Role of Nitric Oxide in Cyclosporine A–Induced Hypertension

Gibson K. Oriji, Harry R. Keiser

Abstract—Cyclosporine A (CsA) is an immunosuppressive agent that also causes hypertension. The effect of CsA on vascular responses was determined in Sprague-Dawley rats and isolated rat aortic rings. Male rats weighing 250 to 300 g were given either CsA (25 mg · kg⁻¹ · d⁻¹) in olive oil or vehicle by intraperitoneal injection for 7 days. CsA administration produced a 42% increase (P < 0.001) in mean arterial pressure (MAP) that reached a plateau after 3 days. Conversely, the levels of both nitrate/nitrite, metabolites of nitric oxide (NO), and cGMP, which mediates NO action, decreased by 50% (P < 0.001) and 35% (P < 0.001), respectively, in the urine. Thoracic aortic rings from rats treated with CsA and precontracted with endothelin (10⁻⁹ mol/L) showed a 35% increase (P < 0.001) in tension, whereas endothelium-dependent relaxation induced by acetylcholine (ACh, 10⁻⁹ mol/L) was inhibited 65% (P < 0.001) compared with that in untreated rats. This response was similar to that of endothelium-denuded aortic rings from untreated rats in which ACh-induced relaxation was completely abolished (P < 0.001), but relaxation induced by S-nitroso-N-acetylpenicillamine (SNAP, 10⁻⁸ mol/L) was unaffected (P < 0.001). ACh-induced formation of both nitrate/nitrite and cGMP by both denuded and CsA-treated aortic rings was inhibited 95% (P < 0.001) and 65% (P < 0.001), respectively, compared with intact aortic rings. The effects of CsA were reversed both in vivo and in vitro by pretreatment with l-arginine (10 mg · kg⁻¹ · d⁻¹ IP), the precursor of NO. There were no changes in MAP and tension in rats treated with l-arginine alone. In summary, CsA inhibits endothelial NO activity, with resulting increases in MAP and tension, and this inhibition can be overcome by parenteral administration of l-arginine. (Hypertension. 1998;32:849-855.)

Key Words: cyclosporine • arginine • endothelin • acetylcholine • nitrates • rats • aortic rings

Cyclosporine A (CsA) is a potent immunosuppressive agent that is associated with the development of arterial hypertension. Numerous reports have indicated that support many possible causes for the hypertension, but none have really clarified the mechanism. As a result, effective treatment of CsA-induced hypertension remains empirical.

Therefore, this study aimed to answer the following questions: (1) What vasoactive system abnormalities, if any, accompany CsA-induced hypertension in the whole animal? (2) Are similar abnormalities found in isolated aortic rings from CsA-treated animals? (3) Does direct measurement of the vasoactive substance(s) produced by the rings provide evidence of the abnormality? (4) Can CsA toxicity be overcome by administration of an agent that should remedy the abnormality? By performing complementary studies in the whole animal, isolated aortic rings, and the fluid that bathed those rings, we found that CsA toxicity is associated with an inhibition of nitric oxide (NO) activity that can be overcome by pretreatment with l-arginine (L-Arg).

Methods

Animals
Male Sprague-Dawley rats weighing 250 to 300 g were housed in individual metabolic cages with free access to water and rat chow (batch 5001 from Purina Mills Inc).

Effects of CsA, L-Arg, Bosentan, and BQ-123 on Blood Pressure
After 3 days of acclimatization, the rats were divided randomly into 8 groups and treated with (1) CsA (25 mg/kg) in 1 mL of olive oil; (2) olive oil (1 mL); (3) L-Arg (10 mg/kg); (4) bosentan (25 mg/kg), an endothelin (ET)α receptor antagonist; (5) BQ-123 (0.1 mg/kg), an ETα receptor antagonist; (6) bosentan (25 mg/kg) plus CsA (25 mg/kg); (7) BQ-123 (0.1 mg/kg) plus CsA (25 mg/kg); or (8) L-Arg (10 mg/kg) plus CsA (25 mg/kg); each drug was given via a separate intraperitoneal injection daily for 7 days (n = 6 for each group). In a preliminary study, we found that daily injections of CsA (25 mg/kg) produced hypertension that was only partially prevented by injections of either BQ-123 (0.1 mg/kg) or bosentan (25 mg/kg), whereas doses of 50 mg/kg bosentan caused progressive toxicity with severe anorexia, weight loss, and death (Figure 2). Not all groups of rats were studied at the same time. However, an appropriate control group was always studied simultaneously with every treated group.

Measurement of Blood Pressure
Mean arterial pressure (MAP) was measured at the same time each day with the tail-cuff method using a model 229 Blood Pressure Amplifier/Pump from IITC Inc.

Collection of Urine
Urine was collected into 0.2 mL of 6N-HCl for the 24-hour period immediately after the last treatment and stored at −70°C until assay for NO, cGMP, and prostacyclin (PGI₂) or their metabolites.

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Preparation of Aortic Rings
On day 7 of treatment, rats were anesthetized by injection of sodium pentobarbital (40 mg/kg IP), and the thoracic aorta was removed and prepared as previously described.4 The aortic rings were stimulated every 20 minutes with KCl (50 mmol/L) until reproducible contractions were obtained. All subsequent contractile responses in each vessel were expressed as a percentage of the last contractile response to KCl, and all data are expressed as mean±SEM.

In Vivo Effects of CsA on Tension
Aortic rings were prepared from intact rings from animals given either CsA (n=4) or vehicle (n=6) and from either intact (n=6) or denuded (n=4) rings from untreated rats. Later, rings were prepared from rats given either CsA plus L-Arg or L-Arg alone (n=6 for each). The doses of drugs or vehicle were the same as those listed above. ET (10⁻⁹ mol/L) was added, and the rings were allowed to contract for 120 minutes without washing out of the agonist. Another set of rings was prepared as above, either acetylcholine (ACh; 10⁻⁹ mol/L) or S-nitroso-N-acetylpenicillamine (SNAP; 10⁻⁸ mol/L) was added 30 minutes after the ET (10⁻⁹ mol/L), and the reaction was observed for 90 more minutes without washing out of either agonist.

In Vitro Effects of CsA on Tension
Aortic rings from a group of untreated control rats were used to study the in vitro effects of CsA on tension and the production of nitrate/nitrite (NO₂⁻/NO₃⁻), cGMP, and 6 keto-PGF₁α. After a 30-minute equilibration period, CsA (10⁻⁹ mol/L) was added to the bath, and the aortic ring was allowed to contract for 120 minutes. Bath fluid was harvested 90 minutes after the addition of CsA and stored at −70°C until assay for NO, cGMP, and PGI₂ or their metabolites.

Measurement of NO, cGMP, and PGI₂
NO was quantified by measurement of its metabolites nitrate/nitrite using Greiss reagent with sodium nitrite as a standard.5 cGMP was assayed via a radioimmunounassay kit (NEX 133, Du Pont). PGI₂ was assayed via a radioimmunounassay kit for its metabolite 6 keto-PGF₁α (Kit 515, Amersham).

Statistical Analysis
All data were generated with paired controls. Values are expressed as mean±SEM. Two-way ANOVA was used for comparisons within experiments. A value of P<0.05 was considered significant.

Drugs and Chemicals
CsA (Sandimmune) was a gift from Sandoz Inc (East Hanover, NJ), and bosentan was a gift from Hoffmann La Roche (Basel, Switzerland). Endothelin, BQ-123, ACh, SNAP, and L-Arg were all purchased from Calbiochem Inc.

Results
Effects of Daily Intraperitoneal Injection of CsA for 7 Days on MAP
In CsA-treated rats, MAP began to increase on day 1 and increased rapidly through day 3, after which it reached a plateau from which it often increased further on day 7. Basal MAP averaged 120±4 mm Hg. By day 3, MAP had increased to 160±3 mm Hg in CsA-treated rats compared with 126±3 mm Hg in untreated rats (P<0.001) (Figure 1). On day 3, MAP in CsA-treated rats was 42% higher than in untreated rats.

Effects of Daily Intraperitoneal Injection of BQ-123 or Bosentan on CsA-Induced Hypertension
In preliminary experiments, we sought to determine whether ET was the cause of CsA-induced hypertension. Despite the fact that either 0.1 mg/kg BQ-123 or 25 mg/kg bosentan prevented the hypertension produced by daily injection of 100 ng/kg of ET, those same doses of either BQ-123 or bosentan failed to prevent CsA-induced hypertension (Figure 2).
Effects of ET on Either Denuded or Intact Aortic Rings and on Aortic Rings of Rats Treated With Either Olive Oil or CsA

To explore the cause of this hypertension, we performed experiments with aortic rings. The maximum tension developed in response to ET increased rapidly to a plateau by 30 minutes, remained at that level through 70 minutes, and then declined slowly. Maximum tension in rings from CsA-treated rats (206 ± 7%) was not significantly different from that in rings denuded of endothelium from untreated rats (212 ± 6%). These values were on average 35 ± 5% (P < 0.001) higher than those in intact rings from either untreated rats (160 ± 6%) or vehicle-treated rats (160 ± 6%) (Figure 3).

Effects of ACh or SNAP on ET-Induced Contractions in Either Denuded or Intact Aortic Rings and on Aortic Rings of Rats Treated With Either Olive Oil or CsA

Thirty minutes after administration of ET, when the ET-induced contraction was maximal, addition of either ACh or SNAP produced a rapid 99.9% (P < 0.001) decrease in contractile tension in both intact and vehicle-treated aortic rings, but ACh produced only a minimal effect in either denuded or CsA-treated aortic rings; the effect of SNAP was similar to that seen in intact aortic rings (Figure 4). ACh-induced formation of both nitrate/nitrite and cGMP in both denuded and CsA-treated aortic rings was inhibited compared with that in intact aortic rings (data not shown).

Acute Effects of Either CsA on Tension, Level of Nitrate/Nitrite, cGMP, and 6-Keto PGF_1α in the Organ Bath

When CsA was added acutely to intact aortic rings from untreated rats, tension increased from 0% to 105 ± 1% of KCl tension (P < 0.01). Concomitantly, nitrate/nitrite production decreased 83 ± 4% (23 ± 1 versus 3.8 ± 0.1 nmol/L per milliliter), cGMP production decreased 90 ± 1% (12.1 ± 1.2 versus 0.0 pmol/L per milliliter), and PGI_2 production increased 40-fold (299 ± 11 versus 3878 ± 65 pg/mL; P < 0.01 for each) (Figure 5).

Because all our data suggested that CsA was inhibiting endothelial NO production, we sought to determine whether added L-Arg could mitigate these effects. In a preliminary experiment, we found that once-daily injections of either 2.5 or 5 mg/kg L-Arg had no effect on CsA-induced hypertension, whereas 10 mg/kg prevented the hypertension and 25 mg/kg reduced blood pressure below the baseline level (data not shown).

Daily injections of L-Arg (10 mg/kg) completely prevented CsA-induced hypertension, whereas daily injections of L-Arg alone had no effect on MAP (Figure 1).

Effects of ET on Aortic Rings of Rats Treated With L-Arg and CsA

Daily administration of L-Arg to rats abolished the CsA-induced increases in maximum tension of aortic rings to ET and normalized the response of aortic rings from rats given either L-Arg or vehicle alone (Figure 6).

Effects of ACh or SNAP on ET-Induced Contractions in Aortic Rings of Rats Treated With L-Arg and CsA

Daily administration of L-Arg to rats restored ACh-induced relaxation in aortic rings from rats treated with CsA to that of rings from rats treated with either L-Arg or vehicle alone (Figure 7). SNAP-induced relaxation in aortic rings from rats was unaffected by any of the treatments (Figure 7).

Effects of Daily Intraperitoneal Injection (7 Days) of CsA, L-Arg, or L-Arg + CsA on Levels of Nitrate/Nitrite and cGMP in Urine

Daily administration of L-Arg to rats prevented CsA-induced decreases of both nitrate/nitrite (Figure 8A) and cGMP.
Discussion

There are several important findings of this study: (1) CsA administration to rats increased MAP and concomitantly decreased levels of both nitrate/nitrite and cGMP in urine. (2) ET antagonists were only partially able to block CsA-induced hypertension. (3) ET-induced tension was increased in aortic rings from CsA-treated rats compared with controls and was the same as that of normal rings denuded of endothelium. (4) Aortic rings from CsA-treated rats did not relax in response to ACh. (5) Aortic rings from CsA-treated rats relaxed normally in response to SNAP. (6) All the effects of CsA, both in vivo and in vitro, were reversed by daily intraperitoneal injections of L-Arg.

The finding that CsA administration causes hypertension in both humans and laboratory animals is well established. A number of possible explanations for the hypertension have been proposed, but none has been definitive. Over the years, data have been presented suggesting that CsA causes time- and dose-dependent endothelial cell injury in vitro, that CsA augments the in vitro contractile response to nerve stimulation, and that chronic CsA affects both endothelium-dependent vasodilation and vascular smooth muscle contraction depending on the stimulus applied.

More recently, the possibility that ET is involved in CsA-induced nephrotoxicity and hypertension has gained interest because of the following observations: (1) CsA-induced glomerular hypoperfusion is associated with a remarkable increase in urinary ET and in some cases in plasma ET; (2) CsA stimulates ET production by several types of renal and nonrenal cells; and (3) CsA-related glomerular hypoperfusion/filtration and exaggerated cellular proliferation can be ameliorated by agents that block ET actions, such as anti-ET antibodies or selective ET antagonists. However, in our own studies in rats, we were unable to prevent CsA-induced hypertension by administration of ET antagonists (ie, either BQ-123 or bosentan).

Our finding that CsA-induced hypertension is accompanied by decreases in urinary NO and cGMP suggested that a vasodilator pathway was suppressed, most likely NO. It is very unlikely that the major effector of

Figure 5. Acute effects in aortic rings of either CsA (10^{-9} mol/L) or olive oil on tension (a) or the level of nitrate/nitrite (b), cGMP (c), or 6-keto PGF_{1α} (d) in the organ bath. See Methods for details. *P<0.01 vs control for a, b, c, and d.

Figure 6. Effects of ET (10^{-9} mol/L) on aortic rings of rats treated with either CsA, L-Arg, L-Arg+CsA, or olive oil by intraperitoneal injection daily for 7 days. See Methods for details. *P<0.001 vs control.

Figure 7. Effects of ACh (10^{-9} mol/L; A) or SNAP (10^{-8} mol/L; B) on ET-induced contractions of aortic rings of rat treated with either olive oil, CsA, L-Arg, or L-Arg+CsA by intraperitoneal injection daily for 7 days. See Methods for details. *P<0.001 vs control.
CsA-induced hypertension is a product of cyclooxygenase, i.e., either the vasodilator PG12 or a vasoconstrictor such as PGF2α, because indomethacin did not alter the level of CsA-induced hypertension in preliminary experiments (data not shown). Acute exposure of aortic rings to CsA, increased PG12 release (Figure 5), and L-Arg reversed the CsA-suppressed endothelium-dependent relaxation of ACh, but pretreatment with indomethacin did not reverse the suppressed ACh endothelium-dependent relaxation in aortic rings treated with CsA (data not shown). This observation is similar to the findings of Kim et al.15 L-Arg completely corrected CsA-induced hypertension, therefore suggesting that perturbations in a pathway other than NO were the principal cause of the hypertension.

We chose ET as our model vasoconstrictor because of its prolonged duration of action. We found that CsA-treated aortic rings developed greater tension in response to ET than untreated rings. This effect is not specific to the agonist ET because angiotensin II also produced greater increases in tension in aortic rings from CsA-treated rats than it did in rings from controls (data not shown). Similar findings have been reported by a number of authors using rat thoracic aorta or tail artery and various agonists.15–18 We findings have been reported by a number of authors using ET because angiotensin II also produced greater increases in tension in aortic rings from CsA-treated rats than untreated rings. This effect is not specific to the agonist ET because angiotensin II also produced greater increases in tension in aortic rings from CsA-treated rats than it did in rings from controls (data not shown). 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cGMP were nearly abolished. CsA did not suppress endothelium-independent relaxation. This implied that CsA does not seriously damage the endothelium or block the production of all vasoactive substances; rather, it appears to interfere specifically with the NO pathway.

There are at least 3 possible explanations for the reduced vasodilator response to ACh in CsA-treated rats: (1) an abnormality of ACh receptor/signal transduction, (2) an abnormality in NO formation, or (3) NO degradation. The first hypothesis seems improbable since administration of L-Arg restored both ACh-induced vasodilation and the activity of NO. The second and third hypotheses that CsA may induce an abnormality in NO formation or degradation, respectively, seem much more likely. Such an abnormality or degradation could occur at any one of the steps in the pathway. At present, it is difficult to assess directly many of these steps in vivo. Therefore, since NO can be synthesized from L-Arg by endothelial cells in culture, we used L-Arg, a substrate for NO, in this study.

L-Arg administration completely prevented the hypertension in CsA-treated rats and normalized the urinary excretion of both NO2/NO3 and cGMP. L-Arg completely restored the excessive tension in response to ET that occurred in CsA-treated rats and it restored completely the vasodilatory response to ACh. Both Gallego et al., who used rat femoral artery strips, and Kim et al., who used rat thoracic aortic rings, found only partial restoration of ACh-induced vasodilation after preincubation of the aortic rings of CsA-treated rats with L-Arg. Both Gallego et al and Kim et al incubated the aortic rings with L-Arg and did not study the effects of L-Arg on MAP, whereas we administered L-Arg by intraperitoneal injection in the whole animal and studied changes in both MAP and tension. The reasons for the differences between their results and ours are unclear, but they may relate to differences in dosage or experimental design. In any case, we found that all the effects of CsA were reversed completely, both in vivo and in vitro, by treatment with L-Arg.

The finding that L-Arg administration can correct the hypertension and vascular toxicity of CsA administration suggests possible therapy for patients who must take CsA as an immunosuppressive agent. It also suggests further experiments to define the precise mechanism by which CsA impairs the NO pathway.

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