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Depressed renal and vascular nitric oxide synthase expression in cyclosporine-induced hypertension

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Depressed renal and vascular nitric oxide synthase expression in cyclosporine-induced hypertension.

Background. Introduction of cyclosporine (CsA) for clinical use has greatly enhanced the outcome of organ transplantation. However, CsA can cause nephrotoxicity and hypertension (HTN). This study was designed to test the hypothesis that CsA-induced HTN is related to depressed nitric oxide (NO) production.

Methods. Urinary excretion of NO metabolites (NOx) and endothelial and inducible NO synthase (eNOS and iNOS) proteins were determined in thoracic aortas and kidneys of CsA-treated (given CsA 18 mg/kg/day for 3 weeks) and placebo-treated rats. In addition, renal tissue eNOS and iNOS mRNA and aorta iNOS activity were measured.

Results. CsA administration resulted in a significant rise in arterial blood pressure (BP) coupled with a steady decline in urinary NOx excretion, suggesting depressed NO production. This was accompanied by a significant reduction in iNOS protein abundance in the kidney and thoracic aorta but no change in eNOS protein abundance. The fall in renal iNOS protein in CsA-treated rats was accompanied by a parallel decline in iNOS mRNA abundance and enzymatic activity.

Conclusion. Administration of CsA for three weeks resulted in a significant rise in BP together with marked reductions in urinary NOx excretion, and renal and vascular iNOS expression. These observations suggest that CsA-induced HTN may be, in part, related to impaired NO production. If true, strategies designed to restore NO availability may mitigate HTN and other vascular complications of CsA therapy.

Cyclosporine (CsA) is a cyclic neutral peptide of fungal origin that possesses potent immunosuppressive properties with practically no bone marrow toxicity. Use of CsA has greatly enhanced the outcome of organ transplantation and as such has become the mainstay of the current anti-rejection regimens [1]. However, CsA administration often leads to a number of side effects that are primarily circulatory and vascular in nature. These include increased renal and systemic vascular resistance, systemic hypertension (HTN), microvascular thrombosis, hypercholesterolemia, and impaired renal function and structure [2–9].

During the past few years, numerous studies have attempted to elucidate the mechanism of CsA-induced HTN and nephrotoxicity. Much of the initial efforts were directed toward the effects of CsA on vasopressor systems including catecholamines, renin-angiotensin-aldosterone system, endothelin, and arachidonic acid metabolites. While CsA was found to alter either production of or vascular reactivity to these vasopressors, the findings have not uncovered a unifying mechanism to account for the diverse vascular complications of CsA. However, increasing evidence has emerged pointing to endothelial injury and dysfunction as the central cause of CsA-induced vascular, renal and circulatory disorders. In particular, CsA appears to impair endothelium-dependent relaxation (EDR) in a variety of *in vivo*, *ex vivo* and *in vitro* models, as well as vascular preparations of different sizes and functional characteristics. These include intact animals treated with CsA [10], aortic rings from CsA-pretreated animals [7, 11–12], normal aortic rings incubated in CsA-containing media [12], femoral arteries from CsA-pretreated rats, mesenteric vascular bed perfused with CsA, afferent and efferent arterioles of the kidneys in CsA-treated rats, epicardial coronary arteries of CsA-pretreated animals, and human subcutaneous arterioles challenged with CsA [12–17].

A number of recent studies point to the L-arginine-NO pathway as the target for CsA-induced impairment of endothelium-dependent relaxation. For instance, CsA-induced HTN in both normotensive and spontaneously hypertensive rats can be prevented by supplementing the animals' diet with L-arginine [10, 18]. These observations indicate that CsA-induced impaired vasorelaxation response to acetylcholine may be due to decreased NO production.

The present study was undertaken to examine the effect of chronic CsA administration on L-arginine-NO pathway

Key words: cyclosporine, nitric oxide, hypertension, nephrotoxicity, transplantation, vasculopathy.

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and expression of endothelial and inducible NO synthase in the kidney and vascular tissues.

METHODS

Animal models

Male Sprague-Dawley rats (Harlan Sprague-Dawley Inc., Indianapolis, IN, USA) with an average weight of 240 g were used. They were randomly assigned to either a CsA group or a placebo-treated control group. A minimum of twelve rats were included in each group. Animals were kept in a climate-controlled light-regulated space with 12-hour light ($\geq$500 lux) and 12-hour dark ($<$5 lux) cycles. They were allowed free access to a low-nitrate basic diet (Purina Mills, Brentwood, MO, USA) and water. Animals in the CsA group received 18 mg/kg/day of cyclosporine (Sandoz Inc., East Hanover, NJ, USA) in olive oil by gastric gavage for three weeks. The control group received the vehicle instead.

Arterial blood pressure was monitored regularly using a tail blood pressure monitor (Harvard Apparatus, South Natick, MA, USA). On each occasion the animal was placed on a heating pad, and a tail cuff was placed and inflated several times to allow the animal to become accustomed to the procedure. Blood pressure was then measured and recorded thrice, and the average of the three values was used.

Five-hour urine collections were obtained over dry ice at baseline and on days 7, 14 and 21 using individual metabolic cages. On each occasion blood samples were drawn by orbital sinus puncture. The procedure was carried out under general anesthesia using pentobarbital, 50 mg/kg i.p. The blood samples were immediately centrifuged, plasma separated and frozen at -70°C until processed.

At the conclusion of the three-week treatment period, animals within each group were divided into two subgroups. One subgroup was used for infusion experiments while the other was used for measurements of renal and thoracic aorta NO synthase (NOS) isotypes. After general anesthesia with i.p. pentobarbital (50 mg/kg), the latter animals were killed by decapitation between the hours of 9 and 11 a.m. Thoracic aorta and kidneys were harvested immediately, cleaned with PBS, snap-frozen in liquid nitrogen and stored at -70°C until processed.

Hematocrit, creatinine and creatinine clearance were determined using standard laboratory methods. All experiments had been approved by the Institutional Animal Care and Use Committee of the University of California, Irvine.

Response to L-arginine, L-NMMA and sodium nitroprusside

Under general anesthesia with inactin (100 mg/kg, i.p.), the left jugular vein and carotid artery were cannulated with polyethylene tubings (PE50). A tracheal cannula was also inserted. Rats were then placed on heating pads. Arterial blood pressure was monitored directly via the arterial catheter which was connected to a Gould P-50 pressure transducer and recorded on a Dynograph R511A recorder (Sensor Medics, Anaheim, CA, USA). Once stable, blood pressure was recorded for five minutes to determine the baseline value. Subsequently, the hypotensive response to bolus injections of NO precursor, L-arginine (100 mg/kg; Sigma Chemical Co., St. Louis, MO, USA) and NO-donor, sodium nitroprusside (SNP, 0.5 μg/kg; Sigma Chemical Co.), was determined. This was followed by bolus injections of L-NMMA (10 mg/kg; Sigma Chemical Co.), a competitive inhibitor of NO synthase. Response to each drug was calculated as peak change in blood pressure from baseline. Mean arterial pressure (MAP) was calculated as the sum of diastolic blood pressure and one-third of the pulse pressure. Each drug was injected at least twice, and the average of the values obtained was used. A thirty-minute recovery period was allowed after each bolus injection except for the L-NMMA injections, which were sixty minutes apart.

RNA isolations

Kidney specimen for analysis of RNA was immediately frozen in liquid nitrogen and then stored at -70°C. Frozen kidney was homogenized by a polytron homogenizer (Kinematica AG, Littau, Switzerland). Total RNA was purified by TRI reagent from Molecular Research Center Inc. (Cincinnati, OH, USA). The total RNA concentration in each sample was determined from absorbance at 260 nm. The quality of each RNA preparation was determined by 1% agarose-formaldehyde gel electrophoresis and ethidium bromide staining.

Northern blot analysis

Twenty micrograms of total RNA were loaded into each lane of 1% agarose-formaldehyde gel. Electrophoresis was carried out at 4 volts per cm gel length. The RNA from the gel was transferred onto positively charged Hybond nylon membrane (Amersham Life Science, Arlington Heights, IL, USA) by capillary transfer. The nylon membrane was UV cross-linked using a UV cross-linker (Fisher Scientific, Pittsburgh, PA, USA). The cDNA clones for rat endothelial eNOS (324 bp) and inducible iNOS (1500 bp) were generously provided by Dr. Thomas Michel (Harvard Medical School, Boston, MA, USA) and Dr. Robert Star (Southwestern Medical Center, Dallas, TX, USA), respectively. The clone for eNOS was linearized by a restriction endonuclease *Xba*I while the iNOS clone was linearized with KPN (Pharmacia Biotech Inc., Piscataway, NJ, USA). The antisence cRNA of eNOS was generated with T7 RNA polymerase (Ambion Inc., Austin, TX, USA), and the antisence cRNA for iNOS was generated by T3 RNA polymerase (Ambion Inc.). The blots were hybridized using $\alpha[^{32}p]$ cytidin triphosphate-labeled eNOS and iNOS riboprobes. The membranes were washed four times in 20

minutes, twice in 2 × SSC with 0.1% SDS at room temperature and twice in 0.1 × SSC with 0.5% SDS at 65°C. Autoradiography was performed by exposing the blots to x-ray film (Reflection; NEN Research Products, Boston, MA, USA) with intensifying screens at −70°C. An internal standard of β-actin cDNA probe (Clontech Laboratories Inc., Palo Alto, CA, USA) was used to assess RNA loading. Quantitation of mRNA signals was performed by densitometric scanning of autoradiographs (Molecular Dynamics, Sunnyvale, CA, USA). The eNOS and iNOS signals were standardized against the β-actin signal for each sample, and the results were expressed as eNOS/β-actin and iNOS/β-actin ratios.

Table 1. Body weight, hematocrit (Hct), serum creatinine (S_{Cr}) concentration and creatinine clearance (C_{Cr}) obtained at the beginning and the end of the study in the cyclosporine-treated (CsA) and placebo-treated control rats

	Control group		CsA group	
	Initial	Final	Initial	Final
Body weight *g*	237 ± 4.7	367 ± 6.3[a]	243 ± 2.3	319 ± 7.6[a,b]
Hct %	44.4 ± 1.1	43.4 ± 1.2	45 ± 1.5	43.7 ± 1.9
S_{Cr} *mg/dl*	0.48 ± 0.01	0.45 ± 0.01	0.43 ± 0.02	0.51 ± 0.01[a,b]
C_{Cr} *ml/min*	1.1 ± 0.1	1.66 ± 0.2[a]	1.16 ± 0.02	1.43 ± 0.1[a]

$N = 12$ in each group.
[a] $P < 0.05$ vs. initial
[b] $P < 0.05$ vs. corresponding value in the control

Tissue preparation

Thoracic aorta and kidney were used for determination of NO synthase (NOS). Rats were sacrificed by decapitation, and thoracic aorta and kidney were immediately excised, cleaned with PBS, frozen in liquid nitrogen and stored at −70°C. Homogenates (25% wt/vol) were prepared in 10 mM HEPES buffer, pH 7.4, containing 320 mM sucrose, 1 mM EDTA, 1 mM DTT, 10 μg/ml leupeptin and 2 μg/ml aprotinin at 0 to 4°C with the aid of a tissue grinder fitted with a motor-driven ground glass pestle. Homogenates were centrifuged at 12,000 g for five minutes at 4°C to remove tissue debris without precipitating plasma membrane fragments [19, 20]. The supernatant was used for determination of NOS activity and protein mass. Protein concentration was determined by using a Bio-Rad kit (Bio-Rad Laboratories, Hercules, CA, USA).

Nitric oxide synthase activity assay

Calcium-independent NOS activity was measured as previously described. [21] In brief, 40 μl of the homogenates (approximately 200 μg protein) were incubated in a buffer (Tris-HCl [50 mM], EDTA [1.7 mM], and EGTA [0.1 mM]) containing cofactors (calmodulin [10 μM], tetrahydrobiopterin [3 μM] and NADPH [1 mM]) and the substrate, L-arginine 100 μM, combined with L-[^{3}H]-arginine (0.2 μCi; sp at 55 Ci/mmol) for 45 minutes at 37°C. The mixture also contained 1 mM L-citrulline to minimize conversion of newly formed L-[^{3}H]-citrulline back to L-[^{3}H]-arginine. After the incubation period, the reaction was quenched by addition of 1 ml of stop buffer [HEPES (20 mM), pH 5.5, EDTA (2 mM), and EGTA (2 mM)]. The reaction mix was applied to a 1-ml column containing Dowex AG 50-WX8 (Na^+ form) resin that had been pre-equilibrated with the stop buffer. L-[^{3}H]-citrulline was eluted twice with 0.5 ml of stop buffer, and radioactivity was determined by liquid scintillation counting. Protein concentration of the homogenates was determined by using a Bio-Rad kit with bovine albumin used as the standard.

Western blot analysis

These measurements were carried out to determine the endothelial and inducible NOS (eNOS and iNOS) protein mass as previously described [19, 20]. Anti-eNOS monoclonal antibody, peroxidase-conjugated goat anti-mouse IgG antibody, anti-Mac NOS-I, human endothelial positive control and mouse macrophage positive control were supplied by Transduction Laboratories (Lexington, KY, USA). Briefly, aorta and kidney tissue preparations (50 μg of protein for the aorta and 100 μg for the kidney) were size-fractionated on 4 to 12% Tris-glycine gel (Novex, San Diego, CA, USA) at 120 V for three hours. In preliminary experiments we had found that the given protein concentrations were within the linear range of detection for our Western blot technique. After electrophoresis, proteins were transferred onto hybond-ECL membrane (Amersham Life Science Inc., Arlington Heights, IL, USA) at 400 mA for 120 minutes using the Novex transfer system. The membrane was prehybridized in 10 ml of buffer A (10 mM Tris hydrochloride, pH 7.5, 100 mM NaCl, 0.1% Tween 20 and 10% nonfat milk powder) for one hour and then hybridized for an additional one-hour period in the same buffer containing 10 μl of the given anti-NOS monoclonal antibody (1:1000). The membrane was then washed for 30 minutes in a shaking bath, changing the wash buffer (buffer A without nonfat milk) every five minutes prior to one hour of incubation in buffer A plus goat anti-mouse IgG-HRP (horse radish peroxidase) at the final titer of 1:1000. Experiments were carried out at room temperature. The washes were repeated before the membrane was developed with a light emitting nonradioactive method using ECL reagent (Amersham Inc.). The membrane was then subjected to autoluminography for one to five minutes. The autoluminographs were scanned with a laser densitometer (Model PD1211; Molecular Dynamics, Sunnyvale, CA, USA) to determine the relative optical densities of the bands. In all instances, the membranes were stained with Ponceau stain which verified the uniformity of protein load and transfer efficiency across the test samples.

Measurements of total nitrate and nitrite

The concentration of total nitrate and nitrite (NOx) in the test samples was determined as described previously [22] using the purge system of a Sievers Instruments Model

270B Nitric Oxide Analyzer (NOA™; Sievers Instruments Inc., Boulder, CO, USA). Briefly, urine samples were diluted ten times in distilled water prior to analysis. A saturated solution of VCl_3 in 1 M HCl was prepared and filtered prior to use. Five milliliters of this reagent were added to the purge vessel and purged with nitrogen gas for 5 to 10 minutes prior to use. The purge vessel was equipped with a cold water condenser and a water jacket to permit heating of the reagent to 95°C using a circulating water bath. The hydrochloric acid vapors were removed by a gas bubbler containing ~15 ml of 1 M NaOH. The gas flow rate into the chemiluminescence detector was controlled using a needle valve adjusted to yield a cell pressure of ~7 torr. The flow rate of nitrogen into the purge vessel was adjusted to prevent vacuum distillation of the reagent.

Samples were injected into the purge vessel to react with the VCl_3/HCl reagent which converted nitrate, nitrite and S-nitroso compounds to NO. The NO produced was stripped from the reaction chamber (by purging with nitrogen and vacuum) and detected by ozone-induced chemiluminescence in the chemiluminescence detector. The signal generated (NO peak and peak area) was recorded and processed by a Hewlett Packard Model 3390 Integrator. In a typical assay, 5 μl of the test sample were injected to the purge vessel, and all samples were run in triplicate.

Standard curves were constructed using various concentrations of NO_3^- (5 to 100 μM) relating the luminescence produced to the given NO_3^- concentrations of the standard solutions. The amount of NO_2^-/NO_3^- in the test sample was determined by interpolation of the result into the standard curve.

Data analysis

Analysis of variance (ANOVA) and a *t*-test were used in evaluation of the data which are presented as mean ± SEM. *P* values less than 0.05 were considered significant.

RESULTS

General data

Data are shown in Table 1 and Figure 1. The CsA group showed a significantly lower body wt, a slight but significantly higher serum creatinine concentration, and a slight and insignificantly lower creatinine clearance at the conclusion of the study when compared with the placebo-treated control group. Hematocrit values in the CsA group were comparable with those found in the control group. As expected, the CsA-treated animals exhibited a significant rise in arterial blood pressure during the study period. In contrast, blood pressure remained virtually unchanged in the placebo-treated control animals.

Response to L-arginine, L-NMMA and nitroprusside

Data are illustrated in Figure 2. The CsA and normal control animals exhibited a transient fall in arterial blood pressure within a few seconds after the bolus injection of L-arginine. However, the magnitude of hypotensive response to L-arginine in the CsA group was significantly less than that seen in the control group ($P < 0.05$).

L-NMMA administration resulted in a rise in arterial blood pressure lasting ten to twenty minutes in the two groups. However, the hypertensive response to L-NMMA was greatly reduced in the CsA group as compared to that seen in the control group ($P < 0.01$). In contrast to L-arginine and L-NMMA, the magnitude of hypotensive response to NO-donor sodium nitroprusside was similar in the two groups.

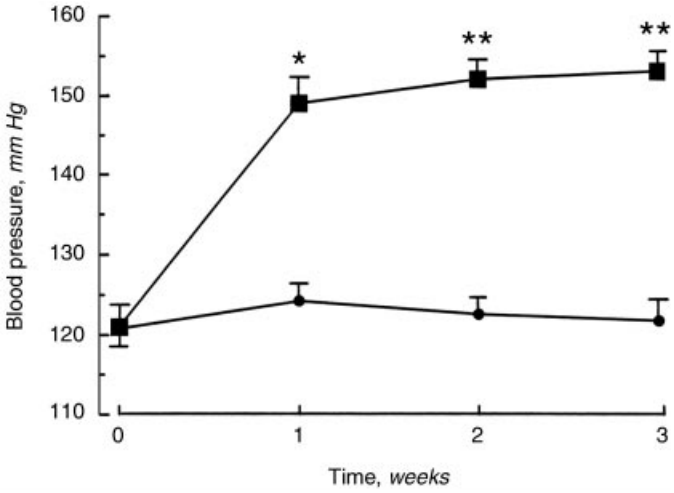

Fig. 1. Measurements of systolic arterial blood pressure in the cyclosporine-treated (CsA; ■) and vehicle-treated control (CTL; ●) animals. $N = 6$ in each group. $^*P < 0.05$. $^{**}P < 0.01$ versus both baseline and control values.

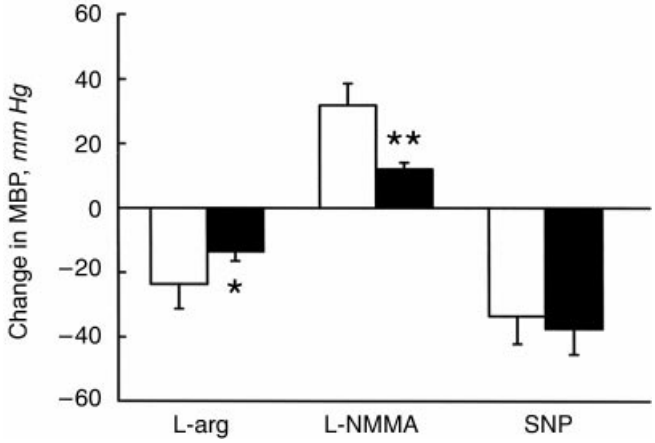

Fig. 2. Change in arterial blood pressure in response to intravenous administrations of L-arginine, N^G monomethyl-L-arginine (L-NMMA) and sodium nitroprusside (SNP) in cyclosporine-treated (■) and vehicle-treated control (□) animals. $N = 6$ in each group. $^*P < 0.05$, $^{**}P < 0.01$ versus control group.

Urinary nitrate/nitrite (NOx) data

Data are depicted in Figure 3. CsA administration resulted in a significant and steady reduction in urinary

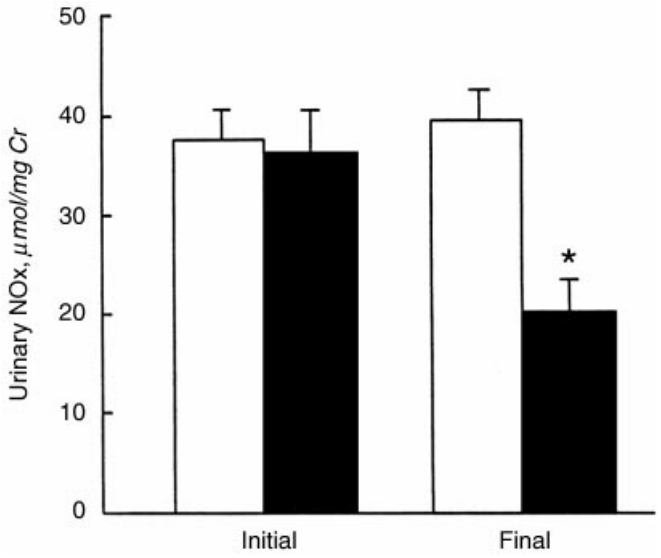

Fig. 3. Urinary excretion of total nitrate and nitrite (NOx) in the cyclosporine-treated (■) and vehicle-treated control (□) groups. $N = 6$ in each group. $^*P < 0.05$ versus control group.

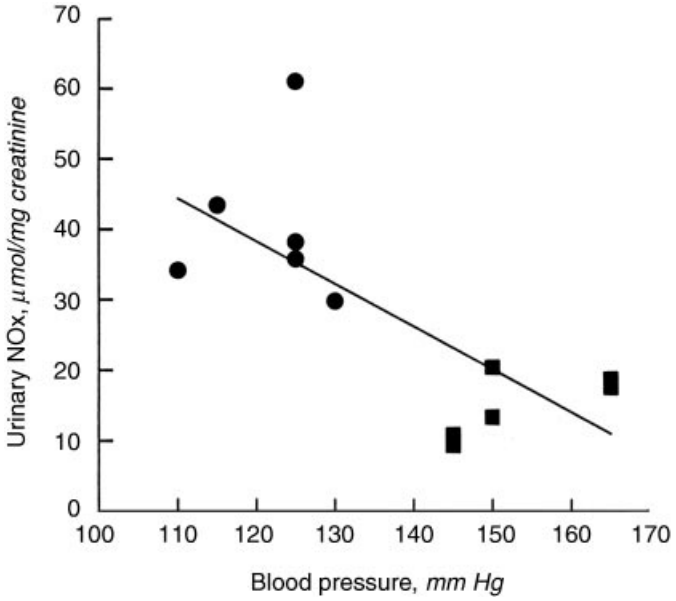

Fig. 4. Correlation between urinary excretion of total nitrate and nitrite (NOx) and systolic blood pressure in the cyclosporine-treated (■) and vehicle-treated control (●) animals ($r = -0.78$; $P < 0.05$; $N = 6$ in each group.

NOx excretion during the study period. In fact, urinary NOx excretion fell by 33% after one week and 62% after three weeks of CsA therapy in the CsA group. In contrast, urinary NOx excretion remained unchanged in the placebo-treated control group.

Kidney and aorta nitric oxide synthase data

Data are illustrated in Figures 5 to 11. The CsA group showed a nearly threefold reduction in the aorta iNOS protein abundance as compared to the normal control group ($P < 0.05$). The reduction in the aorta iNOS protein abundance was accompanied by a parallel decline in Ca^{2+}-independent NOS activity, which signifies depressed iNOS enzymatic activity. However, no significant difference was found in the aorta eNOS abundance between the two groups. As with the aorta, kidney tissue iNOS protein abundance was significantly reduced in the CsA groups when compared with that found in the control group ($P < 0.05$). The reduction in renal tissue iNOS abundance was accompanied by a parallel reduction in kidney iNOS mRNA abundance. No significant difference was found in renal tissue eNOS protein abundance between the CsA and the control groups. Likewise, kidney tissue eNOS mRNA abundance was similar in the two groups.

DISCUSSION

The CsA-treated animals employed in the present study exhibited an expected rise in arterial blood pressure. The rise in arterial blood pressure in the CsA group was accompanied by a steady decline in urinary NOx excretion, suggesting a possible decline in total body NO production during the study period. In addition, the CsA-treated

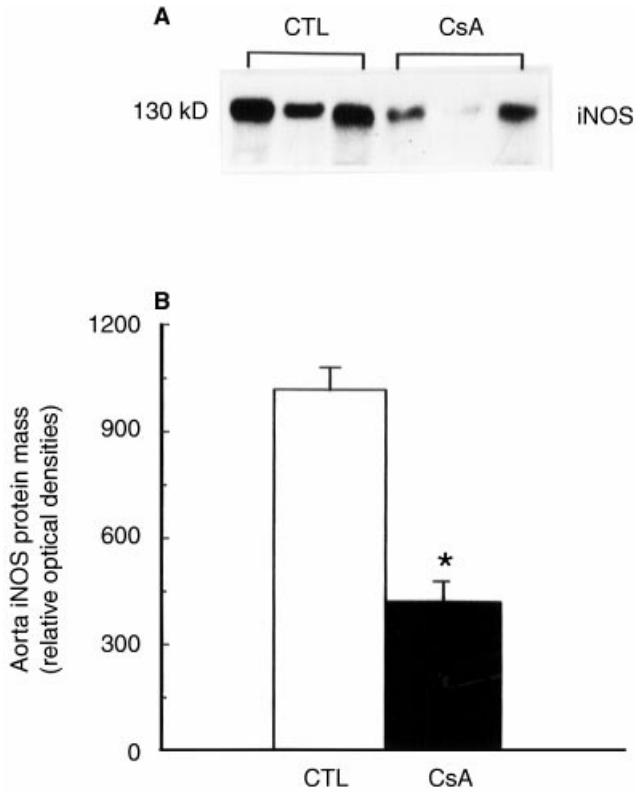

Fig. 5. (*A*) Representative Western blot of aorta iNOS protein in three vehicle-treated (CTL) and three cyclosporine-treated (CsA) rats. (*B*) Group data depicting relative optical densities of aorta iNOS protein bands in the CTL (□) and CsA (■) groups. $N = 6$ in each group. $^*P < 0.05$ versus control group.

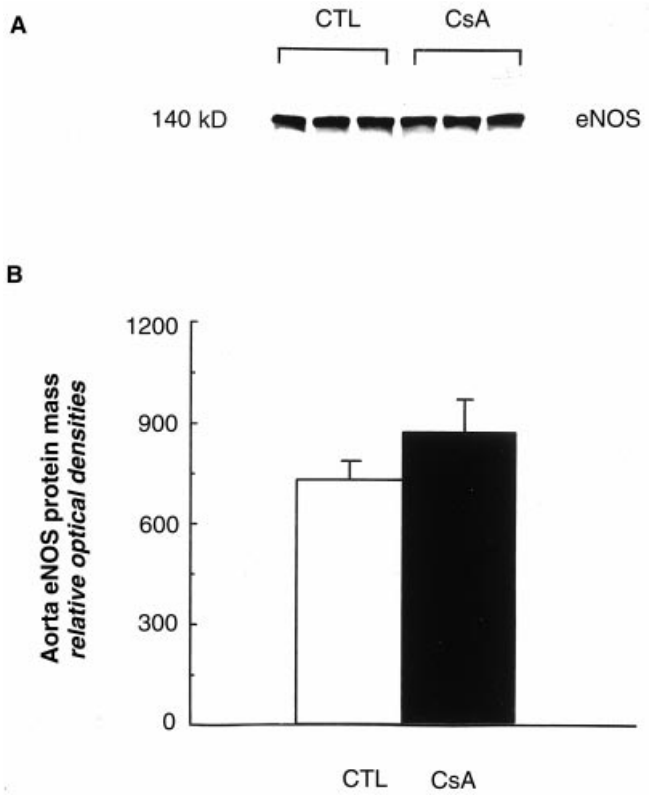

Fig. 6. (*A*) Representative Western blot of aorta eNOS protein in three vehicle-treated control (CTL) and three cyclosporine-treated (CsA) rats. (*B*) Group data illustrating the optical densities of aorta eNOS protein bands in the CTL (□) and CsA (■) groups. $N = 6$ in each group.

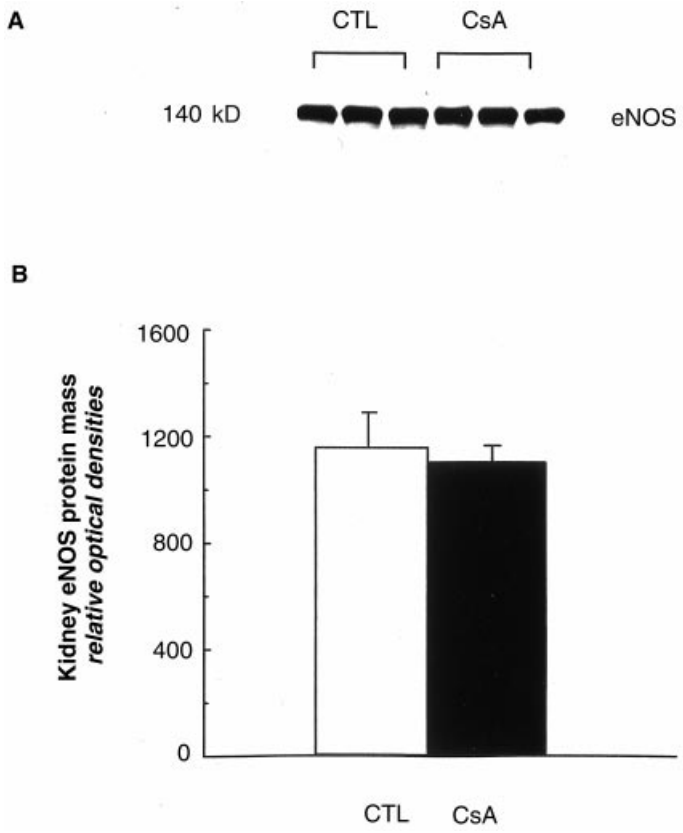

Fig. 7. (*A*) Representative Western blot of kidney tissue eNOS protein in three vehicle-treated (CTL) and three cyclosporine-treated (CsA) rats. (*B*) Group data depicting relative optical densities of kidney eNOS protein bands in the CTL (□) and CsA (■) groups. $N = 6$ in each group.

animals showed an attenuated hypotensive response to L-arginine but a normal response to NO-donor, nitroprusside. The impaired response to NO-substrate despite normal response to exogenous NO suggests that NO synthesis from L-arginine may be diminished in CsA-treated animals. This supposition is supported by significant reduction in vascular and renal tissue expression of iNOS protein in the CsA group. The reduction in kidney tissue iNOS protein in the CsA-treated animals was accompanied by a parallel decline in renal tissue iNOS mRNA abundance. This observation suggests that down-regulation of iNOS in the CsA-treated rats occurs at the level of mRNA. Interestingly, CsA therapy under the given conditions had no discernible effect on eNOS expression in either the kidney or thoracic aorta. It should be noted that the normality of eNOS protein mass shown here does not contradict the reported inhibitory effect of CsA on eNOS enzymatic activity [23]. The latter is due to interference of CsA with Ca-calmodulin-dependent activation as opposed to quantitative modification of eNOS [23]. Consequently, the reduction in urinary NOx excretion and attenuated response to L-arginine in CsA-treated animals could be due to a combination of iNOS deficiency shown here and eNOS inhibition shown by Hutcheson et al [23]. These investigators recently demonstrated a marked inhibition of NOS activity of the 100,000 g fraction of the rat heart by CsA at a wide range of drug concentrations. This observation suggests that CsA is a direct inhibitor of eNOS [23]. Likewise, these investigators found that CsA inhibited Ca^{2+} ATPase activity of the rat heart preparation *in vitro*. Based on these observations, the authors concluded that inhibition of the two Ca^{2+}-calmodulin-dependent enzymes may be due to the drug's interference with Ca^{2+}-calmodulin-mediated events.

Diminished hypertensive response to NOS inhibitor, L-NMMA, in our animals further supports the notion that NO generation is impaired and that contribution of NO to blood pressure homeostasis is reduced in CsA-treated animals. This viewpoint is supported by earlier studies demonstrating that the inhibitory action of methylene blue (a potent NO quencher) on acetylcholine-mediated vasodilation was greatly reduced in CsA preincubated mesenteric resistance vessels as compared to the untreated tissues [13]. Additional indirect evidence for diminished NO production comes from studies by Rego et al which demonstrated that CsA treatment lowers the aorta content of

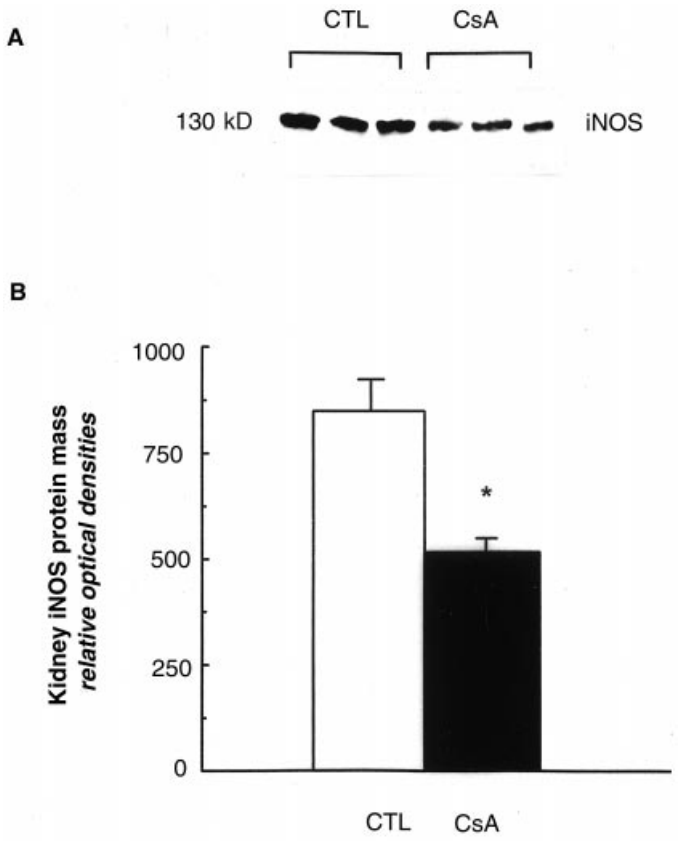

Fig. 8. (*A*) Representative Western blot of kidney tissue iNOS protein in three vehicle-treated (CTL) and three cyclosporine-treated (CsA) rats. (*B*) Group data depicting relative optical densities of kidney iNOS protein bands in the CTL and CsA groups. $N = 6$ in each group. $^*P < 0.05$ versus control group.

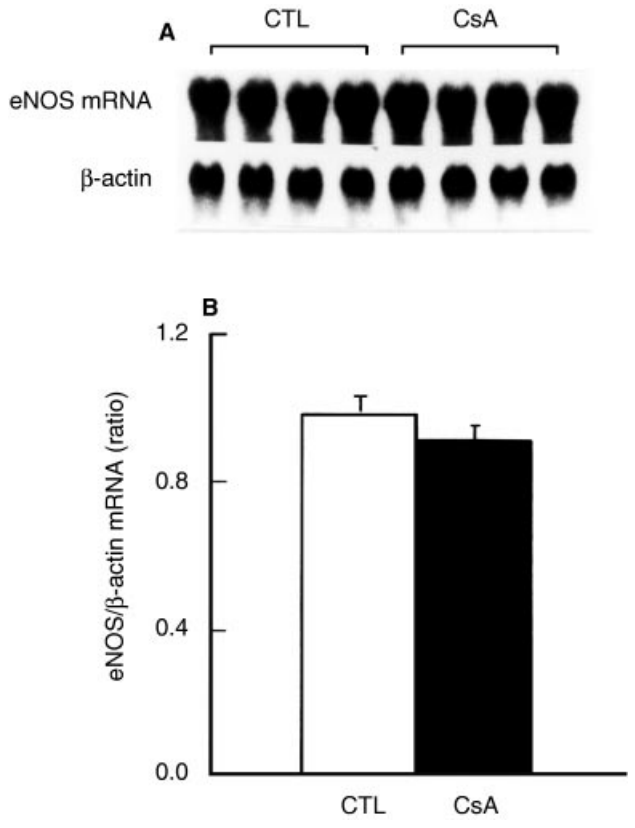

Fig. 9. (*A*) Northern blots of the kidney tissue eNOS and β-actin mRNA in four normal control (CTL) and four cyclosporine-treated (CsA) rats. (*B*) Group data illustrating renal tissue eNOS/β-actin mRNA abundance in CTL (□) and CsA (■) animals. No significant difference was found between the two groups.

cGMP, the second messenger of NO in vascular smooth muscle [12].

Renal production of NO plays an important role in regulation of renal vascular resistance as well as sodium and water transport in the renal tubules [24–30]. For instance, NOS blockade leads to afferent arteriolar vasoconstriction [31, 32], reduced urinary sodium excretion [33–35], and blunted pressure natruresis [36–39]. In contrast, administration of NO-donor lowers blood pressure and promotes natruresis [40]. In addition, impaired vascular NO production necessarily raises systemic vascular resistance by reducing the vasodilatory tone. Thus, by raising renal and systemic vascular resistance and by promoting sodium retention, inhibition of L-arginine-NO pathway can lead to arterial hypertension. Our CsA-treated animals showed a marked reduction in urinary NOx excretion, suggesting depressed renal and systemic NO production. This proposition was supported by the significant reductions in renal and vascular tissue iNOS protein expression shown here and the potent inhibitory action of CsA on eNOS activity shown by Hutcheson et al [23]. It is thus logical to assume that the CsA-induced HTN in this model is, at least, in part, caused by NO deficiency. Given the well-known inhibitory action of NO on platelet adhesion/aggregation, NO deficiency can theoretically contribute to microvascular thrombosis associated with CsA toxicity as well [41]. Thus, inhibition of L-arginine-No pathway can account for several adverse effects of CsA therapy. If true, strategies designed to correct NO deficiency may prove useful in preventing the adverse effects of CsA and other drugs capable of interfering with L-arginine-NO pathway.

The mechanism(s) responsible for down-regulation of iNOS protein in the CsA-treated animals is uncertain. It is of note that contrary to the original views, iNOS is constitutively expressed in a few organs. For instance, in the kidney iNOS is abundantly expressed in the medullary thick ascending limb of loop of Henle, glomeruli, as well as interlobular and arcuate arteries at basal conditions [42–44]. In fact, we have recently shown constitutive expression of iNOS in the kidney and vascular tissues of untreated intact Sprague Dawley rats, Wistar Kyoto rats and spontaneously hypertensive rats (SHR), and its altered expression in various disease states [45–47]. In this regard, we have

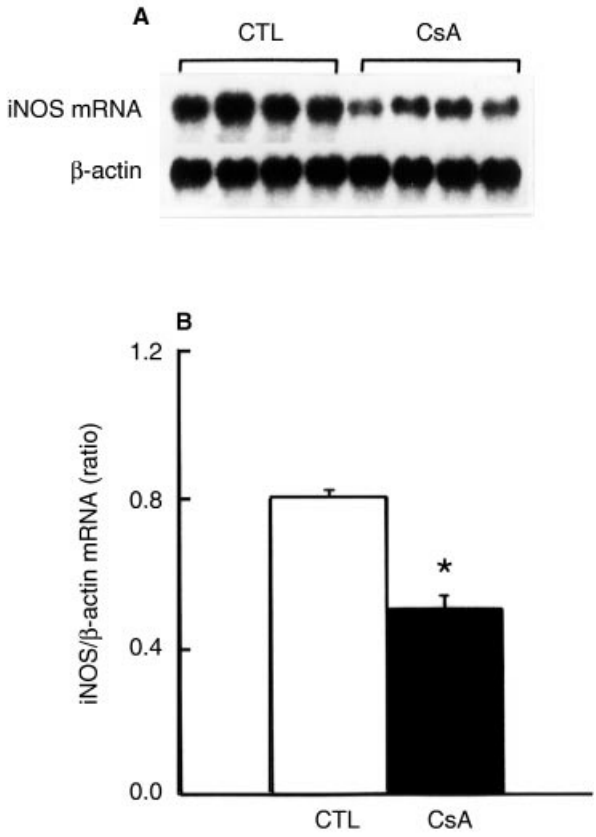

Fig. 10. (*A*) **Northern blots of the kidney tissue iNOS and β-actin mRNA in four normal control (CTL) and four cyclosporine-treated (CsA) rats.** (*B*) Group data illustrating renal tissue iNOS/β-actin mRNA abundance in CTL (□) and CsA (■) animals. $^*P < 0.01$.

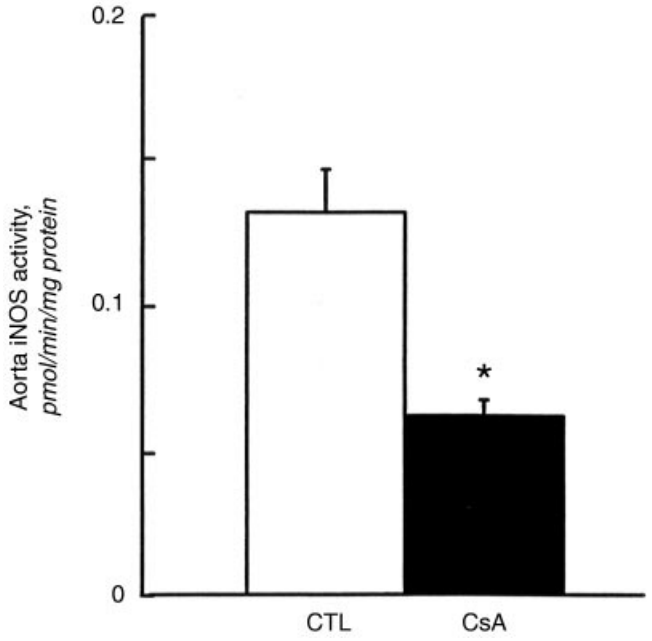

Fig. 11. Aorta tissue iNOS (Ca^{2+}-independent) activity in six cyclosporine-treated (CsA; ■) and six normal control (CTL; □) rats. $^*P < 0.001$.

found up-regulation of iNOS and eNOS in the SHR [45], up-regulation of iNOS but not eNOS in rats with lead-induced hypertension [46], and down-regulation of both in rats with chronic renal insufficiency [47]. The constitutive expression of iNOS in the kidney and other structures suggests its possible role in some homeostatic function in these tissues. While low level expression occurs constitutively in the kidney and certain other tissues, maximum expression of iNOS requires induction by a specific combination of lipopolysaccharide or cytokines.

The CsA animals were treated with oral administration of the drug by gavage. In a pharmacokinetic study, Hedayati, Bernareggi and Rowland demonstrated that 24% of orally administered CsA in olive oil, given by gavage, was absorbed within five hours in the rat. They further found that the absorption ceased after five hours and was unaffected by rat chow [48]. Although we did not study the pharmacokinetics of CsA in the present study, we assume that the general observations reported by these authors may be applicable to the rats employed in the present study.

In an earlier study Rao et al reported increased NOS enzymatic activity in the kidneys of rats treated with CsA for four weeks. In addition, they found increased NOS activity of the normal kidneys in the presence of CsA *in vitro* [49]. They concluded that this phenomenon may be due to alteration of calcium-calmodulin dependent activation of NOS by CsA [49]. In contrast, we found diminished iNOS protein together with decreased urinary NOx excretion in the CsA-treated rats suggesting impaired NO production. The reason for the apparent disparity between the results of this study and those of Rao et al is unclear. It is of interest that in another study, Hutcheson and Rao et al showed a marked inhibition of cardiac tissue NOS activity with CsA *in vitro* [23]. They suggested that the observed inhibitory effect of CsA may be due to interference of CsA with calcium-calmodulin dependent activation of NOS [23]. Thus, in the latter study in which Dr. Rao was a co-author, CsA had an inhibitory action on cardiac NOS [23], whereas in the former study by Rao et al, CsA was found to have a stimulatory effect on NOS [49]. Given the fact that calcium-calmodulin dependent activation of constitutive NOS isotypes is similar in all tissues, we found the disparity between the two studies by these authors somewhat puzzling. It is of note that abundance in the kidney of arginase, an enzyme that like NOS utilizes L-arginine as it substrate, constitutes a major difficulty in assessing the true NOS activity in this organ. For this reason, we abandoned the measurement of NOS enzymatic activity in the kidney. Instead, we used direct measurements of the enzyme protein mass and urinary NOx excretion in our studies. It is conceivable that the apparent increase in NOS activity of the kidney with CsA reported by Rao et al may have been an artifact of CsA interaction with the renal arginase activity. Further studies are required to explore the latter possibility.

In conclusion, CsA administration for three weeks resulted in a significant increase in arterial blood pressure in genetically normotensive Sprague Dawley rats. This was accompanied by a marked reduction in urinary nitrate/nitrite excretion together with marked down-regulation of renal and vascular expression of iNOS but not eNOS. The observed down-regulation of iNOS shown here together with CsA-induced inhibition of eNOS enzymatic activity shown by others can account for the reduction in NO production in the CsA-treated animals. If true, depressed NO production can contribute to CsA-induced hypertension.

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