Evidence That Reduced Renal Medullary Nitric Oxide Synthase Activity of Dahl S Rats Enables Small Elevations of Arginine Vasopressin to Produce Sustained Hypertension

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Abstract—On the basis of observations supporting the functional importance of nitric oxide (NO) in the regulation of renal medullary function, and a reduced nitric oxide synthase (NOS) enzyme activity in the outer medulla of the Dahl salt-sensitive (SS/Mcw) rats, we hypothesized that these inbred rats would have reduced capacity to synthesize renal medullary NO. This reduced capacity would sensitize them to the hypertensive effects of small elevations of circulating arginine vasopressin (AVP). SS/Mcw and Brown Norway (BN/Mcw) rats with implanted arterial and venous catheters were fed a 0.4% salt diet and infused intravenously for 14 days with a subpressor dose of AVP (2 ng/kg per min). Mean arterial pressure (MAP) was measured 2 hours daily in unanesthetized rats maintained in their home cages. MAP in SS/Mcw rats increased during day 1 of AVP infusion from a control level of 127±0.9 mm Hg to an average of 147±1.6 mm Hg after 14 days. MAP did not return to control values during the 3 days after the end of AVP infusion. BN/Mcw rats showed no changes of MAP during 14 days of AVP infusion (90.4±0.6 mm Hg and 92.3±0.4 mm Hg). Northern blot analysis of renal tissue from vehicle (saline) -infused rats demonstrated that NOS I and NOS III mRNA expression was significantly less in SS/Mcw rats in the renal outer medulla compared with BN/Mcw rats. We conclude that small, normally subpressor elevations of plasma AVP can produce chronic hypertension in SS/Mcw rats and that this phenomenon is related to the reduced medullary NOS enzyme activity, which in turn reduces the AVP-stimulated NO synthesis. (Hypertension. 2001;37[part 2]:524-528.)

Key Words: Dahl salt-sensitive rats ■ Brown Norway rats ■ NOS isoforms ■ blood pressure ■ vasopressin

It is well known that chronic elevation of plasma arginine vasopressin (AVP) does not produce sustained hypertension in rats, dogs, and in human,1–4 despite that AVP is one of the most potent circulating vasoconstrictors and a fluid retaining hormone. However, previous studies from our laboratory have found that chronic intravenous infusions of the AVP V1 receptor agonist [Phe2, Ile3, Orn8]VP resulted in sustained hypertension.5 It was found that these hypertensive actions of the V1 agonist were mediated through actions on the medulla of the kidney because chronic infusion of a selective V1R antagonist into renal medullary interstitium prevented the rise of arterial pressure. Additionally, hypertension was also achieved when the V1R agonist was infused directly into the renal medullary interstitium6 at a dose previously shown to reduce blood flow to the renal medulla.7–9

The hypertension achieved with V1R stimulation suggested that the failure of AVP to elevate the blood pressure was related to the stimulation of V2 receptors (V2R), which activated an opposing renal medullary vasodilator response. One such mechanism was identified to be the release of NO. Because AVP increased blood flow within the renal medulla by V2R-mediated production of NO.7,10 Since the V2R mRNA could not be detected within the microvasculature of renal cortex or medulla,10 it was suggested that the elevation of medullary NO concentrations in response to AVP or V2 agonist stimulation11–13 was the result of V2 receptors stimulation most likely in the medullary collecting ducts. The concept that NO responses in the medulla served to buffer AVP-V1 receptor vasoconstrictor actions was validated by recent studies in which N(t)-nitro-L-arginine methyl ester (L-NAME) was infused chronically into the medullary interstitial space of Sprague-Dawley rats to reduce NO activity and the NO response to AVP. Even a moderate reduction of NOS activity in the renal medulla enabled small elevations of circulating AVP to produce sustained hypertension,14 demonstrating the importance of the medullary NO counter-regulatory system. Recently, studies have demonstrated that SS/Mcw rats exhibit reduced vasodilator responses to compounds that stimulate NO release (eg, acetylcholine)15 and that renal medullary nitric oxide synthase (NOS) activity is significant reduced in SS/Mcw rats compared with the Brown Norway (BN/Mcw) rats.16

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Taken together, these findings provided the rationale for the present study. Specifically, studies were carried out to determine whether small elevations of circulating AVP that failed to produce hypertension in normotensive BN/Mcw rats (as shown in Sprague-Dawley rats) would lead to chronic hypertension in SS/Mcw rats even when maintained on a low salt diet (0.4%). Northern blot analyses were performed to determine whether NOS mRNA of the three NOS isoforms were reduced in the medulla of SS/Mcw rats.

**Methods**

Twelve-week-old male inbred Dahl S (SS/JrHsdMcw) and Brown Norway (BN/SSsNMcw) rats from colonies maintained at the Medical College of Wisconsin were used in the experiments. These rats will be referred to as SS/Mcw and BN/Mcw throughout this manuscript. The Brown Norway rat was chosen as the normotensive salt-insensitive control strain because we carried out a genetic linkage analysis using the inbred BN/Mcw and SS/Mcw strains and found differences between these 2 strains that can be combined with the genetic mapping studies to link genes to function. All rats received free access to tap water and were maintained throughout the study on low salt (0.4% NaCl) rat chow (Dyets).

**Renal Regional NOS mRNA Expression Studies in Dahl S and BN/Mcw Rats**

Male adult SS/Mcw and BN/Mcw rats maintained throughout life on low 0.4% salt intake were killed with an overdose of sodium pentobarbital; the kidneys were quickly removed, dissected, and rinsed free of blood with ice cold normal saline. The renal cortex, outer medulla, and inner medulla were separated, and the tissues were frozen quickly on dry ice and transferred to −80°C freezer until use. Total RNA was extracted using TRIzol Reagent (Life Technologies) according to the manufacturer’s protocol. A total of 20 μg of total RNA from cortex, outer medulla, and 10 μg of total RNA from inner medulla were separated on 5% formaldehyde-MOPS denaturing agarose (1%) gel by electrophoresis. The RNA was then transferred onto a positively charged nylon membrane (Amersham Pharmacia) by overnight capillary transfer action that was then UV cross-linked (Stratagene). The specific probes for neuronal NOS (NOS I), inducible NOS (NOS II), and endothelial NOS (NOS III) used in subsequent Northern blot analysis were generated by reverse transcriptase-polymerase chain reaction (RT-PCR) using specific primers described previously. PCR products were purified (PCR purification kit, QIAGEN, Germany), and the probes were labeled with 3P (RepPrime II DNA Labeling Kit, Amersham). The membrane was prehybridized (Rapid-Hyb buffer, Amersham Pharmacia) for 1 hour, and then 25 ng of labeled probe was added to the hybridization solution (Rapid-Hyb buffer, Amersham Pharmacia) and hybridized for 2.5 hours. The membrane was washed with 2× SSC (3.0 mol/L sodium chloride and 0.3 mol/L sodium citrate) and 0.1% SDS (sodium dodecyl sulfate solution) for 20 minutes, then washed twice for 30 minutes with 1× SSC and 0.1% SDS, and 0.1× SSC and 0.1% SDS, respectively. The membrane was then exposed to the x-ray film with intensifying screen, 48 hours for eNOS, nNOS, and 72 hours for iNOS at −80°C, respectively. The signals were analyzed by densitometry scanner and ImagQuant software (Molecular Dynamics).

**Surgical Preparation and Chronic MAP Measurement in Dahl S, Dahl R, and BN/Mcw Rats**

Rats were surgically prepared with the femoral arterial and femoral vein catheters for the measurement of arterial pressure and intravenous infusion as described previously. Rats were allowed to recover for 5 to 7 days while saline was infused intravenously at a rate of 0.25 mL/h throughout the recovery and experimental control period.

**Statistical Analysis**

Data are expressed as means ± standard error. Within-group changes in MAP were evaluated with a 1-way ANOVA for repeated measures and Duncan’s post hoc test. Northern Blots were analyzed using ImagQuant software from Molecular Dynamics. The t test was used to examine the differences in mRNA expression between strains. The level of significance was P < 0.05.
Effects of Long-Term Intravenous Infusion of AVP on MAP in SS/Mcw and BN/Mcw Rats

Figure 3 summarizes the effects of intravenous infusion of subpressor dose of AVP (2 ng/kg per min) on MAP in SS/Mcw and BN/Mcw rats. The SS/Mcw rats showed a significant higher MAP during the control period (averaged 125.0 ± 3.9 mm Hg, n = 7, P < 0.0001) when compared with the control levels of MAP in BN/Mcw rats (90.0 ± 2.7 mm Hg). Moreover, MAP in SS/Mcw rats was increased significantly to an average of 143.6 ± 5.7 mm Hg after 14 days of administration of AVP when compared with the control levels. The blood pressure was increased significantly even after 1 day of AVP infusion. MAP did not return back to the control level for 3 days after ending the AVP infusion, averaging 149.1 ± 7.4 mm Hg during this post-AVP infusion period. BN/Mcw exhibited no significant changes of MAP during the 14 days of AVP infusion. The control MAP averaged 90.0 ± 2.7 mm Hg in BN/Mcw rats (n = 6), and 92.3 ± 2.3 mm Hg during the intravenous AVP infusion. These results clearly indicate different sensitivity to AVP for these two strains of rat.

Discussion

Our previous studies have shown that NO in the renal medulla protects from vasopressin-induced hypertension and that there is a reduced capacity of SS/Mcw rats to produce NO in response to stimuli such as angiotensin II.13,16 The present study was designed to determine whether AVP could produce hypertension in rats that exhibited reduced endogenous medullary NOS and NO production.

Evidence of Reduced Medullary NOS Gene Expression in Dahl S Rats

Northern Blot analysis indicated that NOS III mRNA and NOS I mRNA isoforms were abundantly expressed in both the outer and inner medulla of SS/Mcw and BN/Mcw rats. However, it was found that SS/Mcw rats contained significantly less mRNA for these 2 NOS isoforms in the outer medulla. These observations conform with other preliminary studies from our laboratory, which indicate that NOS III and NOS I enzyme activity and protein expression are also reduced significantly in the outer medulla of SS/Mcw rats compared with BN/Mcw rats.16 Despite the lower NOS enzyme activity and protein expression in SS/Mcw rats, there was no significant difference in NO concentration under basal conditions between SS/Mcw and BN/Mcw rats as measured by microdialysis Hb trapping techniques.16 It therefore appears that there exists sufficient NOS enzyme and substrate, L-arginine (L-Arg), in the medulla to maintain the basal NO concentrations in the renal medulla in SS/Mcw rats. Because the microdialysis technique for the measurement of NO concentration determines NO in the whole medulla, it is also possible that the inner medullary NOS could contribute a disproportionate amount of NO to the assay, which masks the decreased enzyme activity in the outer medulla. We and others have observed that, in Sprague-Dawley rats, both NOS enzyme activity and protein expression were substantially higher in the inner medulla than in the outer medulla.20,21
rats may be responsible for the reduction of NO production induced by different stimuli such as AVP. Previous studies in our laboratory have shown that AVP stimulates NO production in normal Sprague-Dawley rats and that NO counteracts the vasoconstrictor effects of AVP.\textsuperscript{13,16} The present study further defined the significance of this AVP-stimulated NO production in the long-term control of arterial pressure. The results also demonstrated a deficit in the NOS RNA levels in the SS/Mcw rats. Taken together, we conclude that decreased outer medullary NOS expression primarily contributes to a reduction of NO production in response to AVP, which abolishes the counteracting effect of NO on medullary vasoconstriction resulting in AVP-induced hypertension.

Although we and others have reported previously that NOS II mRNA and proteins are present in rat renal medulla,\textsuperscript{20,22} the inducible NOS isoform was not detectable by Northern blot analysis in the present study in either SS/Mcw or BN/Mcw kidneys. The failure to demonstrate the NOS II mRNA in the renal outer and inner medulla in the present study could simply be due to the limitation of tissue mRNA amount (20 \(\mu\)g for outer medulla and 10 \(\mu\)g for inner medulla) in the Northern blot study. The present data indicate only that SS/Mcw and BN/Mcw rats express much less NOS II mRNA than NOS III and NOS I under normal physiological conditions.

In light of other recent data from our laboratory, the present study provides the basis for a more integrated understanding of the overall impairment of the renal NO system in Dahl S rats. Because the concentration of NO does not appear to differ in the medulla of SS/Mcw and BN/Mcw rats\textsuperscript{16} under basal conditions, the deficits in NOS enzyme expression of the medulla seem to attenuate the AVP-stimulated increases of tissue NO concentration. We have also recently found in preliminary studies an inability of angiotensin II to increase medullary NO concentration in SS/Mcw rats, despite significant increases in BN/Mcw rats.\textsuperscript{16} It is this reduced ability of AVP to stimulate medullary NO production in SS/Mcw rats and the lack of this counterregulatory system that we believe enables small elevations of AVP to produce hypertension in this strain of rats.

**Long-Term Elevation of Plasma AVP on Blood Pressure Regulation in SS/Mcw Rats**

The present study demonstrates that small elevations of plasma AVP are capable of producing sustained hypertension in animals that are genetically deficient in NO. Numerous studies in rats, dogs, and human have observed that chronic administration of AVP resulted in either no significant elevation of blood pressure, or elevations that lasted only days despite the elevated plasma AVP.\textsuperscript{1-4} This was also clearly demonstrated in the present study in that BN/Mcw rats were unable to develop hypertension with chronic administration of AVP. Sustained hypertension has been produced with AVP only under special circumstances when the water intake was fixed\textsuperscript{23} or when renal mass was substantially reduced.\textsuperscript{24,25}

Recently, studies by Szentivanyi et al\textsuperscript{14} demonstrated that when medullary NOS activity was selectively blunted by chronic infusion of L-NAME into the renal medulla, intravenous infusion of AVP produced sustained hypertension in Sprague-Dawley rats. Because Park et al\textsuperscript{13} demonstrated that acute administration of AVP directly into the renal medulla resulted in significant elevation of medullary NO concentrations and Szentivanyi et al\textsuperscript{14} found that [NO] levels were sustained elevated throughout a 10-day intravenous infusion of AVP, there is clear evidence that small elevations of plasma AVP normally increase the production of NO in the renal medulla. Taken together, these data indicate that this increased NO production serves as a counterregulatory mechanism to offset the medullary vasoconstrictor and hypertension effects of AVP, because chronic reduction of medullary blood flow results in hypertension.\textsuperscript{26} Because the SS/Mcw rats cannot increase the production of medullary NO with AVP stimulation and are unable to overcome the medullary vasoconstrictor effects of AVP, SS/Mcw rats are susceptible to the hypertensive actions of small elevations of circulating AVP.

The saline infusion study summarized in Figure 4 served as a vehicle control experiment, because we added AVP in saline for the intravenous infusion. With a very low infusion rate (0.25 mL/h), the rats received 54 mg NaCl/24 hours, which amounts to approximately a 0.1% salt diet. Because the diet of these rats was 0.4%, the total daily intake was equivalent to a 0.5% salt diet. It seems that this small amount of intravenous NaCl may contribute to the slight elevation of MAP in these SS/Mcw rats on the last 2 days of the infusion period, but this contribution of the saline (vehicle) to the rapid and sustained increase of MAP in the AVP-infused SS/Mcw rats seems to be minimal in this study.

It is not clear what causes the NO deficiency in SS/Mcw rats. Chen et al\textsuperscript{27} identified a single nucleotide mutation in the NOS II gene in SS/Mcw rats. However, because most investigators find the level of NOS II in the outer and inner medulla is quite low, the functional significance of this variation remains to be determined. Deng and Rapp\textsuperscript{28} reported that NOS III does not segregate with the trait of blood pressure in a genetic linkage study. An alternative hypothesis to explain reduced NO production with AVP stimulation could be a reduced expression of AVP V\textsubscript{2}\textsuperscript{29-31} or V\textsubscript{2}-like receptors,\textsuperscript{32} because they modulate vasodilatory effects in the renal medulla by increased production of NO. However, this would not explain why SS/Mcw rats are also hyperresponsive to angiotensin II\textsuperscript{16} and norepinephrine. These observations would suggest a generally reduced capacity to produce NO for reasons that remain to be determined.

In conclusion, we have shown that SS/Mcw rats, like the medullary L-NAME infused rats, exhibit an abnormal NO system in the renal medulla. This defect in the NO system makes them more vulnerable to vasoconstrictor effect of AVP, such that long-term elevation of plasma AVP causes sustained hypertension.

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