Cyclosporine Produces Endothelial Dysfunction by Increased Production of Superoxide

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Abstract Vasoconstriction and hypertension are major side effects of cyclosporine therapy. The mechanism or mechanisms responsible for the vascular effects of cyclosporine are unclear. The vascular effects of cyclosporine may arise as a consequence of endothelial dysfunction induced by the agent. To test this possibility, we compared in vessels prepared in myographs endothelium-mediated relaxations of mesenteric resistance arteries of Wistar-Kyoto rats treated for 21 to 28 days with subcutaneous injections of cyclosporine (25 mg/kg per day) or vehicle. Endothelium-dependent relaxations in response to acetylcholine were impaired in arteries from cyclosporine-treated rats; the concentrations of acetylcholine required to produce 50% relaxation of norepinephrine activation (pD2) were 31.6±0.1 versus 5±0.1 nmol/L in control arteries (P<.05). Nitro-L-arginine produced comparable 10-fold decreases in sensitivity to acetylcholine in arteries from both rat groups, indicating that the relaxations were mediated by endothelium-derived nitric oxide. Acetylcholine-induced relaxations in cyclosporine-treated arteries were normalized by pretreatment of the arteries with superoxide dismutase (150 IU/mL; pD2, 3.6±0.1; P<.05); superoxide dismutase had no effect on relaxations in control arteries. SQ 29,548, an inhibitor of prostaglandin H2/thromboxane A2 receptors; H-7, an inhibitor of protein kinase C; and indomethacin did not alter relaxations in response to acetylcholine in either group of arteries. Cyclosporine-treated arteries were more sensitive than control arteries to nitroprusside, an agent that induces relaxation via nitric oxide (pD2, 1.3 and 6.2 μmol/L, respectively; P<.05). Thus, cyclosporine impairs relaxations mediated by endothelium-derived nitric oxide in mesenteric arteries via enhanced production of free radicals that inactivate endothelium-derived nitric oxide. (Hypertension. 1994;23[part 2]:957-961.)

Key Words • nitric oxide • cyclosporine • endothelium • superoxide • vascular resistance

Hypertension and nephrotoxicity are major side effects of cyclosporine-induced vasoconstriction. Vasoconstriction induced by cyclosporine is both acute in onset and persistent during chronic administration of the agent.1 Administration of a single dose of cyclosporine to healthy individuals produces an abrupt 20% to 30% decrease in renal blood flow and glomerular filtration rate over the course of 2 hours.1 Similarly, renal blood flow and glomerular filtration rate increase after cyclosporine is stopped in subjects who have received the drug for more than a year.2 The mechanism or mechanisms responsible for cyclosporine-induced vasoconstriction remain poorly understood. Three mechanisms have received the most attention: (1) an increase in intracellular calcium with enhancement of vasoconstrictor responses,4,5 (2) activation of the sympathetic nervous system,6 and (3) cyclosporine-induced endothelial dysfunction.7,8 Studies described in this report will focus on the effects of cyclosporine on endothelial function. Endothelial cells modulate vascular smooth muscle tone in large part by regulated production of endothelium-derived nitric oxide (EDNO) and to a lesser extent by vasodilating prostaglandins.10-12 EDNO modulates vascular smooth muscle tone by regulating the production of cyclic GMP (cGMP).11 Cyclosporine-induced endothelial dysfunction could result from decreased production of EDNO, from increased destruction of EDNO, or from increased production of endothelium-derived constricting factors, which oppose the vascular effects of EDNO. The goals of the present studies were to test the hypothesis that the vascular effects of cyclosporine are a consequence of endothelial dysfunction induced by cyclosporine and to elucidate the mechanism or mechanisms responsible for cyclosporine-induced endothelial dysfunction. EDNO-mediated responses were compared in mesenteric resistance arteries of rats treated with cyclosporine or vehicle. We performed additional experiments to examine the potential role of increased production of prostaglandin endoperoxides and of free radicals in the genesis of cyclosporine-induced endothelial dysfunction.

Methods

Male Wistar-Kyoto rats weighing 230 to 250 g (Harlan Laboratories) were randomized to receive daily subcutaneous injections of cyclosporine vehicle (Cremophor, Sigma Chemical Co) or cyclosporine (Sandimmune I.V., Sandoz Pharmaceuticals Co) at a dose of 25 mg/kg for 21 to 28 days. All procedures were in accordance with institutional guidelines. At the time of death, blood was obtained for measurements of serum creatinine, whole-blood cyclosporine, and hematocrit. Cyclosporine measurements were performed by the Nuclear Medicine Laboratory in the Department of Radiology using a radioimmunoassay with a monoclonal antibody.

Segments of mesenteric artery arcade with and without endothelium were prepared in three myographs (Living Sys-
tem) for measurement of isometric force as previously described. After 30 minutes of equilibration in Krebs-Ringer solution, optimum wall tension was applied, and vessel diameter was determined as previously described. Removal of the endothelium was confirmed by the absence of relaxation of activated arteries in response to acetylcholine (10⁻⁶ mol/L). Endothelium-mediated relaxations were characterized by the responses of norepinephrine-activated arteries (ED₅₀ dose) to acetylcholine (10⁻⁹ to 10⁻⁴ mol/L). The effects of several inhibitors on acetylcholine-induced relaxations were tested by comparing paired responses of arteries with and without inhibitor; inhibitors were added 20 minutes before activation of the artery. Inhibitors tested included (1) indomethacin (5 μmol/L), an inhibitor of the cyclooxygenase pathway; (2) SQ 29,548 (5 μmol/L), a competitive inhibitor of prostaglandin H₂/thromboxane A₂ receptors; (3) H-7 [1-(5-isouquinolinesulfonyl)-2-methylpiperazine, 5 μmol/L], an inhibitor of protein kinase C (PKC); (4) superoxide dismutase (SOD, 150 IU/mL), a scavenger of superoxide anion; and (5) N⁵-nitro-L-arginine (L-NA, 0.1 mmol/L), an inhibitor of nitric oxide production. Acetylcholine-mediated responses (with or without inhibitors) were derived from a single exposure of the study artery to the muscarinic agonist.

In an additional experiment 16 rats injected with cyclosporine were randomized so that 8 rats received 2.5 g L-arginine in their drinking water for the duration of the study.

**Drugs**

Acetylcholine hydrochloride, sodium nitroprusside, L-norepinephrine, bovine SOD (3200 U/mg protein), 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate (CHAPS), L-NA, L-arginine hydrochloride, H-7, and verapamil were obtained from Sigma Chemical Co. SQ 29,548 was a gift from ER Squibb & Sons. Drug concentrations are expressed as moles per liter.

**Calculations and Statistics**

Individual data points, ED₅₀ (expressed as negative logarithm of the concentration of agonist required to produce 50% relaxation, pD₂), the area under the concentration-response curve, and the maximal relaxations of concentration-response curves were used from comparative analyses. Results are presented as mean±SEM; n refers to the number of rats from which vessels were studied. Statistical evaluation of the effects of inhibitors on acetylcholine responses was performed with Student's t test for paired observations. Data comparing responses in arteries from vehicle versus cyclosporine-treated rats were analyzed with one-way ANOVA; significant differences were analyzed with Student's t test for unpaired observations. Means were considered significantly different when the two-tailed probability was <.05.

**Results**

Cyclosporine-treated rats failed to gain weight as rapidly as control rats (mean weights, 272±12 versus 322±16 g for control rats; n=32, P<.05). Mean serum creatinine was 1.45±0.31 vs 1.21±0.24 mg/dL in control rats, P<.05. The mean blood cyclosporine value for treated rats was 3720±226 ng/mL. Vessel dimensions were not different in arteries from control and cyclosporine-treated rats; calculated lumen diameters were 212±12 and 192±14 μm for control and treated rats.

**Endothelium-Dependent Relaxations**

Relaxations in response to acetylcholine were impaired in mesenteric arteries from cyclosporine-treated rats (Fig 1); the pD₂ values were 31.6±0.1 and 5±0.1 nmol/L in cyclosporine-treated and control arteries, respectively (P<.05). The relaxations in response to acetylcholine were abolished by prior removal of the endothelium in arteries from both rat groups (relaxations <10% with 1 μmol/L acetylcholine in both groups, n=4). Preincubation of the arteries with L-NA increased the pD₂ from 5 to 50 nmol/L in control arteries (n=12, P<.05) and from 31 to 200 nmol/L in arteries from cyclosporine-treated rats (n=12, P<.05), indicating that the relaxations induced by acetylcholine were at least in part mediated by nitric oxide in both groups.

Relaxations noted in arteries from cyclosporine-treated rats in response to acetylcholine were normalized to those observed in control arteries after preincubation of the arteries with SOD (Fig 2). The pD₂ value decreased from 31±0.1 to 36±0.2 nmol/L in arteries

**Figures**

**Fig 1**. Line graph shows acetylcholine-induced relaxations of mesenteric resistance arteries from rats injected with cyclosporine (CSA; 25 mg/kg per day for 21 to 28 days) or vehicle (Control) expressed as percentage of baseline contraction with norepinephrine (NE). Relaxations in response to acetylcholine were significantly impaired in arteries from CSA-treated rats. Data points represent mean±SEM. *Significantly different from control arteries, P<.05.

**Fig 2**. Line graph shows effects of inhibitors on acetylcholine-induced relaxations of mesenteric arteries from cyclosporine (CSA)-treated rats. Arteries from CSA-treated rats were preincubated with SOD (5 μmol/L, n=8), a competitive inhibitor of prostaglandin H₂/thromboxane A₂ receptor, or H-7 (5 μmol/L, n=8), an inhibitor of protein kinase C. Arteries from CSA-treated rats with no inhibitor added served as controls for each inhibitor; in the figure, all untreated arteries are pooled to present a single control group. SOD normalized relaxations in response to acetylcholine in arteries from CSA-treated rats. Effects of each inhibitor were analyzed by comparing data points for arteries with and without inhibitor using Student's t test for paired analyses; *P<.05. NE indicates norepinephrine.
pretreated with SOD from cyclosporine-treated rats (n=12, P<.05); the pD2 value for acetylcholine in arteries from control rats was not affected by pretreatment with SOD (4.8 versus 5 nmol/L; n=12). The addition of L-NA (0.1 mmol/L) with SOD during preincubation abolished relaxations in response to acetylcholine in arteries from cyclosporine-treated rats (pD2, >100 μmol/L; n=4; P<.05 versus untreated arteries).

SQ 29,548 failed to improve acetylcholine relaxations in arteries from cyclosporine-treated rats (Fig 2) and had no effect on acetylcholine relaxations in control arteries (pD2, 5.6±0.2 nmol/L; n=6). Similarly, H-7 had no effect on acetylcholine relaxations in arteries from either control or cyclosporine-treated rats (pD2, 5.2±0.1 and 29±0.4 nmol/L, respectively; n=8 rats per group). Arginine supplementation had no effect on acetylcholine relaxations in arteries from cyclosporine-treated rats (pD2, 32±0.4 nmol/L; n=8).

**Endothelium-Independent Responses**

Arteries from cyclosporine-treated rats were slightly more sensitive than control arteries in their relaxations in response to sodium nitroprusside; pD2 values were 1.3±0.1 and 6.2±0.2 μmol/L for cyclosporine-treated and control arteries, respectively (n=12; P<.05). Relaxations in response to nitroprusside were markedly increased and identical in arteries from both rat groups after removal of the endothelium; the pD2 values decreased to 5.1 and 5.2 nmol/L for cyclosporine-treated and control arteries without endothelium, respectively. Relaxations in response to the calcium channel antagonist verapamil were similar in arteries from control and cyclosporine-treated rats (pD2, 2.2±0.1 and 2±0.1 μmol/L, respectively). Arteries from control and cyclosporine-treated rats were equally sensitive to norepinephrine (pD2, 4.6±0.1 and 5.7±0.1 μmol/L, respectively; n=32; P>.05).

**Discussion**

The present study demonstrates that relaxations in response to acetylcholine are impaired in mesenteric resistance arteries from rats treated with cyclosporine. Relaxations in response to acetylcholine were endothelium dependent; prior removal of the endothelium abolished responses to the muscarinic agonist. Pretreatment of arteries from both control and cyclosporine-treated rats with L-NA, an inhibitor of nitric oxide synthase, produced a 10-fold decrease in sensitivity to acetylcholine in both control and cyclosporine-treated vessels. These findings indicate that cyclosporine treatment leads to inhibition of relaxations mediated by EDNO.

Impairment in EDNO-mediated relaxations by cyclosporine therapy could arise from several mechanisms, including (1) decreased production or release of EDNO, (2) increased destruction of EDNO, (3) decreased responsiveness of vascular smooth muscle to EDNO, (4) increased production of an endothelium-derived contracting factor or factors, which oppose the vasorelaxant properties of EDNO, or (5) a combination of these mechanisms. Additional experiments were performed to explore the potential mechanism or mechanisms responsible for the development of cyclosporine-induced endothelial dysfunction. The main finding from these additional studies is the observation that pretreatment with SOD, a scavenger of superoxide anion, normalized EDNO-mediated relaxations in arteries from cyclosporine-treated rats; SOD had virtually no effect on acetylcholine responses in arteries from control rats. Although not conclusive, these findings are consistent with increased production of superoxide in cyclosporine-treated arteries. The SOD-induced normalization of relaxations in response to acetylcholine in arteries from cyclosporine-treated rats was abolished by the addition of L-NA along with SOD. This finding indicates that the enhanced relaxations effected by SOD are mediated by l-arginine-derived nitric oxide. These experiments with SOD provide important findings relative to the mechanism or mechanisms by which cyclosporine induces endothelial dysfunction: (1) Arteries from rats treated with cyclosporine can produce at least normal amounts of EDNO, (2) free radical (superoxide) production is increased in arteries from cyclosporine-treated rats less likely the result of a nonspecific, toxic effect of high levels of cyclosporine achieved in the rats. Other investigators have noted that rats are considerably less sensitive than humans for both immunosuppressive and toxic effects of cyclosporine.14 If arteries from cyclosporine-treated rats in fact produce appropriate amounts of EDNO, why the impaired relaxations in response to acetylcholine? The present findings provide evidence that the increased production of superoxide anion (or related radical) inactivates EDNO in arteries from cyclosporine-treated rats. Two recent reports offer support for this interpretation. Gallego and colleagues15 recently described increased production of nitric oxide by bovine aortic endothelial cells after incubation with cyclosporine (at concentrations similar to those noted in the present study). However, basal and bradykinin-stimulated cGMP production failed to increase in their preparation. The failure of cGMP to increase was attributed to blockade of guanylate cyclase activation induced by cyclosporine. An equally plausible explanation for the findings would be increased inactivation of nitric oxide via increased production of superoxide, as we propose in interpreting our findings. Amore et al17 demonstrated increased nitric oxide synthesis in kidney homogenates of rats treated with cyclosporine. Conclusive demonstration of increased production of free radicals and of intact production of EDNO in arteries from cyclosporine-treated rats will require direct measurements of superoxide and nitric oxide production, which were not performed in the present studies.

The possibility that cyclosporine-induced endothelial dysfunction might be mediated by increased production of EDNO was explored in two ways. PKC has been reported to downregulate membrane phospholipase C activity.3 Muscarinic receptor stimulation activates phospholipase C. H-7, an inhibitor of PKC activity, had no effect on acetylcholine-induced relaxations in either control or cyclosporine-treated arteries (Fig 2), suggesting that PKC was not downregulating responses to the muscarinic agonist in either rat group. Additional experiments with more specific PKC inhibitors will be required for more definitive exclusion of a potential role...
of PKC activation in cyclosporine-treated arteries. Dietary L-arginine supplementation reverses endothelial dysfunction in aortic rings from hypercholesterolemic animals. Recent reports describe improvement in cyclosporine-induced renal vasoconstriction in rats by L-arginine supplementation, whereas in human transplant recipients arginine failed to improve cyclosporine-induced renal constriction. L-Arginine supplementation had no effect on acetylcholine-induced relaxations in isolated mesenteric arteries from cyclosporine-treated rats in the present study, suggesting that L-arginine deficiency was not contributing to the impairment in EDNO-mediated responses. 

EDNO-mediated relaxations are impaired by concomitant production of an endothelium- and cyclooxygenase-dependent contracting factor in conduit arteries of rats treated with cyclosporine. The contractile factor or factors in this setting opposed the vasoelaxant effects of EDNO. We found no support for a similar mechanism in the present studies; neither indomethacin nor SQ 29,548, an inhibitor of prostaglandin H₂/thromboxane A₂ receptors, improved relaxations in response to acetylcholine (Fig 2) in mesenteric resistance arteries from cyclosporine-treated rats. A decrease in sensitivity of vascular smooth muscle to EDNO does not appear to be an important factor in cyclosporine-induced endothelial dysfunction in mesenteric arteries. Relaxations in response to nitroprusside, which are mediated by nitric oxide, were identical in arteries without endothelium from control and cyclosporine-treated rats; cyclosporine-treated arteries with endothelium were slightly more sensitive to sodium nitroprusside than were control arteries. Impairment in nitroprusside responses has been shown in conduit arteries from rats exposed to prolonged or higher doses of cyclosporine.

Thus, the findings in the present study offer the greatest support for increased destruction of EDNO due to enhanced production of free radicals as the major mechanism for the impairment in EDNO-mediated relaxations in mesenteric arteries. What causes the increased production of free radicals in arteries from cyclosporine-treated rats? One possible explanation for the enhanced free radical production would be activation of the arachidonic acid cascade because of cyclosporine-induced increased intracellular calcium. Metabolism of arachidonic acid leads to the production of free radicals. The limited information presented would indicate that the cyclooxygenase pathway is unlikely to serve as the source of the enhanced free radical production; indomethacin did not improve relaxations in response to acetylcholine in mesenteric arteries from cyclosporine-treated rats (pD₂, 33±0.4 nmol/L; n=6). The source of the enhanced free radical production in cyclosporine-treated arteries has not been sufficiently addressed to allow any conclusions from the present studies.

In summary, cyclosporine treatment impairs EDNO-mediated relaxations in mesenteric resistance arteries of rats. The impairment in EDNO-mediated relaxations appears to result from increased destruction of EDNO due to enhanced production of free radicals. The normalization of EDNO-mediated relaxation in cyclosporine-treated arteries affected by SOD provides evidence that superoxide anion may be the responsible free radical. Nitric oxide–mediated relaxations induced by sodium nitroprusside are not altered in arteries from cyclosporine-treated rats, suggesting that alterations in endothelial cell rather than vascular smooth muscle cell function are more important in cyclosporine-induced vasoconstriction. The enhanced production of vasoconstricting prostanoids and of endothelin described in subjects treated with cyclosporine may arise as a consequence of a loss of EDNO-mediated inhibition of the production of these agents. Equally important, a decrease in EDNO activity predictably will lead to enhanced vasoconstriction by angiotensin II.

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References


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