Effects of L-arginine on cyclosporin-induced alterations of vascular NO/cGMP generation

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Abstract

Background. Cyclosporin (CsA)-induced vascular dysfunction has been attributed to a diminished role of the nitric oxide (NO)/cGMP-mediated vasodilator mechanism. The present study was aimed at investigating whether L-arginine, the substrate of NO synthesis, ameliorates CsA-induced vascular dysfunction.

Methods. Male Sprague–Dawley rats were used throughout the study. The thoracic aorta was isolated from normal rats and acutely treated with CsA (10⁻⁴ mol/l, 60 min) in vitro, or the aorta was taken from rats treated with CsA (25 mg/kg/day, i.m., 1 week). The vascular relaxation response to acetylcholine, and tissue levels of NO metabolites and cGMP were determined. The vascular expression of NO synthase (NOS) isoforms was also determined by western blot analysis.

Results. Acute treatment with CsA in vitro markedly attenuated the vasorelaxation response to acetylcholine, which was completely restored by L-arginine. The vascular accumulation of NO metabolites in response to acetylcholine was decreased significantly by CsA, which was prevented by cotreatment with L-arginine. CsA decreased the cGMP accumulation in response to both acetylcholine and sodium nitroprusside. L-Arginine restored, although not completely, acetylcholine-stimulated cGMP generation, whereas it did not affect sodium nitroprusside-stimulated cGMP generation. Following chronic CsA treatment in the whole animal, the vasorelaxation response to acetylcholine was decreased significantly along with tissue levels of NO metabolites; this was preserved by L-arginine-supplementation. Vascular expression of iNOS protein was decreased by CsA treatment along with decreased tissue accumulation of NO metabolites. L-Arginine supplementation did not modify the altered expression of NOS proteins.

Conclusion. These results suggest that CsA causes an L-arginine-sensitive vascular dysfunction which is associated with impaired generation of NO and cGMP.

Key words: cGMP; cyclosporin; L-arginine; nitric oxide

Introduction

Cyclosporin (CsA) has been known to cause a number of side-effects that primarily affect the blood vessels [1,2]. CsA-induced vascular dysfunction has been attributed to an attenuated role for the nitric oxide (NO)/cGMP-dependent vasodilator mechanism [3–5]. Furthermore, L-arginine among others, has been found to reverse CsA-induced vascular dysfunction [6]. However, the precise point of derangement due to CsA among steps ranging from the availability of L-arginine, substrate to NO synthase (NOS), to the generation of NO and cGMP has not been elucidated. Nor have the mechanisms underlying the use of L-arginine been established at the cellular level.

The present study was aimed at investigating the potential point of derangement in the vascular NO/cGMP pathway and examining the cellular effect of L-arginine on the vascular dysfunction due to CsA.

Materials and methods

Cyclosporin treatment

Male Sprague–Dawley rats (200–250 g) were used throughout the study. All procedures were carried out in accordance with Institutional Guidelines for Laboratory Animal Care and Use. In the first series of experiments, the thoracic aorta was isolated from normal rats and acutely exposed in vitro to CsA (Sandimmun, Sandoz; 10⁻⁴ mol/l, 60 min) either alone or in combination with L-arginine (10⁻³ mol/l). In the second set of experiments, two groups of rats were treated with CsA (25 mg/kg/day, i.m.) for 1 week. One group received a supplement of L-arginine in the drinking water (4 g/l) whereas the other did not. A control group of rats was treated with diluent (olive oil) only during the same period.

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Recording isometric tension

The thoracic aorta was isolated and cut into 5-mm rings. The rings were suspended in a tissue bath containing physiological salt solution (PSS) at 37°C, while being continuously bubbled with 95% O₂-5% CO₂ (pH 7.4). The baseline load placed on the rings was 2.0 g, and the changes in isometric tension were recorded using a force-displacement transducer (Grass FT03). The composition (in mmol/l) of PSS used was NaCl 112, KCl 5, NaHCO₃ 25, KH₂PO₄ 1.0, MgSO₄ 1.2, CaCl₂ 2.5 and glucose 11.5. The aortic rings were precontracted with EC80 phenylephrine (3.5 × 10⁻⁶ mol/l), and the relaxation response to acetylcholine was calculated as a percentage of the phenylephrine-induced maximum contraction.

Colourimetric assay of nitrite/nitrate

In the acute experiment, NO production in the thoracic aorta was stimulated with acetylcholine (10⁻⁵ mol/l) for 10 min, after which the reaction was stopped by a quick freeze with liquid nitrogen. In the chronically CsA-treated rats, trunk blood and thoracic aorta were taken to determine the plasma and vascular tissue levels of NO metabolites.

The nitrite/nitrate assay was performed with a colorimetric NO assay kit (Oxford; Oxford, MI, USA). For the spectrophotometric assay of nitrates with Griess reagent, 80 μl MOPS (50 mmol/l) EDTA (1 mmol/l) buffer and 5 μl tissue samples were added to the wells in duplicate. Nitrate reductase (0.01 U) and 10 μl NADH (2 mmol/l) were added to the reaction mixture, and the plate was shaken for 20 min at room temperature. Colour reagents, sulfanilamide and N-(1-naphthyl) ethylenediamine dihydrochloride were added, and the absorbance was read at 540 nm in a microtitre plate reader (Bio-Rad, Model 3550; Hercules, CA, USA). The protein concentration was determined using the method described by Bradford [7] with bovine serum albumin (BSA) as standard.

cGMP measurements

Aortic rings prepared from normal rats were incubated in 15 ml beakers at 37°C, while saturated with mixed gas of 95% O₂-5% CO₂ (pH 7.4). An equilibration period of 1–2 h was allowed to elapse. The aortic rings were then treated with L-arginine (10⁻⁷ mol/l), CsA (10⁻⁴ mol/l), or both for 60 min, along with phenylephrine (3.5 × 10⁻⁶ mol/l) and 3-isobutyl-1-methylxanthine (10⁻⁵ mol/l), the phosphodiesterase inhibitor. They were then treated with acetylcholine (10⁻⁵ mol/l) or sodium nitroprusside (3.5 × 10⁻⁷ mol/l) for 10 min, which was terminated by a quick freeze with liquid nitrogen.

The tissue homogenate made in 10% trichloroacetic acid was centrifuged at 2500 g for 30 min at 4°C. The supernatant was extracted four times with 3 ml of water-saturated ether, and the extract was acetylated and assayed for cGMP using a radioimmunoassay kit (Amersham; Buckinghamshire, UK). The pellet was used for protein assay.

Protein preparation and western blot analysis

The aorta was homogenized with Polytron homogenizer in 3000 r.p.m. in a solution containing 250 mmol/l sucrose, 1 mmol/l EDTA, 0.1 mmol/l phenylmethylsulfonyl fluoride and 20 mmol/l potassium phosphate buffer, at pH 7.6. Large tissue debris and nuclear fragments were removed by two low-speed spins in succession (3000 × g; 5 min; 10000 × g, 10 min). The total protein solution was centrifuged at 100 000 × g for 1 h. The supernatant was used for blotting of iNOS, and the pellet was resuspended for blotting of eNOS.

Western blotting was performed according to the method of Mattson and Higgins [8], with slight modification. Protein samples (100 μg) were size-separated electrophoretically using a discontinuous system consisting of a 6% polyacrylamide resolving gel and 5% polyacrylamide stacking gel, and transferred to a nitrocellulose membrane at 20 V and 100 mA overnight. The membranes were incubated with a 1:2500 dilution of monoclonal mouse anti-NOS antibodies (Transduction Laboratories; Lexington, KY, USA), and a horseradish-peroxidase-labelled goat anti-mouse IgG (1:1000). The bound secondary antibody was detected by enhanced chemiluminescence (Amersham). The relative protein levels were determined by analysing the signals of autoradiograms using the transmitter scanning video densitometer.

Statistical analysis

Results are expressed as means ± SEM. The statistical significance of differences between the groups was determined using ANOVA or unpaired t-test.

Results

Treatment with CsA in vitro significantly decreased the vasorelaxation response to acetylcholine, which was completely restored by L-arginine (Figure 1). The acetylcholine-stimulated accumulation of NO metabolites was decreased significantly by CsA, which was prevented by cotreatment with L-arginine (Figure 2). Figure 3 shows the amount of cGMP accumulated in responses to acetylcholine and sodium nitroprusside. Acetylcholine-stimulated cGMP generation was decreased significantly by CsA-treatment, which was restored, although not completely, by L-arginine. Sodium nitroprusside-stimulated cGMP formation was also decreased significantly by CsA, but was not, however, significantly affected by L-arginine.

Following chronic treatment with CsA in the whole
Fig. 2. Effects of CsA on the vascular tissue NO metabolites. The aortic rings were stimulated with acetylcholine (10^{-5} mol/l) in the presence of CsA alone [CsA] or combined with L-arginine [CsA/L-Arg]. The control group [Control] was stimulated with acetylcholine but without CsA. The number of rats in each group is 6. *P<0.01, compared with control; #P<0.05, compared with CsA.

Fig. 3. Effects of CsA on the vascular cGMP formation. The aortic rings were stimulated with acetylcholine (ACh, 10^{-5} mol/l) or sodium nitroprusside (SNP, 10^{-7} mol/l) in the presence of CsA alone [CsA] or combined with L-arginine [CsA/L-Arg]. The control group [Control] was without CsA. The number of experiments in each column is between 5 and 8. *P<0.05, **P<0.01; compared with control. #P<0.05, compared with CsA.

Fig. 5. Tissue contents of NO metabolites in the thoracic aorta. Treatment as in Figure 4. The number of rats in each group is 7. *P<0.05, compared with control. #P<0.05, compared with CsA.

Animal systolic blood pressure, measured by tail-cuff methods without anaesthesia, did not differ significantly among the groups: 115±4 mmHg in CsA-treated, 118±7 mmHg in the CsA-treated plus L-arginine supplement and 110±4 mmHg in the control (n=5 each). The degree of acetylcholine-induced vasorelaxation was reduced by CsA treatment, which was prevented in part by L-arginine supplementation (Figure 4). The plasma concentrations of NO metabolites were not significantly altered by CsA-treatment (control: 100±15, CsA: 103±23, CsA/L-arginine: 112±16 mmol/l, n=6 each). In contrast, the vascular tissue content of NO metabolites was decreased significantly by CsA-treatment, which was restored by L-arginine supplementation (Figure 5). Figure 6 shows the representative autoradiograms and densitometric data for vascular NOS proteins. Following CsA treatment, the expression of eNOS was not affected, whereas that of iNOS was decreased significantly. L-Arginine supplementation did not have a significant effect on the altered NOS expression.

Discussion

The CsA-induced impairment of endothelium-dependent vasorelaxation has been attributed to a reduced capacity to generate NO [9–11]. The present study also showed that either acute in vitro treatment or chronic administration of CsA in the whole animal impaired the endothelium-dependent vasorelaxation,
Arginine restores the cyclosporin-induced impairment of vascular NO/cGMP generation

Vaziri et al. [11] observed no changes in ecNOS protein abundance in the thoracic aorta following a 3-week treatment with CsA. Amore et al. [14] also reported unaltered de novo synthesis of the NO protein following a chronic administration of CsA. The ecNOS protein mass may not correlate with its enzymatic activity. The diminished NO formation may be related to an interference of calcium-dependent activation of ecNOS by CsA [15].

CsA also significantly attenuated the stimulated formation of vascular cGMP, in response to sodium nitroprusside as well as to acetylcholine. It is likely that the step(s) beyond NO formation are also affected by CsA treatment. Guanylate cyclase activity per se may be inhibited by CsA, resulting in a failure to adequately stimulate cGMP formation in response to NO. Taken together, the mechanisms by which CsA impairs the vascular function should be multifactorial, including decreases in NOS and guanylate cyclase activities.

Interventions have been developed to reverse the CsA-induced vasculopathies. Among them has been the administration of L-arginine, substrate to NOS, which is known to reverse vascular dysfunction due to CsA [6]. In the present study, we also observed that L-arginine reversed the impairment of endothelium-dependent vasorelaxation. Furthermore, L-arginine was able to preserve, at least in part, the vascular capacity to generate NO and cGMP. Therefore, the restoration of normal vasorelaxation by L-arginine may be attributed to increases in L-arginine uptake and subsequent enhancement of the NO–cGMP cascade. It has been suggested that high-affinity L-arginine transport is mediated via a Na\(^+\)-independent system \(B^0\), and a Na\(^+\)-dependent system \(B^\text{int}\) in the pulmonary artery endothelial cells [16]. Further studies will be necessary to determine whether this transport system works in the same manner in the thoracic aorta.

It may be of interest to point out that the attenuated vasorelaxation response to acetylcholine in the presence of CsA was completely restored by L-arginine, despite the incomplete recovery of cGMP response. This finding suggests that the vasorelaxation event is not a direct function of the tissue cGMP levels. Only a certain level of cGMP may be sufficient to elicit a full relaxation. In contrast, L-arginine supplementation could not completely preserve the vasorelaxation response to acetylcholine following the chronic CsA treatment in the whole animal. This finding is in accord with a partial restoration of the CsA-induced impairment of acetylcholine-induced vasorelaxation by L-arginine in different vascular species observed by previous investigators [17,18]. The discrepancy in the effects of L-arginine may be accounted for by the duration or dosage of the treatment. It is also possible that other factors, including increased production of superoxide radicals [19], are also involved in the in vivo study.

In summary, the present study suggests that L-arginine restores the impaired vascular capacity to generate NO and cGMP.
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References


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