁵I] isoprostaglandin F_{2α}-tyrosine methyl ester and 8-isoprostaglandin F_{2α} antibody were kindly supplied by Dr. István Mucha, Institute of Isotopes Co, Budapest, Hungary. Urine excretion and creatinine clearance were expressed per 100 g of body weight.

Western blotting of eNOS and iNOS

Heart, aorta, renal medulla and cortex samples were homogenised in 5 volumes of boiling lysis buffer (1% sodium dodecyl sulfate (SDS), 1.0 mM Na₃VO₄, 10 mM Tris pH 7.4) (Sigma Chemical

Co, St. Louis, MO, USA) and microwaved for 10–15 sec. Homogenates were centrifuged at 12 000 rpm at 15°C for 5 min and the supernatant was saved. The protein content of supernatants was measured according to the method of Lowry *et al.* (31). Proteins (40–50 µg) were resolved by 8% SDS-polyacrylamide gel electrophoresis (PAGE) (Minigel apparatus, Bio-Rad Laboratories, Hercules, CA, USA) according to Laemmli *et al.* (34), whereafter the separated proteins were transferred to nitrocellulose membranes (Hybond-C Extra, Amersham Pharmacia Biotech, Uppsala, Sweden). Membranes were blocked for one hour at room temperature with 5% instant nonfat dry milk (Valio Ltd, Helsinki, Finland) in Tris-buffer pH 7.6 containing 0.1% Tween 20 TBS-T (Fluka, Buchs, Switzerland) and 137 mM NaCl. Membranes were incubated with the appropriate primary antibody mouse monoclonal anti-cNOS IgG₁ (1:2500, Transduction Laboratories, Lexington, KY, USA) or rabbit polyclonal anti-iNOS IgG (1:6000, Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C. Membranes were washed 4 times with TBS-T and incubated with horseradish peroxidase-coupled anti-mouse IgG₁ (1:1000, Zymed Laboratories, San Francisco, CA, USA) or anti-rabbit IgG (1:6000, Santa Cruz Biotechnology) for 1 hour. After washing 5 times with TBS-T, the bound antibodies were detected using an enhanced chemiluminescence (ECL) reagent (Amersham Pharmacia Biotech) and exposed to X-OMAT film (Kodak, Paris, France). Homogenised sample, iNOS electrophoresis standard (Cayman Chemical Co) and prestained molecular marker proteins (Bio-Rad Laboratories) were used as positive controls. Each band was quantified with computer programs (GeneSnap and GeneTools, Synoptics, Cambridge, UK). The intensities of sample bands were compared with the intensity of the positive control, which was given an arbitrary value 100.

TRAP samples were measured by a chemiluminescence method as described earlier (35) using 2,2-azo-bis(2-aminopropane)hydrochloride (ABAP; Polysciences, Warrington, PA, USA) as peroxyl radical producer. A water-soluble tocopherol, Trolox[®] (6-hydroxy-2,5,7,8-tetra-methylchroman-2-carboxylic acid; Hoffmann-La Roche Ltd, Basel, Switzerland) was used as a standard to calculate TRAP values of the unknown samples from the linear regression line for Trolox (36). Urate concentrations in plasma were measured with a colorimetric assay kit (Sigma Chemical Co).

Cell cultures

Smooth muscle cells were taken for cell culture from the aorta of the SHR on high-sodium diet who had received CsA or vehicle for 6 weeks ($n=4$ per group). The viability of the cells was tested in a culture of 7 days. The procedure for obtaining the vascular smooth muscle cells for culture was slightly modified from the explant technique (37). In brief, the thoracic aorta was removed and opened longitudinally. The endothelium was removed mechanically and the remaining tissue was washed with Dulbecco's Modified Eagle's Medium/Ham's F-12 (DMEM/F-12) (1:1; BioWhittaker, Walkersville, MD, USA) containing 300 U ml⁻¹ penicillin, 300 µg ml⁻¹ streptomycin and 3 µg ml⁻¹ amphotericin B. Next, the vessel was incubated in a collagenase solution (2 mg ml⁻¹ collagenase, 0.375 mg ml⁻¹ soybean trypsin inhibitor, 0.125 mg ml⁻¹ elastase, 2 mg ml⁻¹ bovine serum albumin and 15 mM HEPES (Sigma Chemical Co) in DMEM/F-12) for 15 min at 37°C before the adventitial layer was removed with forceps. The medial layer was minced (approx. 4–9 mm²) and plated in a petri dish. Until the first passage, the growth medium (DMEM/F12) contained 15% fetal calf serum, 100U/ml penicillin, 100 µg ml⁻¹ streptomycin and 1 µg ml⁻¹ amphotericin B, whereupon 10% fetal calf serum was used. For the experiment, cells from the third passage were used. The cells from the control and CsA group were plated in a concentration of 10⁶ per petri dish (100 mm) ($n=4$). After 7 days, the cells were detached from the dishes and counted. The number of dead, unattached cells was not counted.

Drugs

Cyclosporine A (Sandimmun[®] 50 mg ml⁻¹, infusion concentrate) was a generous gift from Novartis Ltd (Basel, Switzerland). Cyclosporine A was diluted in a lipid solution (Intralipid[®], Kabi Pharmacia, Stockholm, Sweden). L-arginine, acetylcholine chloride and noradrenaline bitartrate were obtained from Sigma Chemical Co and sodium nitroprusside dihydrate was from F. Hoffmann-La Roche AG (Basel, Switzerland). The compounds used to study the arterial responses were dissolved in water. All solutions were freshly prepared before use and protected from light.

Statistics

The results for cumulative arterial responses were analysed with two-way analysis of variance (ANOVA) with repeated measures for overall treatment effect. Other data were analysed by one-way ANOVA. Tukey's test was used for pairwise comparisons between the treatment groups. The group receiving L-arginine alone was compared separately with the control group to determine the effects of L-arginine. $P < 0.05$ was considered significant. The results are expressed as means \pm s.e. mean.

RESULTS

Body weight, food and water consumption, and urine volume

CsA alone or with L-arginine decreased the body weight gain by 12–15% during the 6 weeks of treatment ($P < 0.01$, vs control, *Table 1*). The intakes of food and water were equal in all experimental groups at 3 weeks and at the end of the experiment (data not shown). CsA alone or combined with L-arginine did not affect the urine volume at 3 weeks but increased it at the end of the study (*Table 1*). L-arginine alone had no effect on these variables.

Blood pressure and heart rate

CsA caused a marked rise in systolic blood pressure of about 30 mmHg after the first week of the experiment (*Fig. 1*). The hypertensive effect was further augmented towards the end of the experiment. At week 6, blood pressure was 48 mmHg higher in CsA group than in the control group ($P < 0.001$).

L-arginine tended to prevent CsA-induced hypertension from week 4 onwards but the difference was not significant ($P = 0.1$ at week 4, *Fig. 1*). L-arginine alone did not affect blood pressure.

Left ventricular hypertrophy

CsA increased the left ventricular mass expressed as a ratio to the body weight ($P < 0.05$, *Table 1*) and the effect was not antagonised by L-arginine. L-arginine alone did not influence the relative left ventricular mass.

Table 1. Effects of CsA and L-arginine on body weight gain, left ventricular weight, kidney weight and renal function ($n = 8-9$ per group.)

Variable	Control	CsA	CsA + L-arginine	P, ANOVA
Body weight, g				
Baseline	224 ± 5	223 ± 5	220 ± 6	0.868
At the end of the study	303 ± 4	256 ± 8 ###	266 ± 8 ###	<0.01
Left ventricle wet weight, g kg ⁻¹	3.31 ± 0.12	3.67 ± 0.09 #	3.50 ± 0.08	0.058
Right kidney wet weight, g kg ⁻¹	3.51 ± 0.15	3.67 ± 0.15	3.55 ± 0.0	0.666
Urine at week 3				
Volume, ml 100 g ⁻¹ d ⁻¹	25 ± 1	33 ± 4	28 ± 2	0.159
Creatinine, μmol 100 ⁻¹ d ⁻¹	38.2 ± 1.3	30.7 ± 1.7 ###	35.2 ± 1.1	<0.01
Proteins mg 100 g ⁻¹ d ⁻¹	11.6 ± 1.0	27.7 ± 7.6	14.9 ± 1.6	0.055
Urine at the end of the study				
Volume, ml 100 g ⁻¹ d ⁻¹	23 ± 2	37 ± 3 ###	34 ± 3 #	<0.01
Creatinine, μmol 100 g ⁻¹ d ⁻¹	36.4 ± 1.3	35.4 ± 0.9	33.2 ± 1.4	0.166
Proteins, mg 100 g ⁻¹ d ⁻¹	11.9 ± 1.4	43.8 ± 8.6 ###	25.0 ± 3.7	<0.01
Serum creatinine, mmol l ⁻¹	48.8 ± 1.5	58.7 ± 2.1 ###	56.4 ± 2.6	<0.05
Creatinine clearance, 100 g ⁻¹ d ⁻¹	0.52 ± 0.02	0.43 ± 0.01 ###	0.41 ± 0.03 ###	<0.01

CsA, cyclosporine A. Values are means ± s.e.mean. # $P < 0.05$, ## $P < 0.01$, vs control.

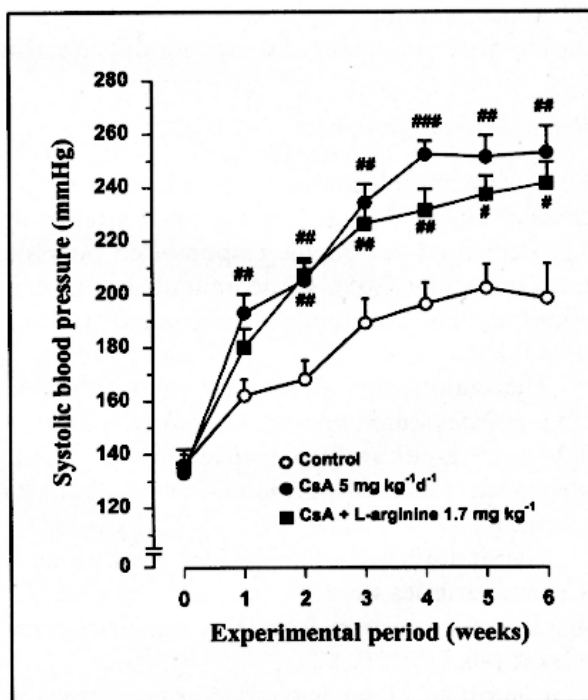


Fig. 1. Effect of cyclosporine A (CsA) and L-arginine on systolic blood pressure in SHR on a high-sodium diet. $N = 8-9$ per group. # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ vs control.

Renal function

There were no differences in the ratio of kidney wet weight to body weight between the treatments (*Table 1*). CsA decreased creatinine excretion at week 3 but not at the end of the study (*Table 1*). At the end of the study, CsA increased serum creatinine concentration and decreased creatinine clearance. The 24-hour urinary protein excretion was more than doubled by CsA at week 3 ($P=0.06$), and at the end of the study the protein excretion was about 4-fold compared to the control (*Table 1*).

L-arginine alone did not affect renal function, but it tended to antagonise the lowering effect of CsA on urinary creatinine excretion at week 3 ($P=0.07$) (*Table 1*). L-arginine also tended to prevent CsA-induced proteinuria at both observation points ($P=0.1$ and $P=0.07$, respectively). Concomitant L-arginine had no influence on serum creatinine concentration or on creatinine clearance at the end of the study (*Table 1*).

Urine electrolytes

Urine excretion of sodium was similar in all groups at weeks 3 and 6 (data not shown). Urine excretion of potassium was about 15% lower in the CsA group than in the control group at week 3 of the study ($P<0.01$). This difference was no longer seen at the end of the study. L-arginine alone or during CsA treatment did not modify electrolyte excretion.

Renal arterial responses

Contractile responses to noradrenaline ($P<0.05$, *Fig. 2*) and to potassium chloride ($P<0.01$, *Fig. 2*) in the renal arteries were impaired in the CsA group. The decreased contractile responses to potassium chloride were improved by L-arginine ($P<0.05$). Concomitant L-arginine also tended to improve the noradrenaline contractions compared to the group receiving CsA alone ($P=0.2$).

The endothelium-dependent relaxation responses to acetylcholine in the renal arteries were impaired by CsA ($P<0.001$, *Fig. 2*). Concomitant L-arginine did not significantly improve the CsA-induced decreased endothelium-dependent relaxation responses ($P=0.4$) but it tended to increase the maximal relaxation ($P=0.08$).

The endothelium-independent relaxations in response to nitroprusside in the renal arteries were also impaired by CsA ($P<0.001$, *Fig. 2*). L-arginine gave significant protection from this impairment of the endothelium-independent relaxations ($P<0.05$).

L-arginine alone had no effect on renal arterial functions.

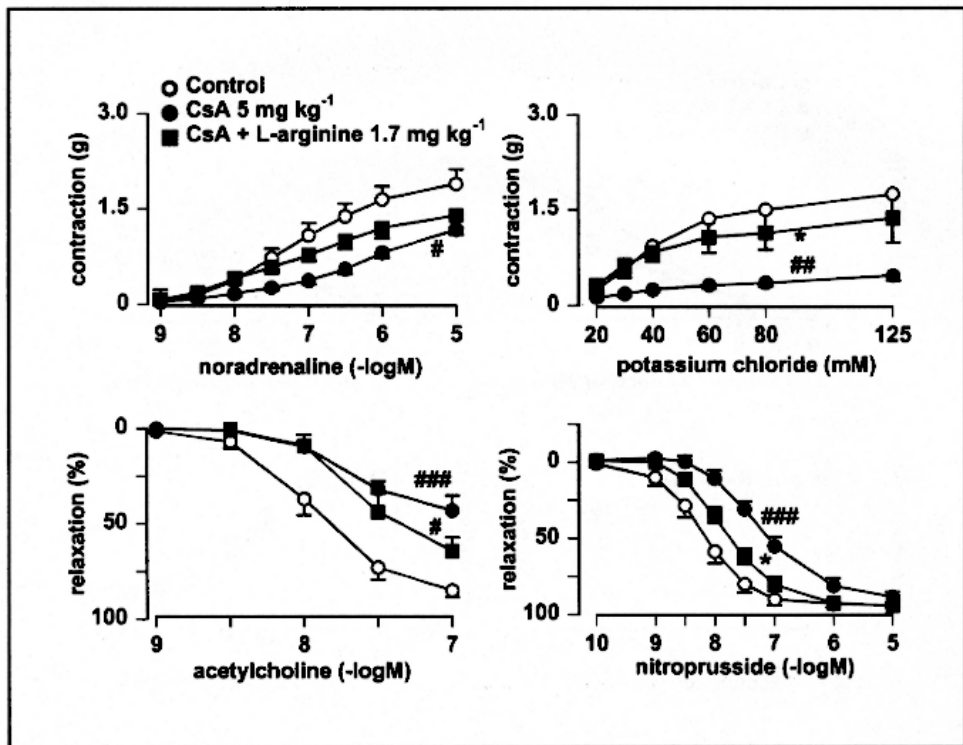


Fig. 2. Renal arteries: Contraction curves for noradrenaline and potassium chloride as well as relaxations responses to acetylcholine and sodium nitroprusside after noradrenaline-precontraction in renal arteries of SHR on a high-sodium diet after the 6 weeks treatment. CsA, cyclosporine A. $N=8-9$ per group. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ vs control. * $P<0.05$ vs CsA.

Mesenteric arterial responses

CsA tended to impair the noradrenaline-induced contractions of the mesenteric arteries ($P=0.2$, Fig. 3) and clearly impaired the potassium chloride-induced contractions ($P<0.01$, Fig. 3). Concomitant L-arginine did not affect the mesenteric arterial contractions compared to the CsA group.

Neither CsA nor L-arginine modified the acetylcholine- or sodium nitroprusside-induced relaxations of the mesenteric arteries (Fig. 3). L-arginine alone did not change mesenteric arterial functions.

Renal histology

In the control group, no glomerular, tubular or interstitial damage was detected. Integrity of intrarenal arteries was mostly preserved.

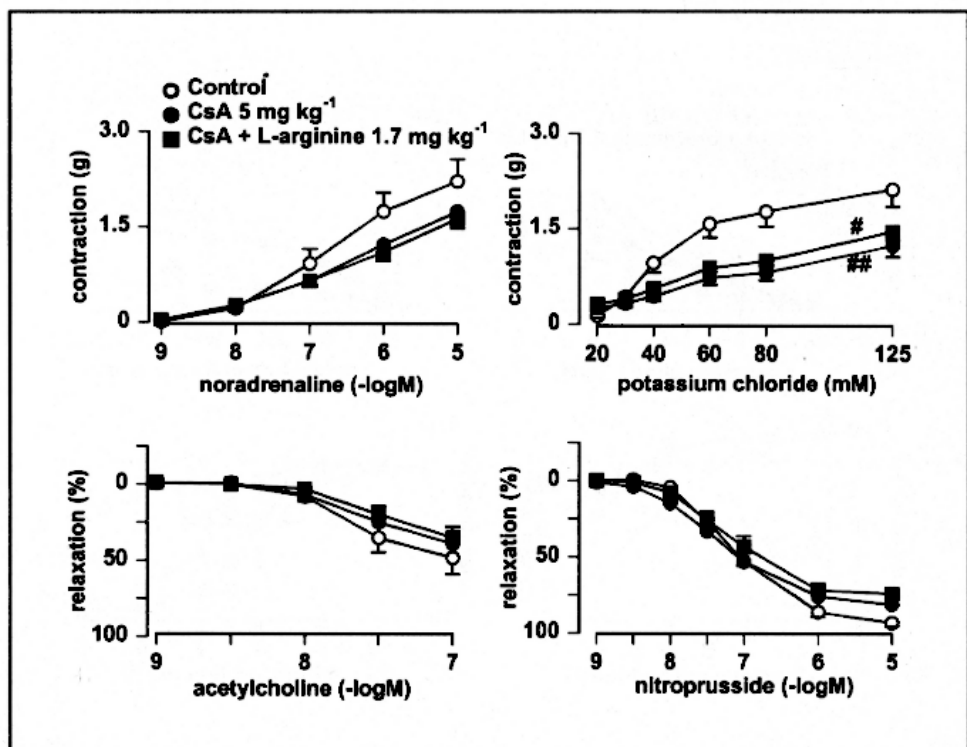


Fig. 3. Mesenteric arteries: Contraction curves to noradrenaline and potassium chloride as well as relaxations responses to acetylcholine and sodium nitroprusside after noradrenaline-precontraction in mesenteric arteries of SHR on a high-sodium diet after the 6 weeks treatment. CsA, cyclosporine A. $N = 8-9$ per group. # $P < 0.05$, ## $P < 0.01$ vs control.

In the CsA group, several glomeruli were damaged and the pathological changes varied from a slight mesangial matrix expansion to a severe necrosis and capillary collapse. Interstitial fibrosis of a striped pattern was seen in the cortex. Tubular atrophy existed in the tubuli surrounded by interstitial fibrotic tissue. Interstitial and perivascular migration of leukocytes was detected. Large intrarenal arteries were mostly normal; only a slight thickening of the adventitia was seen. The smaller intrarenal arterioles had signs of medial changes ranging from minor smooth muscle cell vacuolisation to massive smooth muscle and endothelial cell proliferation, leading to complete occlusion of the vessel (Fig. 4). There was also an increase in the amount of connective tissue surrounding the arterioles.

The changes seen in CsA group were also detected in the rats concomitantly receiving L-arginine. L-arginine alone did not affect renal histology.

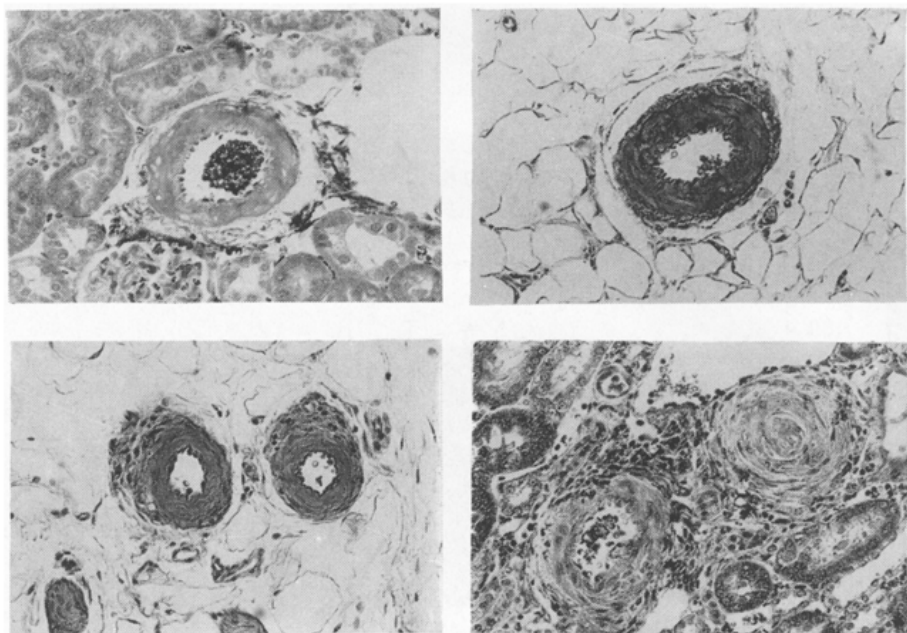


Fig. 4. Small arteries of the kidney (Panel a and b) and of the mesenteric bed (Panel c and d) from the SHR (Masson's trichrome staining). The control group has close to a normal histology (Panel a and c). CsA caused adventitial, medial and intimal changes that are more pronounced in intrarenal vessels (Panel b and d). (Original magnification $\times 250$).

Histology of extrarenal vessels

The structure of the mesenteric arterial trunk, the first and second order branches and smaller arteries and arterioles of the mesenteric arterial bed as well as the renal artery was mostly preserved in the control group.

CsA caused adventitial and perivascular fibrosis and inflammation in small arteries as well as in arterioles of the mesenteric bed, whereas medium-sized arteries were only slightly affected. The larger arteries (mesenteric and renal artery) seemed to be unaffected by CsA. The medial as well as intimal layers remained mostly normal in CsA treated rats. However, there was focal medial hypertrophy in some small arteries and arterioles leading even to occlusion of the vessel (*Fig. 5*).

L-arginine did not give protection from CsA-induced morphological damage to small arteries. L-arginine alone did not affect arterial morphology.

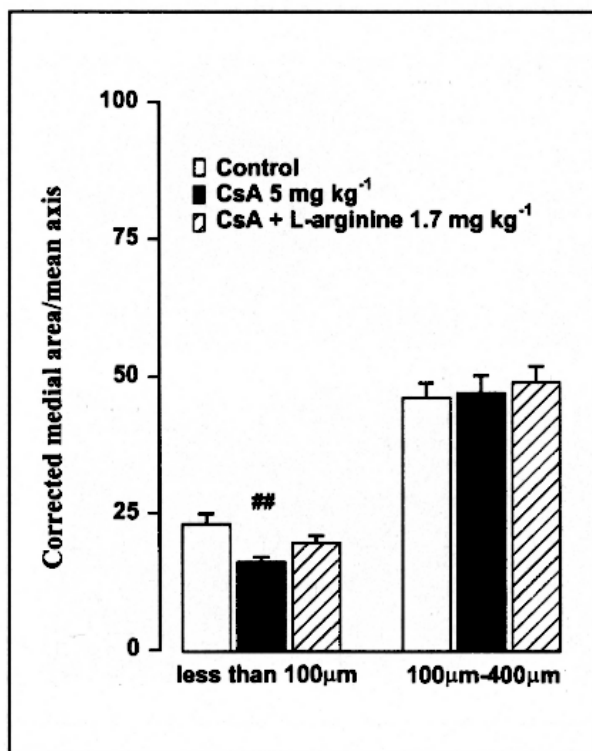


Fig. 5. The corrected medial area relation to the vascular diameter of arteries and arterioles in the mesenteric vascular bed according to their vascular diameter. CsA, cyclosporine A. $N = 24-68$ per group.

Morphometric analysis of vessels from mesenteric bed

CsA reduced the corrected area of the medial layer and its ratio to the mean diameter of the vessel in under 100 μm category by about 30% (Fig. 5). CsA did not affect the medial layer or the medial layer ratio to the diameter of the larger vessels.

Concomitant L-arginine approximately halved the CsA-induced decrease in medial hypotrophy in small vessels (Fig. 4, $P = 0.2$ vs. both Control and CsA group). L-arginine alone had no effect on morphometric values.

NOS expression and urinary cyclic GMP

CsA alone or combined with L-arginine did not affect eNOS expression in the renal cortex, medulla, aorta or heart (Table 2). Expression of iNOS was not detected in any these organs in any of the study groups. L-arginine alone did not affect eNOS or iNOS expression.

Urinary cyclic GMP was regarded as an indicator of the activity of the L-arginine-NO-pathway. CsA or L-arginine alone or combined with CsA did not affect cyclic GMP excretion (Table 2).

Table 2. Effects of CsA and L-arginine on oxidative stress and on the L-arginine-nitric oxide pathway (n=8–9 per group).

Variable	Control	CsA	CsA + L-arginine	P, ANOVA
Urine excretion of 8-isoprostaglandin $F_{2\alpha}$, $\mu\text{mol } 100\text{ g}^{-1} \text{ d}^{-1}$				
At week 3	19.4 ± 2.3	25.4 ± 5.3	27.0 ± 5.3	0.501
At the end of the study	17.3 ± 1.4	15.5 ± 2.1	23.9 ± 4.6	0.127
Cyclic GMP, $\text{fmol } 100\text{ g}^{-1} \text{ d}^{-1}$	9.25 ± 0.97	8.44 ± 8.63	8.63 ± 0.98	0.861
Total radical trapping capacity, μM	629 ± 44	663 ± 39	685 ± 37	0.628
Urate, μM	120 ± 18	120 ± 13	129 ± 17	0.910
Endothelial NOS				
renal cortex	206 ± 10	242 ± 24	233 ± 22	0.438
renal medulla	703 ± 171	716 ± 157	829 ± 170	0.844
aorta	152 ± 14	133 ± 13	108 ± 4	0.067
heart	442 ± 45	488 ± 24	479 ± 75	0.812

CsA, cyclosporine A; NOS, nitric oxide synthase. Values are means ± s.e.mean.

TRAP, serum urate concentration and urinary isoprostaglandin $F_{2\alpha}$ excretion

Neither CsA nor L-arginine affected urinary isoprostaglandin $F_{2\alpha}$ excretion, TRAP or serum urate concentration used as indicators of oxidative stress. (Table 2).

Cell cultures

The viability of the aortic smooth muscle cells was preserved in the control group. After 7 days of culture, the aortic cells from the control group had proliferated to an average number of $4.1 \times 10^6 \pm 0.1 \times 10^6$ per dish. By contrast, the 6-week *in vivo* CsA administration caused a clear decrease in the viability of the smooth muscle cells. After 7 days of culture, only 1 dish out of 4 had a noticeable number (0.6×10^6) of living cells.

DISCUSSION

CsA-induced hypertension and nephrotoxicity were studied in sodium-loaded SHR during the developmental phase of hypertension. The animal model of SHR on a high-sodium diet was chosen on the basis of our previous experience so as to mimic the CsA-induced hypertension and nephrotoxicity seen in humans (3–4). We studied the function and

morphology of arteries of different size and location in renal and cardiovascular systems. The involvement of the L-arginine-NO pathway as well as oxidative stress in CsA toxicity were especially assessed.

CsA impaired renal arterial function in SHR. The dysfunction included both relaxation and contractile responses. The impaired endothelium-dependent responses are in accordance with earlier findings (5-8). Concomitant morphological changes in vascular endothelial cells have been reported (6), but in the present study we were unable to detect any light microscopic alterations in endothelium of renal and mesenteric arteries. However, endothelial proliferation was seen in small intrarenal arteries and arterioles, which indicates that small arteries are more prone to the toxic effects of CsA than larger ones. The endothelial dysfunction and damage may be due to the direct toxicity of CsA found *in vitro* (38). Elevation of blood pressure by CsA *per se* might at least partly participate in endothelial dysfunction and damage (39).

CsA also decreased the endothelium-independent relaxation and contractile responses in renal arteries and, to a lesser extent, in mesenteric arteries. CsA has been reported to impair endothelium-independent relaxations in some (5, 6), but not in all studies (8). When added to the incubation cuvette, CsA induces contraction of renal arterioles *in vitro* (9, 10). Acute (10) and, contrary to our findings, repeated administration of CsA (11) has also been shown to sensitise renal arteries to contractile responses. Histologically, renal and mesenteric arteries of similar sizes that were used in our functional assay did not show any structural changes that could explain the arterial dysfunction described above.

However, a decrease in viability of the cultured aortic smooth muscle cells was apparent in the CsA-treated rats. Long-term CsA treatment affected both the ability of a cell to attach to its growth plate and the probability of proliferation. Despite the nutrient-rich and CsA-free environment of the cell cultures, the toxic effect of CsA seemed to be too deleterious to the survival of the vascular smooth muscle cells. CsA caused morphological damage to the intrarenal small arteries and arterioles and, to a lesser extent, to the mesenteric resistance vessels, where the damage was more focal. Hypertension is usually associated with hypertrophy of resistance vessels (30). In our study, CsA-induced elevation of blood pressure was, however, related to hypotrophy or atrophy of the resistance vessels. The damage was apparently inflammation related with interstitial and perivascular migration of leukocytes. Taken together, these results suggest that the impairment of the smooth muscle cell-dependent nitroprusside relaxation and the contractile responses by CsA in the larger arteries were due to toxic effects on smooth muscle cell structure even though the effect was not detected by light microscopy.

The influence of oxidative stress was investigated to explain CsA-induced hypertension as well as renal and vascular toxicity. TRAP and plasma urate concentration, a major contributor to TRAP, assessed the antioxidant status of the animals (36). Urinary excretion of 8-isoprostaglandin $F_{2\alpha}$ was used as a marker of the generation of oxidative products by CsA (26). In some studies, CsA toxicity has been shown to be at least partly mediated by oxidative stress (23–26). Potential involvement of oxidative stress in our hypertensive animal model with long-term CsA administration, however, seems unlikely, because CsA did not affect urinary excretion of 8-isoprostaglandin $F_{2\alpha}$, plasma urate concentration or TRAP.

The effects of CsA on L-arginine–NO pathway were evaluated. CsA had no effect on urinary cyclic GMP excretion or on eNOS and iNOS expression in the kidney, heart and aorta. L-arginine tended to reduce CsA toxicity somewhat but failed to reverse CsA-induced hypertension, renal and vascular dysfunction, or morphological renal and vascular damage. A defect in the L-arginine–NO pathway caused by CsA has been suggested, because L-arginine has been shown to reduce the renal and cardiovascular effects of CsA in different animal models (5–8, 28). However, in some trials CsA did not affect the L-arginine–NO pathway, but L-arginine reduced the vasoconstrictive effects of CsA, suggesting that CsA toxicity is not mediated by NO deficiency, although NO may attenuate it (40–41). The NO system is up-regulated in young SHR before and after the onset of hypertension (42). This might partly explain the absence of an effect of CsA on the L-arginine–NO pathway and the relatively slight beneficial effects of L-arginine on CsA toxicity in the present study. The absence of effects of L-arginine alone on any parameters further supports this possibility. The present results suggest that even though CsA causes endothelial dysfunction that is related to endothelial damage, a defect in the L-arginine–NO pathway is not the principal mediator of CsA toxicity in this hypertensive rat model.

As in the present study of SHR on a high-sodium diet, CsA induced interstitial fibrosis and arteriopathy in normotensive rats during sodium-deprivation that was related with the presence of inflammatory cells (43). The common feature of these two models of CsA toxicity is the activation of the renin angiotensin system and that antagonism of the renin angiotensin system gives protection against CsA-induced renal damage (4, 43). Therefore, it seems that CsA toxicity is at least partly secondary to activation of the renin angiotensin system. Very recently, however, a different view of the interaction of CsA and the renin angiotensin system has been presented (44). CsA antagonised the harmful effects of overexpression of the renin angiotensin system on end-organ damage in double transgenic rats harbouring human renin and angiotensinogen genes. The protection was regarded to be due to anti-inflammatory properties of CsA. Thus, the interaction of CsA and the

renin angiotensin system is complex. CsA can not only cause renal and cardiovascular toxicity via induction of the renin angiotensin system, but in some experimental models it can also antagonise inflammatory responses to various stimuli, including angiotensin II.

In conclusion, the renal and cardiovascular toxicity of CsA in SHR on a high-sodium diet is associated with arterial dysfunction and morphological changes in small arteries and arterioles. The study does not support the role of oxidative stress or a defect in the L-arginine-NO pathway as a principal mechanism in the harmful renal and cardiovascular effects of CsA.

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