Cyclosporine (CsA) is an immunosuppressor drug. Despite its beneficial effects, however, long-term use is frequently associated with hypertension, nephrotoxicity, and hepatotoxicity.

Nephrotoxicity is the most common complication associated with CsA therapy. It is characterized by renal vasoconstriction that induces a decrease in renal plasma flow and glomerular filtration rate. This side effect is usually reversible when CsA dose is reduced. However, even with normal blood levels or during long-term dosage, CsA can produce chronic hypertension and can be the cause of irreversible impairment of renal function. The mechanism by which CsA induces hypertension remains unknown.

Hepatotoxicity is a less common side effect, which is characterized by cholestasis and metabolic disturbances. In isolated perfused rat liver, CsA administration produced a dose-dependent reduction of bile flow, increased the release of cytosolic and mitochondrial enzymes, and decreased oxygen consumption. High blood levels of CsA in human allograft recipients have been associated with an increase in γ-glutamyl transpeptidase, a liver enzyme that correlates with hepatotoxicity. In one study, hepatic abnormalities developed in up to 32% of patients with endogenous uveitis treated with CsA.

CsA toxicity has been attributed to an imbalance of vasoactive substance release. Recent studies have shown that nitric oxide (NO) is increased during CsA administration by a mechanism that includes changes in the gene expression pattern of NO synthase (NOS) in the kidney. However, it is unknown if the effect of CsA on NOS enzyme gene expression is tissue specific. By using bovine aortic endothelial cells in culture, López-Ongil et al demonstrated that CsA enhances NO production, which correlates with an increase in endothelial NOS (eNOS) mRNA, protein, and activity, suggesting a direct effect of CsA on eNOS expression. On the other hand, we have shown that the intense renal vasoconstriction induced by CsA administration is associated with increased eNOS mRNA in renal cortex and decreased nNOS and iNOS mRNA in renal medulla, suggesting that the effect of CsA on NOS expression could be tissue specific. In addition, it has been well documented that eNOS expression can be activated by shear stress, which may result from vasoconstriction. Thus, hemodynamic changes induced by CsA could be the major mechanism by which this immunosuppressive drug alters NOS isoform pattern of expression. We reasoned that if this were the case, then changes in NOS isoform expression during CsA administration would be
present only in tissues in which CsA induces toxicity. In addition, prevention of hemodynamic changes with a vasodilator drug could also prevent the CsA-induced alterations in NOS enzyme gene expression. Thus, in the present study, we evaluated the effect of CsA on NOS isoform mRNA levels in several tissues and the effect of the vasodilator agent nifedipine on their expression.

Methods
Male Wistar rats, weighing 300 to 350 g, with right nephrectomy, were used for the study. Fifteen days after surgery, animals received daily subcutaneous injections of either vehicle (V, 0.1 mL olive oil) or CsA (30 mg/kg body wt) or oral administration of nifedipine (3 mg/kg). Rats receiving V were pair-fed and served as controls. Studies were performed 7 days after V, CsA, V+nifedipine (V+N), and CsA+nifedipine (CsA+N) administration. All procedures followed were in accordance with our institutional guidelines.

Renal and Hepatic Function
Glomerular filtration rate (GFR) was determined in 8 control and CsA-treated rats as well as in 6 V+N- and CsA+N-treated rats that were anesthetized with an initial intraperitoneal dose of sodium pentobarbital (30 mg/kg), with supplemental doses instilled as required. The rats were placed on a thermostatically regulated table, and temperature was maintained at 37°C. Trachea, both jugular veins, femoral arteries, and the left ureter were catheterized with polyethylene tubing PE-240, PE-50, and PE-10, respectively. Mean arterial pressure was monitored with a pressure transducer (model p23 dh, Gould) and recorded on a polygraph (Grass Instruments). Blood samples were taken periodically and replaced with blood from a donor rat. Rats were maintained under euvoletic conditions by infusion of 10 mL/kg body wt of isotonic rat plasma during surgery, followed by an infusion of 25% polyfructosan solution at 2.2 mL/h (Inutest, Laevosan-Gesellschaft). After an equilibrium period of 60 minutes, urine was collected for 60 to 90 minutes and blood samples were taken at the beginning and end of the urine collection period. Urine and plasma polyfructosan concentrations were determined by the technique of Davidson and Sackner. Blood samples taken at the beginning of GFR determination were used to assess the serum concentration of γ-aspartate amino transferase and were analyzed by a colorimetric method.

NOS mRNA Levels
In additional groups of 5 V- and CsA-treated rats, cerebellum, heart, intestine, liver, renal cortex, and renal medulla were obtained under sodium pentobarbital anesthesia. Renal cortex, renal medulla, and liver were also obtained from 5 V+N- and CsA+N-treated rats. The tissues were frozen in liquid nitrogen and kept at −80°C until used. Total RNA was isolated as previously reported using the guanidine isothiocyanate-cesium chloride method. Total RNA was examined by 1% agarose gel electrophoresis, and RNA concentration was determined by UV-light absorbance at 260 nm (Beckman DU640).

Relative Quantification of NOS mRNA
The relative level of the 3 NOS mRNA expressions was assessed by semiqualitative reverse transcription–polymerase chain reaction (RT-PCR), as we previously described. Briefly, NOS and GAPDH primer sequences were detailed before and were custom obtained (Life Technologies).

RT and PCR reactions were performed as we previously reported. The control gene was coamplified simultaneously in each reaction. To analyze the PCR products, one half of each reaction was electrophoresed in a 5% acrylamide gel. Bands were ethidium bromide stained and visualized under UV light, cut out, and suspended in 1 mL of scintillation cocktail (Ecolume, ICN), and counted by liquid scintillation (Beckman LS6500). Genomic DNA contamination was checked by carrying samples through the PCR procedure without adding RT.

Determination of the Exponential Phase
Knowing the exponential phase for each pair of primers is a critical feature of the semiquantitative PCR reaction in order to avoid false results from analysis during the plateau phase. The amplification kinetics from each NOS isoform and GAPDH were evaluated in all studied tissues from 12 to 45 cycles. Exponential phase was determined as we previously reported.

Statistical Analysis
Statistical significance was defined as 2-tailed P<0.05, and the results are presented as mean±SEM. The results were analyzed with a Student’s unpaired, 2-tailed t test or Mann-Whitney U test as needed.

Results

NOS Isoform mRNA Amplification Kinetics
The amplification kinetics of the 3 types of NOS isoform mRNA as well as the housekeeping gene GAPDH in studied tissues are shown in Figure 1. It is evident in Figure 1 that nNOS mRNA amplification in cerebellum reaches the plateau phase earlier than in the rest of the tissues, indicating higher expression of this isoform in central nervous system total RNA. The tissue with the lower expression of nNOS was the liver. Figure 1 also shows the corresponding iNOS kinetics. Although iNOS has been traditionally known as an inducible isoform, it is clear from this analysis that iNOS is expressed constitutively in all these tissues, with the greater expression in heart and intestine and the lower in renal cortex. Figure 1 depicts the amplification kinetics for eNOS mRNA that was similar among the studied tissues. GAPDH mRNA was expressed in similar extent in all tissues, as shown in Figure 1. Note that amplification kinetics for GAPDH begins at 9 PCR cycles, whereas for the NOS it begins at higher PCR cycle numbers in most tissues, indicating higher levels of expression for the housekeeping gene. From these kinetic curves, we chose the midpoint of exponential phase for each tissue to amplify each NOS isoform as well as the housekeeping gene.

NOS Isoform mRNA Expression in CsA-Treated Rats
The Table shows the results of nNOS and iNOS amplification in all studied tissues, expressed as the ratio between nNOS or iNOS over the housekeeping gene GAPDH, which were obtained in vehicle- and CsA-treated animals. The expression of nNOS was significantly lower in renal medulla from the CsA group, whereas in the rest of the tissues no changes were observed. CsA also affected the iNOS mRNA expression in vessel- and CsA-treated animals. The expression of nNOS was significantly lower in renal medulla from the CsA group, whereas in the rest of the tissues no changes were observed.
the kidney. Although there was no change in the renal cortex, iNOS expression levels were significantly lower in renal medulla from CsA-treated rats.

The ratio between eNOS and GAPDH expression is shown in Figure 2. CsA resulted in a significant increase in eNOS mRNA levels in both renal cortex and liver. Thus, eNOS mRNA levels were 2.4-fold higher in renal cortex and 1.7-fold higher in liver in CsA-treated animals. This increase in eNOS expression induced by CsA in renal cortex and liver was not observed when total RNA from renal medulla, intestine, heart, and cerebellum were analyzed.

Effect of Nifedipine on Alterations Induced by CsA

The fact that CsA altered the pattern of expression of NOS isoforms only in kidney and liver and not in other tissues such as the central nervous system, heart, and intestine supports the hypothesis that this effect might be secondary to a CsA toxic effect rather than a direct consequence of CsA on NOS gene expression. Thus, we evaluated the effect of the calcium channel blocker nifedipine on renal function and on renal and liver NOS enzyme expression in vehicle- and CsA-treated rats. Figure 3 shows the GFR in all studied groups. Nifedipine alone increased GFR from 1.2 ± 0.1 in the V group to 2.2 ± 0.5 mL/min in the V+N group (P < 0.05). In CsA-treated rats, nifedipine administration was able to prevent the GFR reduction induced by CsA. Whereas GFR in CsA-treated animals was 0.7 ± 0.09, in CsA+N rats it was 1.6 ± 0.2. This value is significantly different than the CsA value but is not different than GFR in the control group.

Because nifedipine proved to be an effective drug in preventing renal dysfunction induced by CsA, we also assessed the NOS isoform mRNA levels in those tissues in which CsA modified the NOS pattern of expression; that is, for the eNOS isoform, the renal cortex and liver, and for iNOS and nNOS isoforms, the renal medulla. As Figure 4 shows, nifedipine had no effect on eNOS expression in both, liver and renal cortex total RNA. In contrast, nifedipine administration abrogated the increase in eNOS expression induced by CsA in both tissues. In liver, eNOS/GAPDH ratio in the CsA group was 3.4 ± 0.3; in CsA+N it was 1.9 ± 0.2 (P < 0.01). Similarly, in renal cortex total RNA, the ratios were 1.0 ± 0.2 and 0.6 ± 0.06, respectively (P < 0.05).

<table>
<thead>
<tr>
<th>Tissue</th>
<th>nNOS/GAPDH Ratio</th>
<th>iNOS/GAPDH Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>V</td>
<td>CsA</td>
</tr>
<tr>
<td>Cortex</td>
<td>2.01 ± 0.4</td>
<td>1.92 ± 0.35</td>
</tr>
<tr>
<td>Medulla</td>
<td>0.81 ± 0.08</td>
<td>0.56 ± 0.06*</td>
</tr>
<tr>
<td>Heart</td>
<td>0.63 ± 0.12</td>
<td>0.46 ± 0.02</td>
</tr>
<tr>
<td>Liver</td>
<td>1.02 ± 0.03</td>
<td>1.26 ± 0.61</td>
</tr>
<tr>
<td>Intestine</td>
<td>0.89 ± 0.12</td>
<td>1.3 ± 0.36</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>0.23 ± 0.03</td>
<td>0.26 ± 0.03</td>
</tr>
</tbody>
</table>

*P < 0.05 vs V.
In renal medulla, nifedipine also prevented the changes in NOS isoform expression induced by CsA. Figure 5A shows the mRNA nNOS/GAPDH ratio in renal medulla; the value in the CsA group was significantly different from the other 3 groups. Thus, in renal medulla, nifedipine abrogated the reduction in nNOS expression induced by CsA. Similarly, Figure 5B shows that CsA reduced the iNOS mRNA levels, and nifedipine prevented this reduction.

**Discussion**

The main findings of the present study are that CsA-induced changes in the NOS isoform pattern of expression are a tissue-specific phenomenon and that this effect can be prevented with the vasodilator drug nifedipine.

As expected, after 7 days, CsA administration was associated with renal toxicity, as was evidenced in this study by the marked reduction in GFR in CsA-treated animals. This finding is in agreement with our previous observations8,14 with micropuncture techniques. We showed that 7 days of CsA administration in uninephrectomized rats produced a significant reduction in renal function characterized by severe vasoconstriction caused by a significant increase in both afferent and efferent resistances, resulting in a marked reduction in glomerular plasma flow and single-nephron GFR.8 In this model, we have shown that CsA induces an increase in eNOS in renal cortex and reduction of nNOS and iNOS mRNA levels in medulla.
trigged by CsA, which could result in transcriptional activation of eNOS.

In addition to intracellular production of intermediate signals by CsA, it is well known that one of the main stimuli for eNOS mRNA upregulation is shear stress, which affects eNOS expression at the transcriptional level. A shear stress-responsive element has been characterized in the eNOS promoter region, and shear stress is clearly increased during vasoconstriction, which is the major mechanism responsible for CsA toxicity. Thus, increasing shear stress at the glomerular level could be another mechanism by which CsA increases eNOS mRNA levels. Thus, we reasoned that if shear stress is involved in the CsA effect on eNOS expression, the increased eNOS mRNA levels would be observed only in those tissues in which it is known that CsA produces vasoconstriction, whereas if the effect of CsA on eNOS expression is secondary to an effect of CsA itself on the eNOS gene promoter, then eNOS mRNA in all or most tissues should increase during CsA administration. Our data support the hypothesis that an increase of eNOS expression is associated with vasoconstriction because this effect was observed only in renal cortex and liver, the tissues in which CsA has been reported to induce vasoconstriction. In agreement with our findings, a preliminary report shows that CsA did not change the transcription activity of the human eNOS promoter transfected in rat pulmonary endothelial cells when the eNOS promoter was studied with a reporter gene strategy.

Because eNOS is a Ca\(^{2+}\)/calmodulin-dependent enzyme, an increase in Ca\(^{2+}\) enhances NO production, thus CsA administration may increase NO through its stimulatory effect on intracellular calcium.

In addition to the well-known effect of CsA on the kidney, this immunosuppressive drug is also hepatotoxic in a dose-dependent manner. Bile flow and secretion of bile acid, cholesterol, and phospholipids are reduced during CsA treatment. In the liver, most of the blood flow enters through the portal vein, and the resistance changes are thought to occur in the microvascular anastomosis of the portal and hepatic veins, known as hepatic sinusoids. Shah et al recently reported that in rat liver, eNOS is abundantly expressed in the sinusoidal endothelial cells and that these cells respond to shear stress by increasing NO release. In addition, similar to the glomerular afferent artery, it is known that in the liver, sinusoids function as the resistance vessels. Because we also showed increases in eNOS expression mRNA levels in the liver during CsA administration, our observations suggest that hemodynamic changes induced by CsA could be responsible for this effect.

To study whether the CsA-induced changes in the NOS isoform pattern of expression are related to the hemodynamic effects of this immunosuppressive drug, we evaluated the effect of the concomitant administration of the vasodilator nifedipine, which has been shown to produce renal as well as hepatic vasodilatation. Our results showed that nifedipine was able to prevent renal dysfunction as well as changes in the NOS isoform expression induced by CsA. These findings are in agreement with clinical studies, which suggest that calcium channels blockers are useful to reduce the side effects of CsA in the kidney. In the present study we chose nifedipine because it was previously shown by Naruse et al that this calcium channel blocker has no effect on the eNOS gene expression in the kidney. This observation was confirmed and extended in the present study in which we showed in control animals no effect of nifedipine on neither eNOS mRNA levels in renal cortex and liver nor on nNOS and iNOS in renal medulla.

In CsA-treated animals, nifedipine not only prevented the decrease in renal function but also abrogated the increase in eNOS mRNA levels in renal cortex and liver. These findings suggest that hemodynamic changes induced by CsA appear to be necessary to produce the changes in NOS isoform pattern of expression. Nifedipine administration was associated with increase in GFR in control animals as well as in the group treated with CsA. It is known that nifedipine causes vasoconstriction to renal afferent and efferent arterioles, resulting in an increase in GFR. We have shown that CsA induces a marked increase in both afferent and efferent resistances. Thus, the afferent vasoconstriction induced by nifedipine could be the mechanism by which this calcium channel blocker prevented the reduction in GFR in CsA-treated rats. The fact that renal vasoconstriction induced by CsA is associated with an increase in intracellular calcium concentration suggests that blockage of calcium input from extracellular sources by nifedipine could be the mechanism by which this agent prevents CsA-induced vasoconstriction. Another possibility derived from a recent study by Verhaar et al in which they showed that nifedipine restores the impaired NO availability in hypercholesterolemic patients and reduced superoxide generation in lysates of endothelial cells, is that in our study, nifedipine reduced the free radical production in CsA-treated rats, which in turn increased NO levels, resulting in an improvement of renal function.

As we have shown previously, CsA reduces iNOS and nNOS expression in renal medulla. Furthermore, in the present study, nifedipine administration prevented this reduction in CsA-treated rats. The reduction in iNOS and nNOS mRNA levels in renal medulla could be secondary to the medullary ischemia induced by CsA. Thus, the preservation of nNOS and iNOS mRNA levels in the present study by nifedipine in CsA-treated animals was probably mediated by restoration of the renal blood flow that prevented medullary hypoxia. According to this observation, we previously showed that the renal vasodilation induced by dexamethasone in CsA-treated rats also prevented the decrease in iNOS and nNOS mRNA levels in renal medulla. In addition, it has been suggested that nifedipine prevents interstitial fibrosis in renal transplant allograft from patients treated with CsA.

In conclusion, our data suggest that the changes in the NOS isoform pattern of expression induced by CsA appears to be, at least in part, mediated by the hemodynamic alterations induced by this immunosuppressive agent.

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Nifedipine Prevents Changes in Nitric Oxide Synthase mRNA Levels Induced by Cyclosporine
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