Angiotensin Blockade Reverses Hypertension During Long-term Nitric Oxide Synthase Inhibition

David M. Pollock, James S. Polakowski, Barbara J. Divish, and Terry J. Opgenorth

Blockade of the renin-angiotensin system was studied in male Sprague-Dawley rats during long-term inhibition of nitric oxide synthase. Nitro-L-arginine-methyl ester (L-NAME) was placed in the drinking water for 4 weeks (~100 mg/kg per day). Separate groups of rats were coadministered the angiotensin II antagonist A-81988 in the drinking water ranging from approximately 0.001 to 1 mg/kg per day. Control groups received only tap water or A-81988 alone. Each week, rats were placed in metabolic cages, and tail-cuff blood pressures and blood samples were taken. L-NAME produced a sustained elevation in tail-cuff pressure that was completely prevented by A-81988. No changes in creatinine clearance, sodium excretion, plasma creatinine concentration, or blood urea nitrogen were observed. Food and water intakes were identical in all groups. Water excretion was significantly increased in L-NAME-treated animals regardless of additional inhibitor treatment, suggesting a possible role for nitric oxide synthase in the control of water excretion; this effect was independent of blood pressure. Although less potent than A-81988, the angiotensin II antagonist losartan and the angiotensin converting enzyme inhibitor enalapril also blocked L-NAME-induced hypertension. In a separate series of experiments, rats were not given A-81988 until 2 weeks after hypertension had fully developed in L-NAME-treated rats. Within 1 week of treatment with the angiotensin II antagonist, tail-cuff pressure returned to normal. We conclude from these studies that long-term inhibition of endogenous nitric oxide production produces an angiotensin II-dependent form of hypertension.

Key words • endothelium-derived relaxing factor • nitro-L-arginine-methyl ester • hypertension, essential • angiotensin converting enzyme inhibitors • renin-angiotensin system • kidney • rat studies • renal function

The endothelium-derived relaxing factor nitric oxide (NO) appears to play a significant role in the regulation of renal and systemic hemodynamics. One line of evidence that supports such a role is the observation of increased vascular resistance and blood pressure after acute inhibition of NO synthase, the enzyme responsible for production of NO and L-citrulline from L-arginine and oxygen. Recently, it has been reported that long-term inhibition of NO synthase will produce a sustained hypertension in otherwise normotensive rats or dogs. This prolonged hypertension was associated with a mild degree of renal failure, as evidenced by decreases in glomerular filtration rate (GFR), proteinuria, and glomerular sclerotic injury. In addition to the enzyme present in vascular endothelium (type III), several isoforms of NO synthase have been identified in a variety of tissues, including the brain (type I). Interestingly, type I NO synthase is present in regions of the hypothalamus thought to be important in fluid-volume regulation, which suggests a role for this isoform in the central nervous system regulation of volume balance and arterial pressure. It is possible that the mechanism responsible for producing hypertension during administration of NO synthase inhibitors in a long-term setting may involve isoforms other than the endothelial enzyme. Long-term NO synthase inhibition may induce changes in vascular sensitivity to constrictor agents such as angiotensin II (Ang II), resulting in increased arterial blood pressure. In fact, Ribeiro et al have recently reported that an Ang II receptor antagonist, losartan, can prevent the development of this form of hypertension.

The purpose of the current study was to further investigate the effects of long-term inhibition of NO synthase on blood pressure and renal function in the conscious rat using the arginine analogue nitro-L-arginine-methyl ester (L-NAME). Importantly, we sought to determine the role of the renin-angiotensin system in this model by examining the ability of the Ang II receptor antagonist 2-{(N-propyl-N-[2'-(1H-tetrazol-5-yl)biphenyl-4-yl)ethyl]amino)pyridine-3-carboxylic acid (A-81988) to prevent hypertension produced by long-term L-NAME administration and comparing its effects to another Ang II antagonist, losartan (DuP 753), and angiotensin converting enzyme inhibition with enalapril. Experiments were also conducted to test the idea that Ang II receptor
blockade could reverse established hypertension produced by long-term L-NAME treatment.

Methods
Male Sprague-Dawley rats with an initial body weight of 200–250 g were used for these studies. After a control observation period (week 0), the following groups of rats were placed on special drinking solutions for 4 weeks: 1) regular tap water (n=5), 2) L-NAME at 67 mg/100 mL (—100 mg/kg per day, n=6), 3) A-81988 at 0.67 mg/100 mL (—1 mg/kg per day, n=6), 4) L-NAME plus A-81988 at 0.67 mg/100 mL (—1 mg/kg per day, n=6), 5) L-NAME plus A-81988 at 0.067 mg/100 mL (—0.1 mg/kg per day, n=6), 6) L-NAME plus A-81988 at 0.0067 mg/100 mL (—0.01 mg/kg per day, n=6), 7) L-NAME plus A-81988 at 0.67 µg/100 mL (—1 µg/kg per day, n=6), 8) L-NAME plus losartan at 6.7 mg/100 mL (—10 mg/kg per day, n=6), 9) L-NAME plus losartan at 0.67 mg/100 mL (—1 mg/kg per day, n=6), 10) L-NAME plus enalapril at 6.7 mg/100 mL (—10 mg/kg per day, n=6), 11) L-NAME plus enalapril at 0.67 mg/100 mL (—1 mg/kg per day, n=6), 12) L-NAME for 2 weeks and tap water only for the remaining 2 weeks (n=6), or 13) L-NAME alone for 2 weeks and then in combination with A-81988 at 0.67 mg/100 mL (—1 mg/kg per day, n=6).

The presence of L-NAME, A-81988, losartan, and enalapril had no effect on water intake, so doses could be regulated by varying the concentration of drug in the drinking water. Rats in groups 1-4 were placed in metabolic cages (Nalgene Co., Rochester, N.Y.) 2 days per week over the entire period of study. Each week, the first day served to acclimate the rats to their cages, and quantitative measurements of food and water intake and urine volume were made on the second day. Inconsistencies in the content of the rat chow were prevented by grinding and blending all of the chow before use in this study. The metabolic cages also allowed for a high degree of accuracy in the measurement of food and water intake by the inclusion of spill catches. When rats were removed from the metabolic cages, body weight determinations were made before tail-cuff pressures were measured and a blood sample via an orbital sinus bleed was taken (see below). The rats were maintained in group housing at all other times. At the end of the study, rats in groups 1-4 were anesthetized with Inactin (100 mg/kg i.p., synthesized in-house), and mean arterial pressure was measured directly via a femoral artery catheter.

Weekly estimates of systolic blood pressure were obtained by the tail-cuff method in all groups. Rats were placed in a restraining chamber and warmed to an ambient temperature of approximately 37°C, typically taking about 10–15 minutes. Automatic data collection was performed using MS-DOS-based (Modular Instruments Inc., Malvern, Pa.) or Macintosh-based (Maclab, World Precision Instruments, Sarasota, Fla.) systems. Data acquisition was synchronized to trigger a programmed electrophysmomanometer (Narco Biosystems, Austin, Tex.) used to inflate and deflate the tail cuff to a calibrated pressure at 2-minute intervals. The tail pressure pulsations were detected with a pneumatic pulse transducer (Narco Biosystems). Ten readings were taken for each rat, and the highest and lowest were discarded. Readings that had weak pressure pulsations or a high degree of noise produced by animal movement were also not used. The average was taken of the remaining readings (a minimum total of five) to generate a value for a given rat for that week.

Weekly blood samples were obtained from all rats while under anesthesia produced by exposure to a CO2:O2 gas mixture (60%:40%) in a closed chamber for approximately 30 seconds. A heparinized capillary tube was inserted into the orbital sinus to allow 1–1.5 mL blood to flow into a collection tube containing EDTA or heparin. The rats always regained consciousness within a couple of minutes. The blood samples were centrifuged and plasma was decanted and stored at 4°C until analysis. At the end of the observation period for all groups, rats in groups 1-4 were anesthetized and blood pressure was measured.

FIGURE 1. Line graphs show effect of 4-week treatment with nitro-L-arginine-methyl ester (L-NAME) and A-81988 on tail-cuff pressure (TCP), blood urea nitrogen (BUN), and plasma creatinine (Pcreat) concentrations in rats. Separate groups of rats received either L-NAME alone at approximately 100 mg/kg per day (n=6), A-81988 alone at approximately 1 mg/kg per day (n=6), both drugs at the same doses (n=6), or vehicle (tap water, n=5). *p<0.05 compared with the vehicle group for a given week.
period, rats were anesthetized with Inactin, and a terminal blood sample was taken from the abdominal aorta for determination of plasma renin activity (PRA).

L-NAME and enalapril were purchased from Sigma Chemical Co., St. Louis, Mo. Losartan was a gift from Du Pont Merck, Wilmington, Del., and A-81988 was synthesized in-house. Urinary electrolytes were measured with ion-selective electrodes (EL-ISE, Beckman Instruments, Brea, Calif.). Urinary protein concentration, plasma and urine creatinine concentrations, and blood urea nitrogen were measured by colorimetric methods with the Abbott VP System (Abbott Laboratories, Abbott Park, Ill.). PRA was evaluated by a standard radioimmunoassay method (INCSTAR, Stillwater, Minn.). Standard formulas were used to calculate urinary excretion rates. Statistical analysis to determine significant differences (p<0.05) included analysis of variance for repeated measures to determine week-to-week changes within a group and a factorial design with the Scheffe test for post hoc comparisons of individual means between groups (STATVIEW II, Abacus Concepts, Inc., Berkeley, Calif.). Animal protocols were approved by the Institutional Animal Care and Use Committee and were in full compliance with the National Institutes of Health guide.

Results

Long-term blockade of NO synthase by administration of L-NAME (~100 mg/kg per day) resulted in a persistent hypertension that reached a maximum level within 2 weeks (Figure 1). Coadministration with the Ang II receptor antagonist A-81988 (~1 mg/kg per day) fully prevented the increase in pressure. L-NAME produced no changes in renal hemodynamics as assessed by plasma creatinine concentration, blood urea nitrogen, or creatinine clearance (Figures 1 and 2). A-81988 had no effect on these variables whether given alone or with L-NAME. All four groups displayed an identical pattern of weight gain over the course of the protocol (Figure 3). The relative differences in tail-cuff estimates of arterial pressure were confirmed in these groups by direct measurement of mean arterial pressure in anesthetized animals at the end of the 4-week treatment period. Rats treated with L-NAME alone had a significantly higher mean arterial pressure (126±6 mm Hg) compared with vehicle-treated rats (84±4 mm Hg). A-81988 significantly reduced the effect of L-NAME (101±8 mm Hg), although values were significantly higher than rats receiving A-81988 alone (84±3 mm Hg); the difference between the L-NAME plus A-81988 and vehicle groups did not achieve statistical significance.

Figure 2. Line graphs show effect of 4-week treatment with nitro-L-arginine-methyl ester (L-NAME) and A-81988 on 24-hour urinary protein excretion, urine osmolality (Uosm), and creatinine clearance (Ccreat) in rats. Separate groups of rats received either L-NAME alone at approximately 100 mg/kg per day (n=6), A-81988 alone at approximately 1 mg/kg per day (n=6), both drugs at the same doses (n=6), or vehicle (tap water, n=5).

Figure 3. Line graph shows body weight of animals receiving 4-week treatment with nitro-L-arginine-methyl ester (L-NAME), A-81988, or both. Separate groups of rats received either L-NAME alone at approximately 100 mg/kg per day (n=6), A-81988 alone at approximately 1 mg/kg per day (n=6), both drugs at the same doses (n=6), or vehicle (tap water, n=5).
TABLE 1. Effect of Long-term Inhibition of Nitric Oxide Synthase on Water Excretion and Intake With or Without Coadministration of A-81988 in Conscious Rats

<table>
<thead>
<tr>
<th>Group</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water excretion (mL/day)</td>
<td>30.3±1.5</td>
<td>30.2±1.7</td>
<td>30.2±1.7</td>
<td>30.2±1.7</td>
<td>30.2±1.7</td>
</tr>
<tr>
<td>Control (vehicle)</td>
<td>31.0±1.1</td>
<td>31.1±1.9</td>
<td>29.9±1.9</td>
<td>30.3±1.5</td>
<td>30.2±1.7</td>
</tr>
<tr>
<td>L-NAME alone</td>
<td>30.0±2.4</td>
<td>36.0±3.9</td>
<td>37.0±2.8</td>
<td>34.5±2.9</td>
<td>36.6±4.3</td>
</tr>
<tr>
<td>L-NAME + A-81988</td>
<td>29.6±1.6</td>
<td>30.6±1.4</td>
<td>34.7±3.6</td>
<td>36.5±3.6*</td>
<td>36.8±2.9*</td>
</tr>
<tr>
<td>A-81988 alone</td>
<td>29.3±1.2</td>
<td>30.8±1.9</td>
<td>32.3±2.0</td>
<td>30.8±1.5</td>
<td>34.5±3.4</td>
</tr>
</tbody>
</table>

L-NAME, nitro-L-arginine-methyl ester. Values are mean±SEM. *p<0.05 vs. week 0.

Water excretion and intake in untreated controls and L-NAME-treated rats with and without A-81988 are listed in Table 1. Urine flow rate tended to be higher in animals receiving L-NAME independent of A-81988 treatment. Factorial analysis revealed that, whether or not animals received A-81988, urine flow rate was significantly greater in animals receiving L-NAME (L-NAME alone and L-NAME plus A-81988) compared with the non-L-NAME groups (vehicle plus A-81988 alone) during the first 3 weeks of treatment (p<0.05). This trend was not observed for sodium excretion, because values were similar between groups at each week during long-term L-NAME administration (Table 2).

Urine protein excretion, urine osmolality, and renal clearance of endogenous creatinine were also unaffected by long-term administration of L-NAME for 4 weeks (Figure 2). Administration of A-81988 (−1 mg/kg per day) had no effect on any of these variables.

Up to 100-fold lower doses of A-81988 (~0.01 mg/kg per day) also prevented the hypertension produced by long-term NO synthase inhibition (Figure 4). Similar to A-81988, losartan blocked L-NAME-induced increases in tail-cuff pressure, although it required more than 100-times more drug to block the hypertension; the dose of 10 mg/kg per day completely prevented the rise in blood pressure, whereas 1 mg/kg per day had no effect (Figure 4). Enalapril displayed a dose–response profile similar to that of losartan. Administration of losartan or enalapril had no effect on plasma creatinine levels or blood urea nitrogen in L-NAME–treated rats (data not shown).

At the end of the 4-week study period, PRA was measured in a terminal blood sample from the abdominal aorta in all rats (Table 3). PRA was slightly, but not significantly, lower in the group receiving L-NAME alone. Administration of A-81988, losartan, or enalapril significantly increased PRA levels in a dose-dependent fashion. To further investigate the tendency of PRA to be lower in L-NAME–treated rats, we measured PRA in two separate groups of rats receiving either L-NAME alone or tap water, but this time we collected blood via an orbital sinus bleed to minimize elevations in PRA due to sampling technique. With the use of this method, L-NAME significantly decreased PRA as measured at weeks 2 and 4 (Figure 5).

Another series of experiments was performed to determine if Ang II receptor blockade with A-81988 could reverse the already established hypertension produced by L-NAME. In these studies, administration of A-81988 was delayed until 2 weeks into the protocol, well after hypertension had developed. Once again, A-81988 at approximately 1 mg/kg per day reversed the effect (Figure 4). Enalapril displayed a dose–response profile similar to that of losartan. Administration of losartan or enalapril had no effect on plasma creatinine levels or blood urea nitrogen in L-NAME–treated rats (data not shown).

TABLE 2. Effect of Long-term Inhibition of Nitric Oxide Synthase on Sodium Excretion and Intake With or Without Coadministration of A-81988 in Conscious Rats

<table>
<thead>
<tr>
<th>Group</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium excretion (mmol/day)</td>
<td>2.4±0.1</td>
<td>2.8±0.2</td>
<td>3.0±0.1*</td>
<td>3.2±0.2*</td>
<td>3.2±0.1*</td>
</tr>
<tr>
<td>Control (vehicle)</td>
<td>2.5±0.1</td>
<td>2.9±0.2</td>
<td>3.2±0.1*</td>
<td>3.5±0.1*</td>
<td>3.1±0.1*</td>
</tr>
<tr>
<td>L-NAME alone</td>
<td>2.5±0.1</td>
<td>3.1±0.3</td>
<td>3.2±0.2*</td>
<td>3.4±0.1*</td>
<td>3.4±0.1*</td>
</tr>
<tr>
<td>L-NAME + A-81988</td>
<td>2.5±0.1</td>
<td>2.8±0.1*</td>
<td>3.2±0.1*</td>
<td>3.4±0.2*</td>
<td>3.2±0.1*</td>
</tr>
<tr>
<td>A-81988 alone</td>
<td>3.2±0.1</td>
<td>4.0±0.2</td>
<td>4.1±0.2</td>
<td>4.1±0.2</td>
<td>3.9±0.1</td>
</tr>
<tr>
<td>Sodium intake (mmol/day)</td>
<td>3.9±0.1</td>
<td>3.9±0.1</td>
<td>3.9±0.1</td>
<td>4.0±0.2</td>
<td>4.1±0.1</td>
</tr>
</tbody>
</table>

L-NAME, nitro-L-arginine-methyl ester. Values are mean±SEM. *p<0.05 vs. week 0.
FIGURE 4. Line graphs show effect of 4-week treatment with A-81988, losartan, or enalapril on tail-cuff pressure (TCP) in rats during long-term inhibition of nitric oxide synthase. Separate groups of rats received nitro-L-arginine-methyl ester (L-NAME) at approximately 100 mg/kg per day along with the inhibitor at the indicated dose. *p<0.05 vs. L-NAME alone.

To test the reversibility of L-NAME–induced hypertension, we gave another group of rats L-NAME for 2 weeks and then just tap water for another 2 weeks. Removal of L-NAME from the drinking water resulted in a return to normotensive pressure within 1 week (Figure 6).

FIGURE 5. Bar graph shows plasma renin activity in rats receiving nitro-L-arginine-methyl ester (L-NAME) at approximately 100 mg/kg per day in drinking water at 2 and 4 weeks of treatment. Blood was obtained from the orbital sinus. n=6 for each group. *p<0.05 vs. control.
readily apparent, because PRA was decreased by L-NAME. One possible explanation for the efficacy of Ang II antagonists or angiotensin converting enzyme inhibitors is that long-term blockade of NO synthase activity may result in hypersensitivity to vasoconstrictors, such as Ang II, due to the removal of the normal modulating influence of endothelium-derived NO. It is unlikely that these agents act through nonspecific effects, because all three inhibitors could reverse L-NAME-induced hypertension, albeit with differing potencies. It is also important to note, however, that these inhibitors lower blood pressure in non–renin-dependent forms of hypertension such as the spontaneously hypertensive rat (unpublished observations from our laboratory and Reference 12).

We observed that long-term L-NAME administration decreased PRA, confirming the observations of Ribeiro et al and Arnal et al. Signor et al recently provided evidence that inhibition of NO synthase decreases PRA in vivo as a result of the elevation in renal perfusion pressure and a decrease in β-adrenergic activity despite the fact that NO production may actually inhibit renin release in isolated renal tissue. As expected, inhibition of the renin-angiotensin system and the consequent removal of negative feedback on renin release with or without L-NAME administration resulted in a significant elevation in PRA. Inhibition of NO synthase activity with L-NAME also produces a significant decrease in cyclic GMP content of the arterial wall. The lack of cyclic GMP–producing capability in vascular smooth muscle may also be a mechanism for L-NAME–induced hypertension.

Other investigators have reported a small, but significant, decrease in GFR in the chronically L-NAME–treated rat, suggesting an important role for endothelium-derived NO in long-term regulation of renal hemodynamics. Baylis et al and Ribeiro et al reported a 30–34% decrease in GFR when measured in anesthetized rats after 4–8 weeks of treatment. This was observed despite the fact that the former study used a dose of L-NAME roughly one third of our dose. If, as has been suggested, long-term blockade of endothelium-derived NO production heightens sensitivity to vasoconstrictors, anesthesia and surgical stress may result in a heightened degree of renal vasoconstriction. Preliminary evidence shows that a change in sensitivity to vasoconstrictors is not unique to Ang II, because prazosin also lowers blood pressure in this model. The length of treatment with L-NAME may also be critical to observing an effect on renal function. If this were the case, however, it would still remain to be determined whether blockade of NO production per se or prolonged elevation of arterial pressure was the causative factor. Consistent with measurements of renal hemodynamic indexes, the lack of changes in urinary protein excretion also contrasts with the findings of Baylis et al, who observed a maximal increase in protein excretion, roughly twofold, after 4 weeks at a lower dose of L-NAME. The degree of hypertension does not appear to be different between studies, although this cannot be confirmed because their measurements were made in anesthetized rats, whereas the present investigation used the tail-cuff technique. The rat strain may be another factor in determining the susceptibility to renal vasoconstriction induced by long-term NO synthase inhibition. Baylis et al and Ribeiro et al used Munich-Wistar rats, whereas we used the Sprague-Dawley strain. In any event, small decreases in renal blood flow, GFR, or both may have remained undetected with the methodology used in the present study.

We used a relatively high dose of L-NAME because we wanted to be confident of producing a maximal inhibition of NO synthase. In pilot studies, we observed that higher doses of L-NAME produced no further increases in systolic pressure. This is important because the l-arginine–based inhibitors have varying potencies for the different isoforms of NO synthase. With submaximal doses, one cannot be confident that NO synthase activity in all tissues is inhibited. The dose of 100 mg/kg per day used in the present study was also shown by Arnal et al to produce a maximal increase in arterial pressure.

Several studies have reported that acute intravenous administration of l-arginine analogues will produce a large degree of renal vasoconstriction, suggesting a role for endothelium-derived NO in the maintenance of renal hemodynamics. Several studies have reported that acute intravenous administration of l-arginine analogues will produce a large degree of renal vasoconstriction, suggesting a role for endothelium-derived NO in the maintenance of renal hemodynamics. Several studies have reported that acute intravenous administration of l-arginine analogues will produce a large degree of renal vasoconstriction, suggesting a role for endothelium-derived NO in the maintenance of renal hemodynamics.
diuresis, although the precise mechanism is unknown. Because sodium excretion did not follow a similar pattern as water excretion, the most probable explanation is that L-NAME interferes with release of antidiuretic hormone in the pituitary or antidiuretic hormone action along the collecting duct. Evidence to support the former explanation arises from the observations that NO synthase is present in the posterior pituitary and hypothalamus.\textsuperscript{5,6} To summarize, the present study demonstrated that inhibition of the renin-angiotensin system not only prevents L-NAME–induced hypertension in the rat but also reverses established hypertension in this model. These findings support the hypothesis that Ang II plays a critical role in the development of hypertension resulting from long-term inhibition of NO synthase. We confirmed previous observations that long-term administration of L-NAME results in a sustained hypertension and extended those observations to provide evidence that NO synthase may be important in the regulation of water excretion independent of its relation with Ang II. Future investigations should focus on the mechanism responsible for the interaction between NO synthase activity and the renin-angiotensin system.

Acknowledgments

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References

Angiotensin blockade reverses hypertension during long-term nitric oxide synthase inhibition.
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