Nitric Oxide in the Renal Medulla Protects From Vasopressin-Induced Hypertension

Mátyás Szentiványi, Jr, Frank Park, Celso Y. Maeda, Allen W. Cowley, Jr

Abstract—In the present study, we assessed whether activation of the nitric oxide (NO) system within the renal medulla could serve as a buffer against the chronic hypertensive effects of arginine vasopressin (AVP). NO concentration in the renal medulla of Sprague-Dawley rats was measured with in vivo microdialysis/oxyhemoglobin NO trapping. The results showed that medullary interstitial [NO] was increased after 2 hours of AVP infusion and remained elevated even after 10 days (by 62±8% and 42±13%, respectively). Western blot analysis showed that 2 days of AVP infusion was insufficient to increase protein expression of any of the NO synthase (NOS) isoforms, but after 10 days of AVP infusion, endothelial NOS expression was significantly increased in the inner medulla with no significant changes in noninducible NOS and inducible NOS levels. When renal medullary NOS enzyme activity was blunted with a nonpressor dose of N^G-nitro-L-arginine methyl ester (75 μg · kg⁻¹ · h⁻¹) that was chronically infused locally into the renal medulla, intravenous AVP infusion (which was shown earlier to be suppressor in chronic studies) produced a sustained elevation in arterial pressure (from 107±2 to 121±2 mm Hg). These data indicate that chronic elevations in plasma AVP enhance renal medullary endothelial NOS protein expression, which enables sustained elevations of NO concentrations in this region of the kidney to buffer the hypertensive effects of AVP. (Hypertension. 2000;35:740-745.)

Key Words: arterial pressure ■ kidney ■ vasopressins ■ nitric oxide ■ endothelium ■ nitric oxide synthase ■ arginine

Arginine vasopressin (AVP) is one of the most potent circulating vasoconstrictor hormones,1–6 and it has long been recognized as the antidiuretic hormone.2 Its combined vasoconstrictor and volume-retaining actions suggest that chronic elevations in circulating AVP should result in hypertension. Hypertension would also be predicted on the basis of observations that small elevations in AVP can significantly reduce medullary blood flow (MBF) and blunt pressure natriuresis,4,6 both of which generally lead to hypertension.3 However, AVP does not produce sustained hypertension in normal rats, dogs, or humans3,7,8 maintained on a normal sodium intake.

Recent studies in conscious rats in which changes in regional renal blood flow were determined with laser-Doppler flowmetry provide, in part, an answer to this puzzle. It was found that although AVP produced immediate reductions in MBF, the reductions were not sustained during a chronic 5-day medullary interstitial infusion of AVP, nor was hypertension observed.8 In contrast, a medullary infusion of a vasopressin V₁ receptor (V₁R) agonist produced a sustained reduction in MBF with a corresponding chronically sustained increase in mean arterial pressure (MAP). The results of these studies suggested that a parallel stimulation of both V₁R and vasopressin V₂ receptors (V₂Rs) with endogenous AVP resulted in an increase in a V₂R-induced counterregulatory system. This was shown to be a V₂R-mediated increase in medullary nitric oxide (NO) production.9 These studies also demonstrated that this NO response was important for the normal maintenance of MBF in the face of elevated circulating levels of AVP. Taken together, the results obtained in acute experimental settings suggested that NO-induced vasodilation may also serve to chronically counterregulate AVP-induced decreases in MBF and to prevent the chronic hypertensive effects of AVP.

To address this hypothesis, we infused AVP intravenously during a period of 10 days to determine whether there would be chronic elevations in renal medullary NO synthase (NOS) protein expression and NO concentrations. Renal medullary [NO] was measured after 2 hours and 10 days of AVP infusion, whereas days 2 and 10 of AVP infusion were specifically examined to represent the early and late chronic phases of stimulation of NOS protein expression. We further determined whether blunting of renal medullary NOS activity with a continuous medullary interstitial infusion of N^G-nitro-L-arginine methyl ester (L-NAME, in an amount that did not produce hypertension) would enable chronic (11 days) nonpressor elevations of circulating AVP to produce chronic hypertension. An AVP dosage of 2 ng · kg⁻¹ · min⁻¹ was infused on the basis of results of earlier studies that have shown that this amount of AVP is acutely and chronically nonhypertensive.9–11

Received July 19, 1999; first decision August 12, 1999; revision accepted November 4, 1999.

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Methods

Male Sprague-Dawley rats weighing 280 to 360 g that were obtained from Harlan Inc were used. All animals were given free access to tap water and fed a standard pellet diet (Purina Mills). All protocols were approved by the Medical College of Wisconsin Animal Care Committee.

Acute Effects of AVP Infusion (2 ng · kg⁻¹ · min⁻¹ IV) on Renal Medullary [NO], MBF, and MAP in Anesthetized Rats

Six rats were anesthetized with Inactin (thiobutabarbital sodium; 100 mg/kg IP) and ketamine (50 mg/kg IM) and surgically prepared for measurement of renal medullary NO concentration with the microdialysis/hemoglobin trapping technique described in our previous studies. A microdialysis probe was inserted into the renal medulla (5.5 mm in depth) and equilibrated for 2 hours before the collection of 70 µL of dialysate fluid during two 30-minute intervals for control measurements. A continuous AVP infusion (2 ng · kg⁻¹ · min⁻¹ IV) was begun, and after 2 hours, dialysate fluid was collected again during two 30-minute intervals and the NO concentration was determined as described in detail in an earlier study from this laboratory. To measure changes in renal MBF, an optical fiber was implanted into the renal medulla at a depth of 5.5 mm. MBF was determined with a Perimed PeriFlux PFS Flowmeter as described in previous studies. Arterial blood pressure was measured through an implanted femoral arterial catheter.

Chronic Effects of AVP (2 ng · kg⁻¹ · min⁻¹) on Renal Medullary [NO] and MAP

Seven rats were anesthetized with acepromazine (2 mg/kg IM) and ketamine (100 mg/kg IM) and surgically prepared with a femoral venous catheter implanted for the continuous delivery of AVP or saline. The animals were placed in their homecages as we previously described. Rats were allowed to recover for 5 to 7 days while receiving saline at a rate of 0.25 mL/h IV. One week after surgery in 1 group of rats, AVP was begun at a rate of 2 ng · kg⁻¹ · min⁻¹ IV and infused continuously for 10 days while a control group of rats continued to receive only saline. On day 10, the rats were anesthetized with Inactin (100 mg/kg IP) and ketamine (50 mg/kg IM) and prepared with a microdialysis probe for the determination of medullary interstitial [NO] as in the protocol described earlier. After 2 hours of equilibration, two 30-minute samples were taken for spectrophotometric analysis, and the renal medullary [NO] was calculated as an average of these 2 samples. Arterial blood pressure was measured again with an implanted femoral arterial catheter.

Effect of Short-Term (2 Days) and Long-Term (10 Days) Infusion of AVP on Steady-State Levels of NOS Enzyme Isoforms in the Rat Kidney

In a separate study, 6 rats prepared with venous catheters were infused intravenously with either saline (control) or AVP (2 ng · kg⁻¹ · min⁻¹) for either 2 or 10 days. On day 2 or 10, kidney-soluble protein was isolated as previously described. Kidneys were isolated with a microdialysis probe for the determination of medullary interstitial [NO] as in the protocol described earlier. After 2 hours of equilibration, two 30-minute samples were taken for spectrophotometric analysis, and the renal medullary [NO] was calculated as an average of these 2 samples. Arterial blood pressure was measured again with an implanted femoral arterial catheter.

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Chronic Effect of Subpressor Intravenous AVP Infusion on MAP in Rats With Blunted Medullary NOS Activity

One week after uninephrectomy, 6 rats were prepared with femoral arterial and venous catheters as well as a renal medullary interstitial catheter while under anesthesia with acepromazine (2 mg/kg IM) and ketamine (100 mg/kg IM) as described previously in detail. One week after surgery, daily 2-hour measurements of MAP were begun with an online data collection and analysis system. After 3 stable control days, L-NAME was infused (0.25 mL/h) through the renal medullary interstitial catheter at a dosage of 75 µg · kg⁻¹ · h⁻¹ that was shown earlier to be nonpressor. After 3 days of L-NAME infusion, an intravenous AVP infusion was started at a dosage of 2 ng/kg · min⁻¹, which also was shown earlier in acute studies to be nonpressor. After 11 days, Adrenalin AVP infusion was administered for 11 days, followed by 2 postcontrol days of saline. In a control group of 4 rats, AVP was intravenously infused in rats that received a renal medullary saline infusion (0.25 mL/h).

Statistical Analysis

Data are presented as mean±SEM and were analyzed with 1-way ANOVA. For the analysis of chronic MAP, an ANOVA for repeated measures was performed. As a post hoc test, Tukey’s multiple range test was used. All statistical analyses were performed on the raw data.

Results

Acute Effects of AVP Infusion (2 ng · kg⁻¹ · min⁻¹ IV) on Renal Medullary [NO] and MBF in Anesthetized Rats

Figure 1 shows that a 2-hour subpressor infusion of AVP nonpressor. The intravenous AVP infusion was administered for 11 days, followed by 2 postcontrol days of saline. In a control group of 4 rats, AVP was intravenously infused in rats that received a renal medullary saline infusion (0.25 mL/h).
of 80±4 to 129±7 nmol/L, a 62% (P<0.01) increase. MAP and MBF remained unchanged from mean control values of 122±3 mm Hg and 0.52±0.05 V, respectively (n=6).

**Chronic Effects of AVP (2 ng · kg⁻¹ · min⁻¹ IV) on Renal Medullary [NO]**

Figure 2 illustrates that after 10 days of intravenous AVP infusion, medullary interstitial [NO] was significantly higher (115±7 nmol/L) than that in the control group, in which isotonic saline alone was administered (79±6 nmol/L, a 41% difference; P<0.01). MAP was not significantly different on the 10th day of AVP infusion compared with that of the control animals (115±3 mm Hg, n=7).

**Effect of Short-Term (2 Days) and Long-Term (10 Days) Infusion of AVP on Steady-State Levels of NOS Enzyme Isoforms in the Rat Kidney**

Because of the distinct NOS isoform tissue distribution throughout the organism, 3 different tissues were used to verify the specificity of the NOS antibody (Figure 3, top). The following tissues were used as positive control: cerebellum for nNOS (expected size ~160 kDa), whole kidney homogenate for eNOS (~140 kDa), and lipopolysaccharide-stimulated liver tissue for iNOS (~130 kDa). Figure 3 demonstrates that iNOS protein was not detected in the rat inner medulla (115±3 mm Hg, n=7).

Figure 3 (2 bottom panels) summarizes the Western blot analysis for nNOS and eNOS responses to AVP in the outer and inner medulla of tissue obtained from 6 rat kidneys. The nNOS protein did not change significantly after either 2 or 10 days of AVP infusion within the outer and inner medulla, although there was a tendency to increase in the inner medulla after 10 days of AVP infusion. In the outer medulla, eNOS protein was not significantly increased after 2 and 10 days of AVP infusion, although there was a slight increase in AVP-infused eNOS protein after 2 days (45.3% higher than control). The major change occurred in the renal inner medulla, in which despite the absence of a significant change in the eNOS expression at day 2 of AVP infusion (46.7% higher than control), eNOS expression after 10 days of AVP infusion showed a nearly 4-fold increase (P<0.05, n=6). iNOS isoform also was not found in the renal medulla of AVP-infused rats.

**Chronic Effect of Subpressor Intravenous AVP Infusion on MAP in Rats With Blunted Medullary NOS Activity**

To determine whether AVP-induced NO release in the renal medulla plays a key role in buffering the hypertensive effect of AVP, medullary NO production was blunted with a continuous medullary interstitial infusion of L-NAME (75 μg · kg⁻¹ · h⁻¹), and a subpressor dose of AVP (2 ng · kg⁻¹ · min⁻¹ IV) was chronically infused. As seen in Figure 4 (top), during the 3-day control period before L-NAME infusion, mean MAP was 104±2 mm Hg compared with 108±1 mm Hg during the 3-day period of L-NAME administration alone. If MAP did tend to rise on the first day of
L-NAME infusion, adjustments in the L-NAME infusion rate were made and the control period was extended until a stable 3-day control period was achieved. It is important to recognize that the goal of this study was not to totally inhibit NOS activity but rather to moderately blunt the ability of AVP to stimulate NO production.

In the presence of L-NAME, as summarized in Figure 4, intravenous AVP infusion (2 ng·kg⁻¹·min⁻¹) significantly elevated MAP after 24 hours of AVP infusion. This AVP-induced hypertension was sustained throughout the 11 days of infusion at a level that averaged nearly 15 mm Hg higher than that during the L-NAME control period (P<0.05, n=6). On cessation of the AVP infusion (with maintenance of the medullary L-NAME infusion), MAP returned to a level that was not significantly different than that during the L-NAME control period. Figure 4 (bottom) shows the control experiments in which AVP was infused intravenously at the same dose but with a renal medullary infusion saline infusion. Under these conditions, AVP increased MAP only on the first 3 days of infusion and returned to normal values thereafter.

**Discussion**

In the present study, we addressed 3 issues: (1) whether intravenous AVP infusion could acutely and chronically increase renal medullary NO concentration, (2) which isoforms of NOS are involved in the increase in renal medullary [NO], and (3) whether AVP could produce hypertension if renal medullary NOS activity was reduced.

**AVP Stimulatory Effect on Renal Medullary NO**

There has been indirect evidence that AVP may stimulate the release of NO as indicated by AVP-induced increases in urinary cGMP concentrations and by observations that the inhibition of NOS acutely enhances pressor responses to AVP. The first direct evidence was obtained by Park et al., who applied the in vivo microdialysis/oxyhemoglobin NO trapping technique used in the present study to demonstrate that the acute medullary interstitial infusion of AVP increased medullary NO concentration. In the present study, AVP was administered systemically via intravenous infusion rather than into the renal interstitium at a rate of 2 ng·kg⁻¹·min⁻¹, which results in elevations of plasma AVP within the physiological range and a normal route of peptide delivery to the renal medulla. We have previously determined that this rate of AVP infusion to conscious rats increases plasma AVP concentrations from normally hydrated control levels of 3 pg/mL to 20 pg/mL, levels that are achieved with 36 hours of water restriction. As seen in Figure 1, this increase in circulating AVP produced a significant increase in medullary [NO]. Importantly, the stimulation and elevation of medullary [NO] were not attenuated during the 10 days of AVP infusion. Control studies were performed in which saline was infused instead of AVP since surgically prepared rats would be expected to have elevated plasma levels of AVP. The results show that both acute and chronic AVP infusion resulted in elevations of medullary [NO], suggesting that the increase in medullary [NO] was sustained throughout the 10 days when AVP was infused.

The acute rise of [NO] has been shown to be mediated through V₂R stimulation. Furthermore, in the presence of V₁R inhibition, medullary infusion of a selective V₂R agonist resulted in a significant rise of MBF that was prevented by medullary interstitial infusion of L-NAME delivered in the same low concentrations as in the present study. It remains to be established whether the sustained elevation of medullary [NO] in the present study was mediated solely by V₂R stimulation, as was seen acutely. Park et al. found that water restriction leads to a time-dependent downregulation of V₂R mRNA and protein expression in the rat kidney during 48 hours. If such a downregulation occurs with chronic AVP administration, a chronic elevation of [NO] within the renal medulla could be related to AVP-induced transcriptional enhancement of NOS enzyme synthesis, as discussed later.

**Increased Expression of Endothelial NOS in the Renal Inner Medulla**

Having demonstrated that AVP could produce a sustained elevation in renal medullary [NO], the mechanisms for the chronic rise of [NO] became of interest. Only the constitutive isoforms of NOS (nNOS and eNOS) were found in the renal medulla, and as reported earlier, both tissue NOS enzyme activity and NOS protein expression were much higher in the renal medulla than in the renal cortex. Importantly, eNOS protein expression was significantly elevated in the inner white medulla after 10 days of AVP stimulation, and there was a tendency for an elevation in nNOS.

Both nNOS and eNOS mRNAs have been found to be expressed in isolated medullary vasa recta and collecting ducts. V₂R mRNA, in contrast, has been found only in the tubular epithelium of the medullary thick ascending limbs and collecting ducts. The results of the present study therefore suggest that the elevation of medullary [NO] in response to chronic elevations of AVP may be explained by an increased expression of eNOS in the medullary collecting ducts.
ducts. If reduced medullary expression of V2R protein occurs with chronic AVP, as was found with water restriction, the increased expression of NOS protein may offset such changes and maintain sustained elevations of medullary [NO]. The increase in eNOS might be a common pathway in response to different vasoconstrictors because an increase in total renal eNOS protein has been observed after the chronic (10-day) administration of angiotensin II. We propose that it is the increase in medullary [NO] that probably explains why AVP is unable to chronically elevate arterial blood pressure. Support for this hypothesis was also found in the results of the chronic studies that were performed.

iNOS protein expression was not found in the renal outer and inner medulla of normal or AVP-infused rats. It is widely accepted in the literature that the message for iNOS is present in the renal medulla. However, the Western blotting technique often is not sufficiently sensitive to show the protein even if it translated, which may explain why some investigators have found iNOS protein with Western blotting, whereas others have not.

### Chronic AVP Administration With Reductions in Renal Medullary NOS Activity

The results of this study show that when NOS activity is selectively blunted in the renal medulla, elevations in circulating AVP can result in a sustained elevation in arterial pressure. The control studies confirmed in our laboratory and by others that elevated circulating levels of AVP fail to produce sustained elevations in arterial pressure in normal rats. L-NAME infused chronically into the renal medulla has been shown to produce hypertension when infused at a dose higher than that used in the present study. However, the goal of the present study was to produce only a partial inhibition of NOS activity, which might simulate naturally occurring pathological variations of NOS activity. We have shown previously that the dose of L-NAME that was chosen neither reduced MBF nor resulted in chronic hypertension.

Previous studies have shown that in the absence of a severe reduction in functional renal mass, the chronic administration of AVP is unable to produce sustained hypertension in rats, dogs, or humans. This has remained puzzling because AVP is a potent circulating vasoconstrictor, and several recent studies in our laboratory by Franchini and colleagues have shown that the renal medullary circulation is highly sensitive to small physiological elevations of circulating AVP. These reductions in MBF by AVP were accompanied by substantial blunting of the pressure-natriuretic relationship, a vasopressin V1R–mediated response. The long-term importance of stimulation of the medullary vasopressin V1R independent of the V2R was shown by the sustained hypertension produced in rats administered a chronic intravenous infusion of a specific V1R agonist. This hypertension was in turn prevented by infusion of a selective V1R antagonist into the renal medullary interstitium. Hypertension was also produced when the V1R agonist was infused directly into the renal medullary interstitium at a dosage that was shown to reduce blood flow to the renal medulla. Recently, it was found that the inability of AVP to produce chronic hypertension was related to the inability of this peptide to produce a sustained reduction in MBF, in contrast to the V1R-specific agonist, which produced hypertension with a sustained reduction in blood flow to the renal medulla.

Taken together, these results suggest that the failure of AVP to produce a sustained reduction in MBF and hypertension may be due to the stimulation of V1R and increased production of NO in the renal medulla, which serves as a counterregulatory mechanism to offset the V1R-mediated effects of the endogenous peptide. This idea is consistent with observations that [NO] and NOS enzyme expression and activity are significantly higher in the renal medulla than in the renal cortex. Because chronic reductions in MBF can lead to hypertension, as reviewed in detail elsewhere, the NO counterregulatory response to AVP may play an important role in stabilization of the arterial pressure, whereas vasopressin performs important osmoregulatory functions.

In summary, the results of the present study show that small increases in circulating AVP can produce chronic elevations of renal medullary [NO] and that this is accompanied by an increase in the expression of eNOS within the inner medulla of rats. Chronic intravenous infusion of AVP produced a sustained elevation in arterial pressure when medullary NOS activity was partially reduced by medullary interstitial L-NAME infusion. These observations indicate that the NO/NOS pathway plays an important long-term role in modulation of the vasoconstrictor effects of AVP and may normally prevent AVP from producing hypertension during conditions of elevated plasma levels of AVP.

### Acknowledgments

This work was supported by National Institutes of Health grant HL-49219. Mátéys Szentiványi, Jr, was visiting postdoctoral fellow from Semmelweis University of Medicine (Budapest, Hungary), with support from OKTA T030245. The authors thank Meredith M. Skelton for careful review of this manuscript.

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Hypertension. 2000;35:740-745
doi: 10.1161/01.HYP.35.3.740

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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