Local Renal Medullary L-NAME Infusion Enhances the Effect of Long-Term Angiotensin II Treatment

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Abstract—We hypothesized that the relatively high doses of angiotensin (Ang) II required to produce hypertension in rats were related to stimulation of renal medullary nitric oxide production, which in turn blunted reductions in medullary blood flow and the development of hypertension. Ang II was infused (5 days at 3 ng · kg⁻¹ · min⁻¹ IV) to uninephrectomized Sprague-Dawley rats in the presence and absence of a continuous medullary interstitial N⁶-nitro-L-arginine methyl ester (L-NAME) infusion. Renal cortical and medullary blood flows were determined with the use of implanted optical fibers and laser-Doppler flowmetry. Ang II in the absence of medullary nitric oxide synthase inhibition did not change cortical or medullary blood flow or mean arterial pressure. A threshold dose of L-NAME was determined (75 μg · kg⁻¹ · h⁻¹) that did not produce significant short- or long-term changes in medullary blood flow and mean arterial pressure. In rats with blunted medullary nitric oxide synthase activity, Ang II infused intravenously resulted in a 30% reduction in medullary blood flow (from 1.3 to 0.9±0.2V) and a 20 mm Hg increase in mean arterial pressure with Ang II infusion over 5 days. During 70 minutes after the start of intravenous Ang II, there was an immediate reduction in medullary blood flow, with no changes in cortical blood flow or mean arterial pressure. We conclude that the relative insensitivity of rats to long-term elevations of circulating Ang II is due to the potent counterregulatory actions of the nitric oxide system, specifically within the renal medulla. The results provide novel insights of how the organism attempts to protect itself from the hypertensive effects of Ang II. (Hypertension. 1999;33[part II]:440-445.)

Key Words: hypertension, renal kidney angiotensin II renal blood flow nitric oxide nitric oxide synthase

It is well recognized that elevations of circulating angiotensin (Ang) II cause resetting of the pressure-natriuresis relationship to elevated levels of arterial blood pressure through the renal retention of sodium and volume expansion.¹ Yet considerable species differences have been found in the concentrations of Ang II required to produce chronic hypertension. We have found that Ang II infused long term at concentrations of 3 to 5 ng · kg⁻¹ · min⁻¹ produce a 30 mm Hg increase in mean arterial pressure (MAP) over a period of 1 week in mongrel dogs maintained on a normal daily NaCl diet.² Humans exhibit even greater pressor sensitivity to prolonged infusions of Ang II.³ In contrast, prolonged infusion of Ang II at 3 to 5 ng · kg⁻¹ · min⁻¹ in Sprague-Dawley rats does not lead to a significant increase in MAP,⁴ and investigators generally administer Ang II at rates of 30 to 60 ng · kg⁻¹ · min⁻¹ to induce prolonged hypertension in rats.⁵

A number of studies have demonstrated that the medullary vasculature of Sprague-Dawley rats is relatively refractive to the vasoconstrictor effects of Ang II compared with the cortical circulation.⁶,⁷ Subpressor doses of Ang II administered intravenously that can significantly reduce renal cortical blood flow (CBF) fail to change medullary blood flow (MBF).⁶,⁷ Yet there appears to be an abundance of Ang II receptors within the renal medulla,⁸ and Pallone⁹ has shown that Ang II is a potent constrictor of isolated-perfused rat medullary vasa recta vessels. Recent studies in our laboratory have demonstrated that medullary vascular insensitivity is in large measure a result of Ang II–stimulated medullary nitric oxide (NO) production, which effectively offsets the vasoconstrictor effects of Ang II.¹⁰ Because a number of studies in our laboratory also have shown that long-term reductions in renal MBF can result in hypertension,¹ we explored in the present study the possibility that the relative insensitivity of rats to elevations of circulating Ang II could be a consequence of the relative insensitivity of the renal medullary circulation. More specifically, we examined the hypothesis that elevations of circulating Ang II stimulate production of NO specifically within the renal medulla, which in turn buffers the medullary actions of this peptide and reduces its hypertensive effects.

Ang II can stimulate at least several counterregulatory vasodilator systems within the renal medulla. Pretreatment with cyclooxygenase⁶ or NO synthase (NOS) inhibitors¹⁰ greatly enhances the medullary vascular constrictor actions of

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Ang II in anesthetized Sprague-Dawley rats. NOS protein expression, enzyme activity, and NO concentration have been found to be significantly greater in the renal medulla compared with the cortex of Sprague-Dawley rats. Studies were then carried out to determine whether subpressor infusions of Ang II (5.0 ng·kg⁻¹·min⁻¹ IV) resulted in more than a doubling of medullary NO, consistent with observations by others using more indirect indexes of NO production, including measurements of cGMP and nitrate and/or nitrite.

The present study was designed to reduce NOS activity only within the region of the renal medulla. Furthermore, the objective was to only moderately reduce NOS activity in this region by an amount that blunts the counterregulatory actions of NO but not to the extent that would lead to a significant reduction in MBF and hypertension. Rather, the goal was to see whether slight reductions in NO production, specifically within the renal medulla alone, would sensitize the animal to small elevations of circulating Ang II and lead to hypertension. Uninephrectomized Sprague-Dawley rats were instrumented with arterial and venous catheters and a small catheter implanted into the renal medulla for long-term delivery of the NOS inhibitor L-NAME (L-NAME). Optical fibers implanted in the superficial cortex and inner medulla were used for daily measurements of changes in blood flow to these regions with laser-Doppler flowmetry. The effects of long-term intravenous infusion of Ang II (3 ng·kg⁻¹·min⁻¹) on MAP, CBF, and MBF were then determined. An Ang II dose of 3 ng·kg⁻¹·min⁻¹ was chosen when it was predetermined that a similar dose did not lower MBF over the short term or result in hypertension during 3 to 5 days of intravenous administration. Studies were then carried out in which a subpressor dose of L-NAME was infused continuously into the interstitial space of the inner medulla while the effects of the same intravenous dose of Ang II were determined. All experiments were carried out in unanesthetized rats in their home cages, with daily measurements determined at the same time each day.

**Methods**

**Chronic Surgical Preparation and Experimental Procedures**

Experiments were performed on Sprague-Dawley rats (250 to 350 g; Harlan Sprague Dawley Inc). All animals were housed individually and maintained on normal rat chow and water ad libitum. All procedures were approved by the Institutional Animal Care Committee. To eliminate compensatory responses from the contralateral kidney, all rats were unilaterally nephrectomized with ketamine (30 mg/kg IM) and xylazine (2 mg/kg IM) anesthesia. All surgeries were performed under aseptic conditions, and 7 to 10 days was allowed for recovery.

A second surgery was then carried out for catheter and optical fiber implantations. The femoral artery and vein were exposed for insertion of the in-dwelling arterial and venous catheters as described previously. The left kidney was then exposed via a flank incision, and an extruded polyethylene interstitial catheter (tip size, ~100 μm in diameter) was implanted in the renal medulla to a depth of ~5.5 mm as described previously. Optical fibers were then implanted in the renal cortex (2-mm depth) and inner medulla (5.5-mm depth) by use of techniques developed in our laboratory.

**Daily Measurement of MAP, CBF, and MBF**

During the 1 week after surgery, the rats were trained to rest in a tubular Plexiglas restrainer within their home cages for 2 hours each day. Daily measurements of MAP, CBF, and MBF were then begun by use of an on-line data collection system (Pall A.K.A.) and analysis software (A.L.). The flow signals from the renal cortex and medulla were measured and processed by a 2-channel laser-Doppler flowmeter (Transonics, Inc). Continuously recorded signals were transformed to minute averages for analysis.

**Experimental Protocols**

**Protocol 1: Effect of L-NAME Infusion Into the Renal Medulla**

After 1 week of recovery from surgery, MAP was measured in a group of Sprague-Dawley rats (n=4) at the same time each morning to obtain values from 3 stable control days. A medullary interstitial infusion of L-NAME (75 μg·kg⁻¹·h⁻¹) was then started, and MAP was measured daily for 10 days.

**Protocol 2: Long-Term Effects of Subpressor Infusion of Ang II (3.0 ng·kg⁻¹·min⁻¹ IV) on MAP, CBF, and MBF in the Absence of Medullary L-NAME Infusion**

After 1 week of recovery from surgery and after 3 days of stable control MAP, CBF, and MBF measurements, an infusion of Ang II (Ile₅-angiotensin II, Sigma Chemical Co) was continued for 5 days while daily measurements of MAP, CBF, and MBF were obtained. A similar dose has been shown previously to be nonhypertensive over a period of 1 week of continuous infusion in Sprague-Dawley rats fed normal rat chow. On the morning that the Ang II infusion was begun, control measurements were obtained for the following 2 days. Isotonic saline was infused at a rate of 0.5 mL/h throughout the experiment as a vehicle control for protocol 3 in which L-NAME diluted in isotonic saline was infused at the same dose.

**Protocol 3: Effect of Subpressor Dose of Intravenous Ang II Infusion on MAP, CBF, and MBF in Rats Receiving a Continuous Infusion of L-NAME Into the Renal Medulla**

After recovery from surgery and 3 days of stable control MAP, CBF, and MBF measurements, an infusion of Ang II was begun at a dose of 3 ng·kg⁻¹·min⁻¹ IV (n=7). The infusion of Ang II was continued for 5 days while daily measurements of MAP, CBF, and MBF were obtained. A similar dose has been shown previously to be nonhypertensive over a period of 1 week of continuous infusion in Sprague-Dawley rats fed normal rat chow. On the morning that the Ang II infusion was begun, control measurements were obtained during the first hour of recording and throughout the first 70 minutes after the start of Ang II infusion. After 5 days, the Ang II infusion was ended, and daily measurements were obtained each morning for the following 2 days. Isotonic saline was infused at a rate of 0.5 mL/h throughout the experiment as a vehicle control for protocol 3 in which L-NAME diluted in isotonic saline was infused at the same dose.
pressure and renal blood flows were obtained for 2 additional days while medullary L-NAME infusion was continued.

Statistical Analysis
Data are presented as mean ± SEM. For statistical comparisons, 1-way ANOVA with repeated measures was used, and Duncan’s multiple range test as a post hoc test was carried out. All statistical analyses were performed on the raw data. We considered P < 0.05 to be statistically significant.

Results

Protocol 1: Effect of L-NAME Infusion Into the Renal Medulla

Infusion of L-NAME into the renal medulla (n = 4) had no significant effect on MAP measured for 10 days. MAP averaged 107 ± 4 mm Hg during saline control and never rose >4 mm Hg over the 10 days of L-NAME infusion. On day 10 of L-NAME infusion, MAP averaged 110 ± 1 mm Hg. These results indicate that the dose of L-NAME used in these studies did not reduce medullary NO production or MBF sufficiently to cause hypertension but rather merely blunted the Ang II–induced increase in NO production as we have measured directly and previously reported.10

Protocol 2: Immediate- and Long-Term Effects of Subpressor Dose of Ang II (3.0 ng · kg⁻¹ · min⁻¹ IV) on MAP, CBF, and MBF in the Absence of Medullary L-NAME Infusion

The effects of long-term administration of Ang II (3.0 ng · kg⁻¹ · min⁻¹ IV; n = 7 rats) in the absence of medullary administration of L-NAME are summarized in Figure 1. MBF, CBF, and MAP were not significantly changed during the 5-day period of Ang II infusion, although there was a tendency for MAP to rise above the control value of 114 ± 2 mm Hg to an average of 121 ± 2 mm Hg during days 4 and 5. MBF averaged 1.3 ± 0.2 V and CBF averaged 3.6 ± 0.8 V over the 3 days of unanesthetized control measurements.

The immediate responses during the first hour after the start of the intravenous Ang II are summarized in Figure 2. In the absence of medullary L-NAME, when only saline was infused into the renal medulla, Ang II resulted in no significant changes in MBF, CBF, or MAP. Resting control values of MAP averaged 113 ± 2 mm Hg, CBF averaged 3.0 ± 0.7 V, and MBF averaged 1.2 ± 0.4 V in these unanesthetized rats (n = 6).

Protocol 3: Immediate- and Long-Term Effects of Subpressor Dose of Ang II (3.0 ng · kg⁻¹ · min⁻¹ IV) on MAP, CBF, and MBF in Rats Receiving a Continuous Infusion of L-NAME Into the Renal Medulla

Figure 3 summarizes the effects of long-term intravenous Ang II infusion on MBF, CBF, and MAP of rats (n = 5) receiving an infusion of medullary L-NAME. The results in this figure indicate that the amount of L-NAME administered to these rats was very near the threshold dose required to lower MBF and elevate MAP. That is, the average MBF control value of 1.5 ± 0.15 V was reduced slightly but not significantly to 1.3 ± 0.1 V, and the MAP of 116 ± 2 mm Hg rose to 119 ± 3.5 mm Hg during the 3 days of medullary L-NAME infusion before intravenous Ang II infusion. Greater changes than this were prevented if necessary by slight adjustments of the L-NAME infusion rate during this period because the goal of this study was to only moderately blunt the ability of Ang II to stimulate NO production, as would be more likely in naturally occurring pathological states.

The most important observation summarized in Figure 3 is that the 3.0-ng · kg⁻¹ · min⁻¹ IV dose of Ang II, which was subpressor in the absence of medullary L-NAME administration (Figures 1 and 2), resulted in significant elevations of MAP, which rose to levels averaging 137 ± 4 mm Hg on days
The renal hemodynamic responses recorded during the initial 70 minutes after the initiation of the intravenous Ang II infusion (Figures 2 and 4) generally confirmed the previous results of both Mattson et al.\(^6,7\) and Zou et al.,\(^10\) which were consistent with recent studies by Zou et al.,\(^10\) who found that the medullary circulation of anesthetized Sprague-Dawley rats was very refractory to Ang II vasoconstrictor actions. Specifically, infusions of Ang II at concentrations of 20 ng \( \cdot \) kg\(^{-1} \) \( \cdot \) min\(^{-1}\) IV produced significant reductions in CBF, whereas papillary blood flow was not significantly altered. Indeed, Nobes et al.\(^20\) reported increases in papillary blood flow when even higher concentrations of Ang II (300 ng \( \cdot \) kg\(^{-1} \) \( \cdot \) min\(^{-1}\)) were infused.

The relative insensitivity of the medullary circulation to Ang II may be explained in part by the stimulation of medullary prostaglandin\(^6\) or kinin production,\(^20\) although the long-term effects of these pathways on MBF remain to be explored. The present study, however, demonstrates that Ang II-induced NO production contributes importantly to the hypertensive actions of circulating Ang II. These results are consistent with recent studies by Zou et al.,\(^10\) who found that subpressor doses of Ang II (5.0 ng \( \cdot \) kg\(^{-1} \) \( \cdot \) min\(^{-1}\) IV) stimulated renal medullary NO production and that acute medullary interstitial administration of L-NAME in amounts similar to those used in the present study greatly enhanced the ability of Ang II to reduce MBF, independent of changes in CBF.

The renal hemodynamic responses recorded during the initial 70 minutes after the initiation of the intravenous Ang II infusion (Figures 2 and 4) generally confirmed the previous results of both Mattson et al.\(^6,7\) and Zou et al.,\(^10\) which were novel insights into how the organism attempts to protect itself from the hypertensive effects of this potent pressor peptide. Although the role of Ang II in the regulation of whole-kidney hemodynamics and glomerular-tubular functions has been studied extensively,\(^18,19\) far less is known about the role of Ang II in deep nephrons and the renal medullary microcirculation, which have been technically more difficult to study. MBF has been reported to increase,\(^20\) decrease,\(^21\) or remain unchanged\(^22\) during Ang II infusion to the anesthetized rat. More recently, by using laser-Doppler flowmetry techniques enabling the direct and continuous recording of changes in MBF, Mattson et al.\(^6,7\) found that the medullary circulation of anesthetized Sprague-Dawley rats was very refractory to Ang II vasoconstrictor actions. Specifically, infusions of Ang II at concentrations of 20 ng \( \cdot \) kg\(^{-1} \) \( \cdot \) min\(^{-1}\) IV produced significant reductions in CBF, whereas papillary blood flow was not significantly altered. Indeed, Nobes et al.\(^20\) reported increases in papillary blood flow when even higher concentrations of Ang II (300 ng \( \cdot \) kg\(^{-1} \) \( \cdot \) min\(^{-1}\)) were infused.

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obtained in anesthetized rats. That is, only rats receiving the medullary infusion of L-NAME responded to the intravenous suppressor dose of Ang II with significant reductions in MBF. It is apparent from the immediate reduction in MBF at the start of the Ang II infusion that these changes preceded the increase in MAP, indicating that the reduction in blood flow to the renal medulla initiated the hypertension. These initial responses were not determined in the first 3 rats used for the long-term studies, which accounts for the smaller number analyzed to determine the immediate changes. The rats were examined on the first day with the recognition that the within-day variation in flow measurement is less than the between-day variation. We believe that this accounts for the statistically significant reduction in MBF seen during the first 70 minutes of infusion that was not fully apparent in the long-term study until greater reductions of flow had occurred by day 3 of Ang II infusion.

**Initial Responses to Intravenous Ang II Infusion**

An important implication of this study is that very small elevations of circulating Ang II can stimulate the production of medullary NO, which serves to buffer the vasoconstrictor actions of Ang II in the medullary circulation for the long term and protect against hypertension. We have found that the renal medulla is particularly rich in NOS enzyme activity, protein expression, and NO concentration, which are much higher compared with the cortex.10–12 Small suppressor doses of Ang II were found to result in significant elevations of medullary [NO] as determined by microdialysis oxyhemoglobin-NO trapping techniques.10

Short-term studies by others also support the conclusions that Ang II releases NO and that inhibition of NOS activity enhances the renal vasoconstrictor actions of Ang II in rat kidneys.23 Ang II infusion has been reported to elevate renal excretion of nitrate and/or nitrite14 and increase cGMP concentration in renal cortical interstitial fluid.13 Madrid et al24 have shown that renal NO production buffers Ang II effects on pressure-natriuresis and on the CBF and papillary blood flow.25 Taken together with the initial hemodynamic responses to Ang II seen in the present study, these results indicate that when medullary NOS activity is only moderately reduced, vasoconstrictor actions of Ang II within the renal medulla of rats are greatly enhanced.

**Long-Term Effects of Ang II on MBF and the Role of Medullary NO in Buffering These Actions**

The major contribution of the present study was the demonstration that long-term reduction in NOS activity, specifically in the renal medulla, renders the animal vulnerable to very small elevations of plasma Ang II, resulting in sustained reductions in MBF and hypertension. Given the variance of laser-Doppler measurements between days and the relatively small number of rats used in these studies, this reduction in MBF was not statistically significant until the third day of Ang II infusion. However, 6 of the 9 rats showed a clear reduction in MBF >25% on the morning after the start of Ang II infusion (eg, 22 hours). The sequence of events leading to the slow increase in arterial pressure was not determined in the present study, although it is presumed that the reduction of MBF leads to enhanced tubular reabsorption of sodium, leading to volume expansion and hypertension. Direct tubular actions of deep nephron segments and the collecting duct cannot be excluded. Long-term infusion of Ang II at 5.0 ng·kg⁻¹·min⁻¹ IV has been shown to increase plasma Ang II concentrations of Sprague-Dawley rats from 11.3 to 19.7 pg/mL,4 whereas plasma Ang II concentrations increased from 3.9 to 18.8 pg/mL when rats were switched from a 4.0% to 0.4% salt diet. This indicates that changes in plasma Ang II within the physiological range can have important consequences on MBF and MAP in situations in which medullary NO production is blunted. The reason for the slow return of MBF and MAP toward control levels after the cessation of Ang II infusion is unclear. CBF remained unchanged, and morphological examination of the kidneys at the end of the study did not reveal sufficient fibrosis surrounding the infusion catheter or optical fibers to explain these events. It is possible that the continued medullary infusion of L-NAME after cessation of the Ang II infusion retarded the loss of sodium and water.

As seen in the present study, moderate reductions in medullary NOS enzyme activity alone may be insufficient to produce hypertension but can make the organism vulnerable to the hypertensive actions of Ang II. As shown clearly in this study, Ang II infused long term at these low concentrations did not normally produce hypertension (Figure 1). It is also important to recognize that the results of our studies indicate that L-NAME infused long term into the renal medulla did not escape in sufficient amounts to exert vasoconstrictor actions on the systemic vasculature. Neither short- nor long-term reductions in CBF nor significant elevations of MAP were observed with medullary infusion of L-NAME. If recirculation of L-NAME had occurred, one would have expected reductions in CBF during the first 4 hours of the medullary L-NAME infusion, changes that were not observed. The renal cortical vasculature is more sensitive than the medullary vessels to Ang II,6,7,15 so blunting of NOS activity in the renal cortex should have amplified these differences. The enhanced vasoconstrictor effects of Ang II were confined strictly to the renal medulla. By the same reasoning, if L-NAME escaped from the kidney in sufficient amounts to influence the systemic circulation, one would have anticipated an increase in MAP during the first several hours of medullary infusion.

Changes in the endogenous renin-angiotensin system are not likely to be a reason for the different sensitivity to Ang II in L-NAME–pretreated and nontreated rats. L-NAME has been shown to increase plasma Ang II concentrations,26 which in itself would be expected to reduce the pressor actions of infused Ang II. Exogenous Ang II even at this low dose (3 ng·kg⁻¹·min⁻¹) has been shown to suppress plasma renin activity so that plasma Ang II levels are only slightly increased.4 The same would be expected to occur in the L-NAME–treated rats. Thus, the hypertension in the present study appears to have been caused by the inability of medullary NO to buffer the vasoconstrictor actions of Ang II and not by other mechanisms.

In summary, the present study indicates that the renal medullary production of NO serves as an important counter-
A regulatory mechanism to buffer the hypertensive effects of elevations in circulating Ang II. The great responsiveness of the medullary NO system to Ang II in rats appears to account in large measure for the relative insensitivity of this species to the hypertensive effects of Ang II.

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