Increase in Renal Medullary Nitric Oxide Synthase Activity Protects From Norepinephrine-Induced Hypertension

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Abstract—Studies were performed in conscious Sprague-Dawley rats to determine the role of the $\alpha_2$-adrenergic receptor–mediated increase in the renal medullary nitric oxide synthase (NOS) activity as a counterregulatory mechanism of blood pressure control in response to increased renal adrenergic stimulation. A subpressor dose of norepinephrine (NE, 8 $\mu$g · kg$^{-1}$ · h$^{-1}$) was infused intravenously, and NOS activity was determined with arginine-citrulline conversion by high-performance liquid chromatography in renal cortical and outer and inner medullary tissues. It was found that after 7 days of intravenous NE infusion, NOS activity was significantly higher in both the outer and inner medullary tissues (158±45 versus 30±24 pmol · mg$^{-1}$ · h$^{-1}$ [outer medulla] and 5.1±0.7 versus 2.0±0.5 nmol · mg$^{-1}$ · h$^{-1}$ [inner medulla]) for NE-treated versus control rats, respectively. To determine whether the increase of NOS activity was mediated through renal medullary $\alpha_2$-receptors, the receptor antagonist rauwolscine (RAU, 1 $\mu$g · kg$^{-1}$ · min$^{-1}$) was infused via an implanted renal medullary interstitial catheter, and the consequences of intravenous NE administration were evaluated. NOS activity was significantly lower in the RAU-infused animals and did not increase with infusion of NE. To determine the systemic effects of the renal medullary $\alpha_2$-receptors, studies were performed to determine the consequences of chronic intravenous infusion of subpressor amounts of NE in the presence and absence of renal medullary $\alpha_2$-receptor inhibition. Under conditions in which RAU was continuously infused into the renal medulla, the same subpressor dose of NE caused sustained and reversible hypertension (mean arterial pressure increased from 120±3 to 131±3 mm Hg). Chronic blunting of the renal medullary NOS activity with $N^\gamma$-nitro-$L$-arginine methyl ester (75 $\mu$g · kg$^{-1}$ · h$^{-1}$) also enabled NE to produce a significant rise in mean arterial pressure (from 117±2 to 134±4 mm Hg). We conclude that the hypertensive effects of moderate elevations of renal adrenergic activity were chronically buffered by the $\alpha_2$-receptor–mediated increase in NOS activity within the renal medulla. (Hypertension. 2000;35[part 2]:418-423.)

Key Words: norepinephrine $\bullet$ receptors, adrenergic, alpha $\bullet$ rauwolscine $\bullet$ nitric oxide synthase $\bullet$ rats

It has been found that small subpressor amounts of intravenously infused norepinephrine (NE) can stimulate renal medullary nitric oxide concentration (NO) in anesthetized Sprague-Dawley rats.¹ This NE-stimulated medullary nitric oxide (NO) release significantly counteracted the medullary vasoconstrictor actions of NE, suggesting an important counterregulatory role of the adrenergic-NO interactions. Sai et al² showed that in isolated perfused outer medullary vasa recta, $N^\gamma$-nitro-$L$-arginine methyl ester (L-NAME) increased the NE response. These observations are of relevance to the long-term control of arterial blood pressure³ because the renal medullary circulation plays an important role in the mechanism of pressure natriuresis.⁴⁻⁷ Chronic reductions of blood flow to the renal medulla induced by a variety of mechanistically different stimuli have been shown to result in hypertension in rats.⁸⁻⁹ There is evidence that NO may serve to protect the renal medulla from the chronic vasoconstrictor effects of angiotensin II and vasopressin.⁹,¹⁰ However, the chronic actions of NE on renal medullary NO production have not been studied previously, and the interaction of these pathways in the long-term control of arterial pressure is unknown. Therefore, one of the major goals of the present study was to determine whether a reduced ability of NE to increase medullary NO would render Sprague-Dawley rats more susceptible to the hypertensive effects of small, normally nonhypertensive, elevations of circulating NE.

The second major goal of the present study was to determine the role of the $\alpha_2$-adrenergic receptor subtype in mediating the chronic actions of NE on renal medullary NO production. There is evidence that NE-induced NO production is mediated by $\alpha_2$-adrenergic receptors in various regions of the body.¹¹ We have found that acute renal medullary interstitial infusion of the $\alpha_2$-receptor inhibitor rauwolscine (RAU) blocks the NE-induced increase in medullary [NO]...
and consequently leads to reductions of medullary blood flow (MBF), which were not observed in the absence of RAU with NE administered at the same dose. It was also found that the α₂-mRNA colocalized to the same renal medullary microves- 
sels (eg, glomeruli and vasa recta) and tubular structures (eg, medullary thick ascending limb and collecting ducts) that contain the greatest NOS enzyme activity. The chronic interaction of this adrenergic receptor and the NO pathways in the renal medulla has not been studied and was therefore the focus of the present experiments.

We hypothesized that chronic stimulation of the renal medullary α₂-receptors associated with a continuous intravenous infusion of NE would result in an increase of renal medullary NOS activity, which would, in turn, act to buffer a rise of arterial pressure. To test this hypothesis, Sprague-Dawley rats were uninephrectomized, and the remaining kidney was instrumented with a chronically implanted renal medullary interstitial catheter. This small medullary catheter was used to continuously deliver either RAU, L-NAME, or isotonic saline during the chronic intravenous administration of NE through an implanted femoral venous catheter. Concentrations of these respective inhibitors were predetermined on the basis of observations that these doses did not result in hypertension when chronically administered alone into the renal medulla. Arterial pressure was measured daily in conscious rats by use of implanted aortic catheters. Renal cortical and medullary NOS activities were determined by use of a high-performance liquid chromatography (HPLC) arginine-citrulline conversion assay technique.

### Methods

**Male Sprague-Dawley rats (12 weeks old, 260 to 320 g)** were used in all studies. They were provided a normal diet and given free access to drinking water. All protocols were approved by the Medical College of Wisconsin Animal Care Committee.

#### Surgical Instrumentation Procedures

For all surgical procedures, acepromazine (2 mg/kg IM) and ketamine (100 mg/kg IM) were used in combination as anesthesia. In the renal medullary infusion experiments, rats were uninephrectomized to avoid possible regulatory effects from the contralateral kidney. One week after uninephrectomy, catheters were implanted into the femoral artery for blood pressure measurement and into the femoral vein for infusion of NE or saline, as previously described in detail. An additional indwelling catheter was placed into the single remaining kidney deep (≈5.5 mm) in the renal medulla to infuse L-NAME, RAU, or saline. All catheters were tunneled subcutaneously to the back of the neck, where they were exteriorized through a midscapular incision and passed through a spring for protection. After surgery, penicillin G (Crysticillin, 300 000 U/kg IM) and buprenorphin (Suppelco LC18-D8 column, and a Packard Beta-flow A100 detector. NOS activity was determined from the ratio of the total [H]arginine converted to [H]citrulline and the amount of total arginine in the reaction. As we previously described, the total L-arginine in the reaction was adjusted to 23 μmol/L in each sample to equalize the starting substrate concentra-

### Experimental Protocol

#### Protocol 1: NOS Enzyme Activity in the Renal Cortex and Outer and Inner Medulla of Sprague-Dawley Rats Receiving Intravenous NE Infusion for 7 Days

Six rats prepared with a venous catheter were infused intravenously with NE (Arterenol, 8 μg · kg⁻¹ · h⁻¹, Sigma Chemical Co) for 7 days. This dose of NE was shown in acute studies in our laboratory to have no effect on MBF and mean arterial pressure (MAP) and did not produce chronic hypertension. In the control group of 6 rats, intravenous saline infusion was continued for 7 days. On day 7 of NE or saline infusion, rats were anesthetized, kidneys were removed, and NOS enzyme activity was measured as described above.

#### Protocol 2: NOS Enzyme Activity in NE-Treated Rats After α₂-Receptor Blockade

Six uninephrectomized rats were prepared with a venous catheter and a renal interstitial catheter. This small medullary catheter was used to continuously deliver either RAU, L-NAME, or isotonic saline during the chronic intravenous administration of NE. NOS enzyme activity was measured in the rats receiving NE in the presence and absence of RAU with NE given intravenously. In the control group of 6 rats, infused with saline, renal medullary activity was measured. As we previously described, the total L-arginine in the reaction was adjusted to 23 μmol/L in each sample to equalize the starting substrate concentra-

### Measuring NOS Enzyme Activity

Kidneys were removed from pentobarbital-anesthetized rats (50 mg/kg IP), and the renal cortex and outer and inner medulla were dissected, snap-frozen, homogenized, and centrifuged (16 000g). The supernatant was retained, and the protein concentration of the supernatant was determined by the Coomassie method (Pierce). The sample was then used immediately for the measurement of NOS enzyme activity, which was determined by arginine-citrulline conversion assay with reverse-phase HPLC as described in detail elsewhere. Briefly, 50 μg of inner medulla protein and 100 μg of cortex or outer medulla were incubated with 2 mM/L CaCl₂, 1 mM/L NADPH, 25 μmol/L flavin adenine dinucleotide, 1.25 μg/mL calmodulin, 10 μmol/L tetrahydrobiopterin, and L-[H]arginine (≈30 000 cpm, specific activity 68 Ci/mmol) in 100 μL of 20 mM/L HEPES buffer, pH 7.2, at 37°C. The duration of the incubation was 30 minutes for the inner medullary samples and 2 hours for outer medullary and cortical samples. The arginine and converted citrulline were separated by isocratic reverse-phase HPLC with a system consisting of a Hitachi 7250 Auto Sampler, Hitachi L-7100 gradient pump, Suppelco LC18-D8 column, and a Packard Beta-flow A100 detector. NOS activity was determined as the ratio of the total L-[H]arginine converted to L-[H]citrulline and the amount of total arginine in the reaction. As we previously described, the total L-arginine in the reaction was adjusted to 23 μmol/L in each sample to equalize the starting substrate concentra-

#### Protocol 3: Chronic Effect of Subpressor Intravenous NE Infusion on MAP in Rats With Blunted Medullary NOS Activity

Seven uninephrectomized rats were prepared with an arterial and venous catheter and a renal interstitial catheter. One week after surgery, daily 2-hour measurements of MAP were begun by using an on-line data collection and analysis system as described previously. After 3 stable control days, L-NAME (Sigma) was infused through the renal medullary interstitial catheter at a dose (75 μg · kg⁻¹ · h⁻¹) that was shown earlier to be nonpressor. After 3 days of L-NAME infusion, intravenous NE infusion (8 μg · kg⁻¹ · h⁻¹) was started and continued for 7 days, followed by 2 postcontrol days of saline. Another similarly prepared group of rats (n = 7) was studied as a control group to determine the influence of chronic NE infusion in the absence of medullary L-NAME administration. A medullary interstitial catheter was implanted, and the rats were infused at the same rate with isotonic saline rather than L-NAME throughout the entire protocol.
Protocol 4: Chronic Effect of Subpressor Intravenous NE Infusion on MAP in Rats With α₂-Adrenergic Blockade

To investigate the role of the renal medullary α₂-receptors in the regulation of arterial blood pressure, 2 additional groups of rats were studied. In the first group, 7 rats were prepared with an arterial and venous catheter and a renal interstitial catheter. After 3 stable control days, the α₂-receptor blocker RAU (1 mg·kg⁻¹·min⁻¹) was continuously infused into the renal medulla through the implanted interstitial catheter for the remainder of the experiment. Three days after starting RAU, intravenous NE (8 μg·kg⁻¹·h⁻¹) was started and continued for 7 days with RAU renal interstitial infusion. As a time control, we infused RAU into the renal medulla of another group of rats (n=5) for 9 days while saline rather than NE was infused intravenously.

Statistical Analysis
Data are presented as mean±SEM. For statistical comparisons of the chronic blood pressure measurement, 1-way ANOVA with repeated measures followed by the Tukey multiple range test as a post hoc test was used. To determine the differences in the NOS enzyme activity, 1-way ANOVA was used. All statistical analyses were performed on the raw data. A value of P<0.05 was considered statistically significant.

Results
Protocol 1: NOS Enzyme Activity in Renal Cortex and Outer and Inner Medulla of Sprague-Dawley Rats Receiving Intravenous NE Infusion for 7 Days
Figure 1 summarizes the results of the arginine-citrulline conversion assay and shows that systemic administration of a nonpressor dose of NE (8 μg·kg⁻¹·h⁻¹) increased NOS enzyme activity 5-fold in the renal outer medulla and 2.5-fold in the inner medulla. NOS enzyme activity was not detectable in the renal cortex in either group (n=6).

Protocol 2: NOS Enzyme Activity and Protein Expression in NE-Treated Rats After α₂-Receptor Blockade
The effect of RAU on NE-induced NOS activity was measured in uninephrectomized rats in which the left kidney was instrumented with a renal interstitial catheter to infuse RAU or saline. As seen in Figure 2, NOS enzyme activity was significantly lower in rats receiving NE infusions in the presence of RAU than in rats receiving NE with renal interstitial saline infusion in place of RAU (n=6).

Protocol 3: Chronic Effect of Subpressor Intravenous NE Infusion on MAP in Rats With Blunted Medullary NOS Activity
The top panel of Figure 3 shows that intravenous infusion of NE (8 μg·kg⁻¹·h⁻¹) resulted in a sustained and reversible hypertension in rats pretreated with renal medullary L-NAME at a subpressor dose (75 μg·kg⁻¹·h⁻¹). The average rise in MAP was 17 mm Hg (n=7). The L-NAME dose was a threshold dose and did not significantly increase MAP. We have previously reported that this L-NAME dose...
infused into the renal medulla did not produce hypertension even if given for 11 days. In control rats, in which saline was infused into the renal medullary interstitial space instead of L-NAME, intravenous NE in the same dose did not significantly change MAP during 1 week of continuous infusion (Figure 3, bottom panel).

**Protocol 4: Chronic Effect of Subpressor Intravenous NE Infusion on MAP in Rats With α2-Adrenergic Blockade**

Because RAU blocked the acute NE-induced rise in medullary [NO], we predicted that the hypertensive effects of NE would be enhanced when RAU was chronically infused into the renal medulla, as was seen with L-NAME. As shown in the top panel of Figure 4, RAU (1 μg·kg⁻¹·min⁻¹), administered locally to the renal medulla, also enabled subpressor doses of chronic intravenous NE (8 μg·kg⁻¹·h⁻¹) to moderately, but significantly, increase MAP by 11 mm Hg (n=7). This hypertension was sustained and reversible, as was the case with the L-NAME–NE hypertension. Renal interstitial infusion of RAU alone at the same dose (Figure 4, bottom panel) did not cause any significant elevation of MAP throughout the 9 days of infusion (n=5).

**Discussion**

Three important observations were made in the present study. First, we showed that NE increases NOS enzyme activity in the renal medulla. Second, the increase of NOS activity is mediated through stimulation of α₂-adrenergic receptors.

Third, reducing the responsiveness of renal medullary NOS to NE by infusion of the α₂-receptor blocker into the renal medulla enabled nonpressor doses (8 μg·kg⁻¹·h⁻¹) of NE to produce a sustained and reversible hypertension, as was seen with the reduction of NOS activity by L-NAME.

**NE Stimulation of Renal Medullary NOS Activity**

The dose of NE used in the present study (8 μg·kg⁻¹·h⁻¹) was shown previously to produce only small physiological increases of plasma NE levels from 0.2 to 1.77 ng/mL; furthermore, it is a nonpressor dose when administered either acutely or chronically. The action of NE on renal NOS activity could therefore be studied in the absence of systemic hemodynamic changes.

The results of the present study have demonstrated that sustained elevations of circulating NE result in a chronic elevation of NOS enzyme activity in the renal medulla but not in the cortex. The vasculature of the renal medulla has been reported to have greater adrenergic tone than that of the cortex; thus, to protect this region of the kidney from excessive vasoconstriction, it may require a stronger counter-regulatory system. The NO system in the renal medulla has been shown to be an effective counterregulatory mechanism against the vasoconstrictor actions of angiotensin II and vasopressin. Elevated plasma levels of these agonists were shown to increase renal medullary [NO], which buffered the expected medullary vasoconstriction and prevented arterial hypertension. We have also shown that small chronic elevations in plasma angiotensin II or vasopressin upregulate
endothelial NOS in the renal medulla. Others have found that noradrenergic neurons are capable of generating NO through an increase in neuronal NOS, the isoform that plays a role in arterial blood pressure regulation as well. NE-induced activation of NO by the NOS isoforms with a resulting increase in [NO] may play an important role in the long-term control of arterial blood pressure and serve as a counterregulatory mechanism against the hypertensive actions of NE.

Role of \( \alpha_2 \)-Receptors in NO Release

There is evidence reported by others that the NE-induced NO release occurs through the \( \alpha_2 \)-receptors and that \( \alpha_2 \)-activation increases the influence of NO in the glomeruli and proximal tubules of rats. It has also been shown that in a model of hypertension induced by inhibition of NOS activity, rat vessels become more sensitive to \( \alpha_2 \)-receptor stimulation. We have reported previously that blockade of the renal medullary \( \alpha_2 \)-receptors with RAU completely abolishes NE-induced increases in the renal medullary [NO], whereas blockade of the \( \alpha_1 \)-receptor with the specific \( \alpha_1 \)-antagonist, prazosin, does not. Furthermore, \( \alpha_2 \)-receptors and NOS enzyme isoforms are colocalized in the inner medullary collecting ducts and in the vasa recta, suggesting that the \( \alpha_2 \)-receptor–mediated NO release takes place in these structures of the kidney. Taken together, it appears that the NE-induced NO release is through \( \alpha_2 \)-receptors.

Chronic Antihypertensive Actions of Medullary NO Mediated by Medullary \( \alpha_2 \)-Receptors

The major finding of the present study is that the renal medullary \( \alpha_2 \)-receptors play an important role in long-term blood pressure regulation. These results are consistent with our previous observations that \( \alpha_2 \)-receptor stimulation increases renal medullary [NO] and buffers the \( \alpha_2 \)-receptor–induced vasoconstriction of medullary vessels. MBF can play an important role in long-term arterial blood pressure regulation, and it has been shown that an increase in blood flow to the renal medulla can enhance sodium and water excretion and reduce arterial blood pressure. The effect of the \( \alpha_2 \)-receptor inhibition on arterial blood pressure appears to be confined to renal medullary structures. A number of earlier studies in our laboratory have confirmed that renal medullary infusion of various drugs into the medullary interstitial space at the same rate as in the present study resulted in trapping of the compounds in the renal medulla with little escape. In addition, in acute studies from our laboratory, even higher amounts of RAU, infused into the renal medulla, did not alter the cortical hemodynamics. This would indicate that RAU neither diffused to the cortex nor reached the cortical circulation by recirculation in a sufficient amount to have any substantial effects.

A number of studies have suggested that \( \alpha_2 \)-receptors may be important in arterial blood pressure regulation. For example, \( \alpha_2 \)-receptors have been implicated in the regulation of renal tubular ion transport activity. However, the role of \( \alpha_2 \)-receptor stimulation on renal function is clearly quite complex. It has been reported that \( \alpha_2 \)-receptor stimulation can produce renal vasoconstriction and inhibit sodium and water excretion, an effect opposite those found in the present study. More consistent with the results of the present study, several genetic hypertensive rat models (spontaneously hypertensive, Dahl salt-sensitive, and Sabra rats) have been reported to exhibit markedly increased density of renal \( \alpha_2 \)-receptors, which could represent compensatory changes in response to hypertension. Genetic linkage analyses have failed to demonstrate a cosegregation of \( \alpha_2 \)-receptors with blood pressure in Dahl salt-sensitive rats. This observation also suggests that the increased receptor density might represent an antihypertensive compensatory mechanism in the same manner that the \( \alpha_2 \)-receptors respond after stimulation with NE in the present study. Taken together, we would propose that renal medullary \( \alpha_2 \)-receptors exert a negative-feedback controller against the hypertensive effects of NE. Blockade of the medullary \( \alpha_2 \)-receptors made NE unable to increase renal medullary NOS enzyme activity, and the lack of this important counterregulatory mechanism resulted in NE-induced hypertension. The present results provide the first evidence for a direct connection between renal medullary \( \alpha_2 \)-receptors and arterial blood pressure.

We conclude that the hypertensive effects of moderate elevations of renal adrenergic activity can be chronically offset by \( \alpha_2 \)-receptor–mediated stimulation of NOS activity within the renal medulla. The \( \alpha_2 \)-receptor–mediated increase in NO activity in this region of the kidney appears to buffer reductions of MBF mediated by \( \alpha_2 \)-receptors and by the vasoconstrictor effects of NE and serves as a potent protective mechanism against the hypertensive effects of NE.

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