Mechanisms of salt-sensitive hypertension: role of renal medullary inducible nitric oxide synthase

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Tian, Niu, Anthony W. Gannon, Raouf A. Khalil, and R. Davis Manning, Jr. Mechanisms of salt-sensitive hypertension: role of renal medullary inducible nitric oxide synthase. Am J Physiol Regul Integr Comp Physiol 284: R372–R379, 2003. First published October 24, 2002; 10.1152/ajpregu.00509.2002.—The goal of this study was to determine the role of renal medullary inducible nitric oxide synthase (iNOS) in the arterial pressure, renal hemodynamics, and renal excretory changes that occur in Dahl R and S rats. Studies were conducted in Dahl R and S rats, Rapp strain, using chronically implanted arterial, venous, and renal medullary catheters, during a 5-day control period and a 5-day period of iNOS inhibition with continuous interstitial infusion of aminoguanidine (AG; 3.075 mg·kg⁻¹·h⁻¹). After 5 days of AG, mean arterial pressure increased to 132 ± 2% control in the S rats with high Na intake and intravenous AG. AG did not change arterial pressure in R rats. AG also caused little change in renal hemodynamics, urinary Na, or H₂O excretion or ACh-induced aortic vasorelaxation in R or S rats. The data suggest that during high Na intake, nitric oxide produced by renal medullary iNOS helps to prevent excessive increases in arterial pressure. NO production by iNOS that decreased salt sensitivity and cardiovascular function was measured in Dahl R and S rats but not in the R rat. Renal hemodynamics; endothelial function

About one-half of the population of human hypertensive patients is salt sensitive, which refers to the dependence of blood pressure on Na intake (38), but the cause of the salt sensitivity is not clearly understood. We recently showed that nitric oxide (NO) produced by inducible nitric oxide synthase (iNOS) helps to prevent salt-sensitive hypertension in Dahl salt-resistant (R) rats and decreases the salt sensitivity in the salt-sensitive (S) rats (32). In this study, the highly salt-resistant Dahl R rat became hypertensive when a high Na intake was combined with systemic iNOS inhibition with aminoguanidine (AG). Also, the arterial pressure of the Dahl S rat on high Na intake and systemic AG was significantly higher than the S rat on high Na intake alone. Yet, the major source of NO produced by iNOS that decreased salt sensitivity in the Dahl R and S rats in this study is not known.

Renal NO production has been shown to be important in the regulation of arterial pressure and renal hemodynamics (14), and renal medullary tissue has a much greater capacity to synthesize NO than the renal cortex (3, 34, 36, 39, 40). Also, the NO concentration in the renal medulla is higher than in the cortex (40), especially during high salt intake. Therefore, the renal medulla is a likely source of NO in Dahl R and S rats during increases in Na intake.

Recent studies have shown that increases in Na intake cause a variable response of NOS proteins in the kidney. Some investigators have found that an increased Na intake caused an increase in renal medullary iNOS protein in Sprague-Dawley rats (20), and others found a decrease in renal NOS proteins in Sprague-Dawley (27) and the outbred Brookhaven strain of Dahl S rats (26). In preliminary studies we found that renal medullary iNOS proteins increased after a 1-wk exposure to a high-Na diet in Dahl R/Rapp rats but did not change after 2 or 3 wk on high Na intake, and there were no significant changes in Dahl S/Rapp rats (35). Although several investigators have measured iNOS protein mass and in vitro activity in renal tissue, only a few have studied the function of iNOS protein in the kidney (5, 22), and there is almost no information available about the function of iNOS in the renal medulla. We hypothesize that the NO produced by iNOS in the renal medulla in the Dahl R and S rats helps to prevent salt sensitivity, and thus iNOS inhibition in the renal medulla will increase the salt sensitivity of both R and S rats. Studies were conducted in Dahl R and S rats, Rapp strain, using chronically implanted arterial, venous, and renal medullary interstitial catheters, during a 5-day control period and a 5-day period of iNOS inhibition with continuous intramedullary or intravenous infusion of AG at 3.075 mg·kg⁻¹·h⁻¹. Rats were subjected to either low or high Na intake, and cardiovascular and renal functional measurements were made throughout the experiment.

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METHODS

Animal preparation, experimental measurements, and instrumentation. Experiments were conducted in 40 conscious 7- to 8-wk-old male Dahl R or S rats, Rapp strain (Harlan Sprague Dawley, Indianapolis, IN). The project had the approval of the local Institutional Animal Committee and followed the newest guiding principles for research (2). Rats were received when they were 5–6 wk old, and surgery was performed when the rats reached a weight of 200 g. Using aseptic surgery with isoflurane anesthesia (1%), the right kidney of these rats was removed first in all groups.

Systemic studies. Chronic arterial and venous catheters were implanted through the femoral artery and vein, respectively. Both catheters were exteriorized at the dorsal nape of the neck. The animals were then connected to a swivel apparatus that allowed the rats free movement without tangle of the catheters. A catheter from the swivel was connected to a Cobe transducer (Lakewood, CO) for measurement of arterial pressure and in turn was connected to a digital amplifier. Arterial pressure and heart rate were determined from pulsatile arterial pressure signals sent to a digital computer at 500 Hz for 4 s of each minute throughout the entire 24-h period.

Intramural studies. In addition to the femoral arterial and vein catheters, an intramedullary catheter (polyethylene no. 10 material) was inserted directly into the medullary interstitial space of the left kidney through a 26-gauge needle hole. The tip of the catheter was placed at or near the intersection of outer and inner medulla (at a depth of 3–4 mm) as previously done (17, 19, 21). The catheter also passed through mersilene mesh on the surface of the kidney, and the mersilene mesh was secured with cyanoacrylate glue. The intramedullary catheter exited the body at the nape of the neck together with the arterial and venous catheter, and it was connected to a swivel apparatus that allowed the rats free movement without tangling of the catheters. A catheter from the swivel was connected to a Cobe transducer (Lakewood, CO) for measurement of arterial pressure and in turn was connected to a digital amplifier. Arterial pressure and heart rate were determined from pulsatile arterial pressure signals sent to a digital computer at 500 Hz for 4 s of each minute throughout the entire 24-h period.

### Intramedullary studies

In addition to the femoral arterial and venous catheters, an intramedullary catheter (polyethylene no. 10 material) was inserted directly into the medullary interstitial space of the left kidney through a 26-gauge needle hole. The tip of the catheter was placed at or near the intersection of outer and inner medulla (at a depth of 3–4 mm) as previously done (17, 19, 21). The catheter also passed through mersilene mesh on the surface of the kidney, and the mersilene mesh was secured with cyanoacrylate glue. The intramedullary catheter exited the body at the nape of the neck together with the arterial and venous catheter, and it was used for continuous intramedullary infusion of chemicals and antibiotics. The intravenous and intramedullary infusion rates were 10.4 μl/min. At the end of experiment, the kidneys were examined, and rats with improperly placed catheters or significant renal damage were excluded from the study.

All rats were placed in a temperature-controlled room with a 12:12-h light-dark cycle. Starting 0.5 h after the catheters were implanted and continuing throughout the experiment, the rats received prophylactic antibiotics (Levaquin, 8.75 mg/kg) in a continuous intravenous or intramedullary infusion using a Harvard apparatus syringe pump (South Natick, MA). All rats were allowed to recover for 7 days before the control period began when the rats weighed ~220 g.

Water intake, urinary volume output, and food intake were measured daily. Urine Na concentration was determined by flame photometry, and Na and water balances were calculated for each experiment day. Urinary Na plus nitrite excretion (UNO₃) was determined every 3–4 days using the Griess reaction and nitrate reductase from Escherichia coli to reduce nitrate to nitrite as we have done before (15, 32, 33).

Glomerular filtration rate (GFR) and effective renal plasma flow (ERPF) were determined every 3–4 days in conscious rats as we have done before (32, 33) by measuring the radioactivity and aminohippurate (30) concentration of a 4-h fasted plasma sample after a 12-h period of intravenous infusion of [¹⁵⁶]iodothalate and aminohippurate Na (4, 6).

Experimental protocols. The following six groups of Dahl rats were studied; the intramedullary groups were R-high Na alone (n = 7), R-high Na, AG (n = 7), S-high Na alone (n = 5), and S-high Na, AG (n = 7); the intravenous groups were R-high Na, AG (n = 7) and S-high Na, AG (n = 7). All these groups received a low-Na (0.3%) diet for the first 5 days of the recovery period and a high-Na (8%) diet for the last 2 days of recovery and the entire experimental period. Immediately after the recovery period, data were collected during a 5-day control period followed by a 5-day period of either vehicle (0.9% isotonic Na chloride solution) infusion or iNOS inhibition with intramedullary or intravenous infusion of AG at 3.075 mg·kg⁻¹·h⁻¹.

To confirm our AG results in R rats, a highly specific iNOS inhibitor, N-[3-(aminomethyl)benzyl]acetamidine HCl (1400W), was infused intravenously in two R rats and into the renal medulla in two R rats at 0.004 mg/h (29). The rats had measurements taken over a 5-day control period and a 4-day experimental period and received an 8% Na intake. 1400W has a 285- and ~7,000-fold selectivity for iNOS than neuronal (nNOS) or endothelial NOS (eNOS), respectively (13, 25). ACh dose-vasorelaxation curves on aortic segments were run on the rats using the protocol described below.

Specificity of iNOS inhibition. At the end of the experiment, to test the selectivity of iNOS inhibition by AG, aortic segments were taken for measuring their relaxation in response to ACh stimulation. On the day of the experiment, the rats were anesthetized by inhalation of isoflurane. The thoracic aorta was rapidly excised, placed in oxygenated Krebs solution, and cleaned of connective tissue. The aorta was cut transversely into 3-mm-wide rings. Aortic rings were cut open into strips. Extreme care was taken throughout the procedure to avoid injury to the endothelium.

One end of the aortic strip was attached to a glass hook using a thread loop, and the other end was connected to a force transducer (Grass FT03). Aortic rings were cut transversely into 3-mm-wide rings. Aortic rings were cut open into strips. Extreme care was taken throughout the procedure to avoid injury to the endothelium.

Statistical analysis. Comparison of data from animals on low- and high-salt diets were first performed using two-way ANOVA for repeated measures followed by a one-way ANOVA for repeated measures for each group and a Fisher least significant difference test for post hoc analysis at each experimental time point. Differences were considered to be statistically significant if P < 0.05. All data are expressed as means ± SE.

RESULTS

Arterial pressure responses to iNOS inhibition. Figure 1A shows that during high Na intake, intramedullary iNOS inhibition significantly increased mean arterial pressure in Dahl S rats compared with the high Na intramedullary group or the AG-intravenous group. By day 10, arterial pressure reached a value of 132 ± 2% control in the AG-intramedullary group and at this
time pressure was 121 ± 4% control (P < 0.05) in S-high Na alone rats. Meanwhile, the same dose of AG in the intravenous group did not increase the arterial pressure compared with the S-high Na alone group, and the pressure in the intravenous group was 121 ± 2% control. Figure 1B shows that there were no significant changes in mean arterial pressure when comparing the R-high Na alone intramedullary group, the R-high Na, AG-intramedullary group, and the R-high Na, AG-intravenous group, and mean arterial pressure values on day 10 were 102 ± 1, 101 ± 1, and 102 ± 2% control, respectively.

Table 1 shows the absolute control values for mean arterial pressure, GFR, and ERPF. Except for the ERPF in the S rats, there were no significant changes in arterial pressure or GFR in the high Na intramedullary group, the high Na, AG-intramedullary group, and the high Na, AG-intravenous group in either R or S rats. There were also no significant changes among these three groups in R rats in their ERPF values.

In the rats that received 1400W to inhibit iNOS, the control arterial pressure in the intravenous group was 93 ± 4 mmHg, and the value after 4 days of 1400W infusion was 93 ± 0 mmHg. In the intramedullary group, control pressure was 95 ± 2 mmHg and after 4 days of 1400W infusion it was 97 ± 6 mmHg. The aortic ACh-vasorelaxation curves were not changed by 1400W in either the intravenous or intramedullary groups compared with a high Na alone group.

GFR and ERPF responses to iNOS inhibition. Figure 2, A and B, and Figure 3, A and B, show that GFR and ERPF in all six groups did not significantly change.
during iNOS inhibition. By day 9, neither GFR nor ERPF of the S or the R rats in the high Na, AG-intramedullary group was significantly different from either the corresponding S or R high Na intramedullary group or high Na, AG-intravenous group.

**Urinary Na output responses to iNOS inhibition.** Figure 4 shows that AG did not significantly affect the urinary Na excretion in Dahl S rats. Likewise, except for day 8, the urinary Na excretion in the R-high Na, AG-intramedullary group was not significantly different from the R-high Na intramedullary or the R-high Na, AG-intravenous groups.

**Urinary volume output responses to iNOS inhibition.** Figure 5 shows that AG did not significantly affect the urinary volume output in the Dahl S groups. Likewise, except for day 8, the urinary volume output in the R-high Na, AG-intramedullary group was not significantly different from the R-high Na intramedullary or the R-high Na, AG-intravenous groups.

**Heart rate and \( U_{\text{NO}} \) excretion responses to iNOS inhibition.** Table 2 shows that heart rate was not significantly affected by iNOS inhibition in either the Dahl R or S rats. Urinary nitrate plus nitrite excretion, an index of whole body NO production, tended to decrease in all AG groups, but the changes did not reach significance.

**Changes in thoracic aorta vasorelaxation during iNOS inhibition.** Figure 6 shows that the ACh-vasorelaxation curve was significantly depressed in the S-high Na group compared with the R-high Na group, suggesting endothelial dysfunction. Inhibition of iNOS had no significant effect in any of the R or S groups.

**DISCUSSION**

The major new finding in this study is that iNOS inhibition in the renal medulla increases the salt sensitivity of the Dahl S rat but not the Dahl R rat. This indicates that NO produced by iNOS in the renal medulla normally prevents some of the increase in arterial pressure that occurs during high Na intake in S rats. Indeed Fig. 1 shows that mean arterial pressure in the S-high Na, AG-intramedullary group was more than the S-high Na alone group.

In a previous study from our laboratory, mean arterial pressure increased in Dahl S rats with intravenous...
AG treatment, suggesting that NO produced by iNOS prevented some of the increase in arterial pressure that occurs in these rats during increased Na intake (32). However, this previous study did not indicate which area of the body produced the iNOS-dependent NO. The present study suggests that during high Na intake the renal medulla of Dahl S rats is a source of iNOS that actively produces NO, thus preventing excessive increases in arterial pressure.

The previous study in our laboratory also showed that intravenous AG treatment made the salt-resistant R rats sodium sensitive (32). However, renal medullary infusion of AG in R rats in the present study did not affect salt sensitivity. There are several possibilities why systemic but not intramedullary AG did not increase salt sensitivity. First, combining the previous results with those from the present study suggests that NO produced by iNOS is important in preventing salt-sensitive hypertension in the R rat, but the source of this NO derived from iNOS is unlikely to be in the renal medulla. This is confirmed by our preliminary studies that showed that renal medullary iNOS protein was upregulated after a 1-wk exposure to a high-Na diet but not after a 2- or 3-wk high-Na diet. At the end of the present study, the rats had been exposed to high Na for 12 days, and iNOS protein in the medulla may not be upregulated at this time. Second, the increased pressure during systemic AG in R rats could have been caused by a direct effect on the renal cortex or by a systemic or central effect, which in turn had an effect on the kidney.

To confirm our AG intramedullary studies in R rats, a highly selective iNOS inhibitor, 1400W, (13, 25, 29) was infused intravenously and into the renal medulla of R rats using the same protocol as in the present experiment, and no increase in arterial pressure occurred. Also, no change occurred in the ACh-vasorelaxation curve in aortic segments. This is further evidence that our AG inhibited iNOS effectively in R rats without affecting other NOS isoforms.

In S rats both systemic and intramedullary AG increased arterial pressure, suggesting that part of the increase in arterial pressure during systemic AG is due to blockade of renal medullary iNOS. The arterial

### Table 2. Responses of HR and $U_{NOX}$ excretion

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<th>High Na IMED</th>
<th>High Na, AG-IMED</th>
<th>High Na, AG-IV</th>
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<tr>
<td><strong>HR, beats/min</strong></td>
<td></td>
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<tr>
<td>Day 5 control R rats</td>
<td>422 ± 3</td>
<td>423 ± 5</td>
<td>407 ± 20</td>
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<tr>
<td>Day 10</td>
<td>395 ± 5</td>
<td>393 ± 4</td>
<td>399 ± 6</td>
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<tr>
<td><strong>$U_{NOX}$, nmol/day</strong></td>
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<tr>
<td>Day 5 control R rats</td>
<td>10,696 ± 2,166</td>
<td>11,860 ± 2,803</td>
<td>7,254 ± 123</td>
</tr>
<tr>
<td>Day 10</td>
<td>10,757 ± 1,886</td>
<td>7,101 ± 483</td>
<td>6,418 ± 431</td>
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<td><strong>All data are means ± SE. HR, heart rate; $U_{NOX}$, urinary nitrates + nitrites. There were no significant changes in the high Na IMED, high Na, AG-IMED, and high Na, AG-IV groups on day 5 of control or on day 10. Day 5 of control is the last day of the control period; day 10 is the last day of the experimental period.</strong></td>
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pressure of the S rat seems to be highly dependent on NO produced by renal medullary iNOS protein. The increase in salt sensitivity in the S rats treated with AG occurred without any change in GFR, renal plasma flow, urine volume, or urinary volume output. However, a higher arterial pressure was required to excite the high-Na diet, indicating that a change in pressure natriuresis occurred.

Changes in renal NOS, including changes in renal iNOS, can have profound effects on renal excretory ability and thus arterial pressure (7, 15, 22, 33). Evidence that renal iNOS may play an important role in salt-sensitive hypertension has been provided by both biochemical and physiological studies. mRNA for iNOS is located in renal tubular and vascular segments. The highest level of iNOS mRNA is in the medullary thick ascending limb and the inner medullary collecting duct (1, 31), indicating that a decrease in medullary iNOS could increase Na reabsorption, resulting in salt-sensitive hypertension. Indeed, Mattson et al. (22) found that intravenous infusion of AG in Sprague-Dawley rats decreased renal medullary iNOS activity and urinary Na excretion for 2 days. Therefore, in the present experiment, medullary iNOS inhibition in the S rat may have increased Na retention in the medullary part of the nephron, thus increasing blood volume. Arterial pressure in turn would increase, thus increasing Na excretion back to normal. Therefore, iNOS inhibition would result in normal Na excretion but at an elevated arterial pressure, and, indeed, this occurred in the present experiment, because an increased arterial pressure in the S rat was necessary to excite the high Na load during medullary AG infusion. These data suggest that NO produced by iNOS in the renal medulla of S rats prevents excessive increases in arterial pressure in the Dahl S rat subjected to high Na intake.

An interesting finding in this study is that the S rats show considerable endothelial dysfunction. The release of NO by ACh during the high-Na diet significantly decreased in the aorta of S rats compared with R rats. N^\text{\textsuperscript{5}}\text{-nitro-L-arginine methyl ester (L-NAME; Refs. 18, 28) but not \text{n-NAME (18)} blocks the vasorelaxation to ACh in aortic rings, indicating that this response is mediated by NO. Because ACh causes the eNOS in endothelial cells to produce NO, this suggests that the release of NO by eNOS is decreased in the S rat.

Another important result that was found is that AG treatment of R rats did not significantly change the ACh-vasorelaxation curve in the intramedullary and intravenous groups. Likewise, AG treatment of S rats also did not significantly change the ACh-vasorelaxation curve in the intramedullary and intravenous groups. Importantly, this likely shows that iNOS inhibition did not affect aortic eNOS production of NO in the R and S rats.

Even though the present study is the first to examine the functional role of renal medullary iNOS in Dahl salt-sensitive hypertension, others have studied other aspects of iNOS in salt-sensitive hypertension. Molecular genetic linkage analysis showed that the locus for iNOS but not constitutive NOS (12) cosegregates with blood pressure in Dahl S rats. In a followup study, Deng (11) found that intraperitoneal injection of L-arginine prevents the decrease in blood pressure in Dahl S rats. This study shows that at low concentrations, which we used in this study, AG does not block eNOS, because it has a K_m that is 32- to 52-fold less for iNOS than for eNOS (24). In the present experiment, AG did not significantly affect the renal plasma flow, which also suggests that eNOS activity was unaffected by AG. Other groups have also shown that AG can selectively inhibit.
iNOS without affecting Ca-dependent NOS activity. AG infusion into Sprague-Dawley rats for 40 min, at a rate 20 times the rate we used in the present experiment, caused no change in arterial pressure or renal cortical blood flow (22). A 6-day intravenous infusion of AG at 10 mg·kg⁻¹·h⁻¹ decreased renal medullary Ca-independent NOS activity without affecting Ca-dependent activity (22).

Other studies have shown that AG at low doses does not affect nNOS. In a previous study in our laboratory, cerebellar Ca-dependent NOS activity, which is mainly nNOS, was unaffected by intravenous AG infusion (33). However, there is a possibility that the reason that this cerebellar NOS activity was unaffected was that AG did not cross the blood-brain barrier. Yet, systemically administered AG caused significant cerebroprotection in rats after brain injury due to either concussion or ischemia (10, 37), indicating that AG crosses the blood-brain barrier. In addition, a 6-day intravenous infusion of AG in rats did not affect Ca-dependent NOS activity in the renal medulla (22).

Renal medullary infusion technique. Several studies have shown that the renal medullary interstitial infusion technique is a powerful tool for assessing the role of the renal medulla. However, proper controls must be used to eliminate potential effects from systemic over- or recirculation of AG. This suggests that the cardiovascular-renal changes that occur after renal medullary infusion of AG are due to effects in the renal medulla and not caused by overflow and recirculation of AG.

In summary, iNOS inhibition by AG infusion into the renal medullary interstitial space caused an increase in salt sensitivity of Dahl S rat but not Dahl R rats. During high Na intake in S rats, AG increased arterial pressure but caused little change in heart rate, renal hemodynamics, and renal volume or Na excretion. AG did not affect ACh-induced vasorelaxation of the aorta and did not significantly change urinary nitrate plus nitrite excretion. These data suggest that during high Na intake, NO produced by iNOS in the renal medulla helps to prevent excessive increases in arterial pressure in Dahl S rats but not in Dahl R rats. NO produced by iNOS is important in preventing salt-sensitive hypertension in the R rat, but this iNOS is not likely to be located in the renal medulla.

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REFERENCES


