Inducible Nitric Oxide Synthase and Blood Pressure

David L. Mattson, Celso Y. Maeda, Timothy D. Bachman, Allen W. Cowley, Jr

Abstract—In the present studies, the influence of inducible nitric oxide synthase (NOS) inhibition with aminoguanidine on renal function and blood pressure was examined in rats. Intravenous aminoguanidine infusion (60 mg·kg⁻¹·hr⁻¹) for 40 minutes to anesthetized Sprague-Dawley rats (n=7) resulted in no significant changes in mean arterial pressure or renal cortical blood flow, while medullary blood flow was slightly increased. Despite minimal effects on renal blood flow, urine flow was significantly decreased from 14.2±2.7 to 10.4±2.3 μL·min⁻¹·g kidney wt⁻¹ during aminoguanidine infusion. To examine the possible effects of inducible NOS on blood pressure, aminoguanidine (10 mg·kg⁻¹·h⁻¹ IV) was infused chronically into uninephrectomized rats maintained on a high salt (4.0% NaCl) diet. Mean arterial pressure significantly increased from 104±2 to 118±3 mm Hg after 6 days of aminoguanidine infusion (n=7) and returned to levels not different from those in the control group after 2 days of postcontrol infusion. Calcium-independent NOS activity in the renal medulla, a tissue that expresses inducible NOS in normal rats, was significantly decreased by 49% in the aminoguanidine-infused group (n=6) compared with that activity in the vehicle-infused control animals (n=6). In contrast, calcium-dependent NOS activity in the renal medulla was not significantly altered by aminoguanidine infusion, indicating specificity of aminoguanidine for inducible NOS in these experiments. In a final group of rats (n=5), oral 1-arginine administration in drinking water (2% wt/vol) increased plasma arginine levels from 118±5 to 232±16 μmol/L and blocked the increase in arterial pressure after 6 days of aminoguanidine infusion. The present experiments provide evidence supporting a role for inducible NOS in the control of arterial pressure, possibly by renal tubular effects. (Hypertension. 1998;31[part 1]:15-20.)

Key Words: renal medulla ♦ nitric oxide ♦ blood flow

Several recent studies demonstrated that total NOS inhibition with arginine analogs leads to a blunting of the pressure natriuretic response, increased renal vascular resistance, and the development of hypertension during chronic treatment. Because nNOS, iNOS, and eNOS are all present in the normal rat kidney, it is not clear which NOS is/are responsible for these functional effects. Of interest is the presence of iNOS in the normal rat kidney. In situ hybridization and reverse transcription–polymerase chain reaction of microdissected vascular and tubular segments have identified iNOS mRNA in arcuate and interlobular arteries, glomeruli, proximal tubules, thick ascending limbs, and collecting ducts. The greatest amount of iNOS message was observed in the medullary thick ascending limbs and the inner medullary collecting ducts; this observation is consistent with protein blotting experiments that demonstrate greater amounts of iNOS immunoreactive protein in renal medullary than in renal cortical tissue homogenates. Despite the morphological localization of iNOS in the kidney, the role of this isoform in the control of normal renal function is unclear.

Several indirect studies suggest that iNOS may participate in the regulation of renal function and arterial pressure. The genetic locus containing the gene for iNOS cosegregated with the high blood pressure phenotype in F₉ populations of two crosses with the inbred Dahl salt-sensitive (SS) rat: [Dahl SS X Milan normotensive] and [Dahl SS X Wistar Kyoto]. Interestingly, the Dahl SS rat may be deficient in NO because arginine feeding (the substrate for NO formation) lowers blood pressure and normalizes the chronic renal function curve in Dahl SS rats. These data indicate that a defect in iNOS in Dahl SS rats may be partly responsible for the hypertension in these rats.

To date, there is limited functional data that describe the importance of iNOS in the normal control of renal function and blood pressure. The present studies were designed to investigate the influence of a selective iNOS enzyme inhibitor on sodium and water excretion, intrarenal blood flow distribution, and blood pressure in normal Sprague-Dawley rats.

Methods

Experiments were performed on male Sprague-Dawley rats (300 to 350 g) obtained from Sasco (Madison, Wisc). The rats were housed in the Animal Resource Center at the Medical College of Wisconsin with normal rat chow and tap water provided ad libitum, except where noted. All animal procedures were approved by the Medical College of Wisconsin Animal Care Committee, and the rats were closely monitored to ensure that none experienced undue stress or discomfort.

Protocol 1: Influence of Chronic Intravenous Infusion of Aminoguanidine on Blood Pressure in Conscious Rats

Rats (n=7) were anesthetized with an intramuscular injection of ketamine (100 mg/kg) and acepromazine (2 mg/kg), and the right
kidney was removed. A right nephrectomy was performed so that the remaining kidney was the sole determinant of renal function. A second surgery was performed 7 to 10 days after the nephrectomy to instrument the rats with chronic indwelling catheters. Catheters were placed in the abdominal aorta below the left renal artery via the femoral artery and in the vena cava via the femoral vein, tunneled subcutaneously, and exteriorized at the back of the neck in a piece of stainless steel spring.10,18,19 The spring was attached to a swivel device that allows the animal to move freely in its cage while being continuously infused. The rats received a postoperative injection of penicillin (40,000 U IM) to prevent infection.

After a 6- to 8-day recovery period from surgery, during which time the rats were continuously infused with saline (0.5 mL/h IV), daily blood pressure measurements were made during a 2- to 3-hour period. After 2 stable control days, aminoguanidine was added to the infusion to deliver 10 mg·kg⁻¹·h⁻¹. Daily blood pressure measurements were obtained as the aminoguanidine infusion was continued for 6 days. When the aminoguanidine infusion was stopped, blood pressure was measured on 2 postcontrol days, during which saline was infused intravenously.

To test for nonspecific effects of aminoguanidine on blood pressure, excess dietary l-arginine was administered to an additional group of rats (n=5) that were nephrectomized and instrumented with femoral arterial and venous catheters, as described above. These animals were maintained on high sodium chow (4.0% NaCl) and were given 2% excess dietary l-arginine was administered to an additional group of rats (n=5) that were nephrectomized and instrumented with femoral arterial and venous catheters, as described above. These animals were maintained on high sodium chow (4.0% NaCl) and were given 2% excess dietary l-arginine. Catheters were placed in the abdominal aorta below the left renal artery via the femoral artery and in the vena cava via the femoral vein, tunneled subcutaneously, and exteriorized at the back of the neck in a piece of stainless steel spring.10,18,19 The spring was attached to a swivel device that allows the animal to move freely in its cage while being continuously infused. The rats received a postoperative injection of penicillin (40,000 U IM) to prevent infection.

After a 6- to 8-day recovery period from surgery, during which time the rats were continuously infused with saline (0.5 mL/h IV), daily blood pressure measurements were made during a 2- to 3-hour period. After 2 stable control days, aminoguanidine was added to the infusion to deliver 10 mg·kg⁻¹·h⁻¹. Daily blood pressure measurements were obtained as the aminoguanidine infusion was continued for 6 days. When the aminoguanidine infusion was stopped, blood pressure was measured on 2 postcontrol days, during which saline was infused intravenously.

An arterial plasma sample was obtained from rats immediately before the administration of oral l-arginine in the drinking water and at the end of the experiment to determine the changes in plasma arginine after long-term l-arginine intake. Amino acids were separated by reverse-phase HPLC and quantified by fluorometric detection, using an adaptation of previously described methods.25 Before HPLC separation, the plasma samples were deproteinized with an equal volume of 0.14 mol/L sulfosalicylic acid containing a known concentration of L-β-alanine, which served as an internal standard. The samples then were mixed well, centrifuged at 10,000 g for 15 minutes to remove the precipitated protein, and derivatized with an equal volume of o-phthalaldehyde (1 mg/mL). The individual samples were separated by reverse-phase HPLC with a system consisting of a Bio-Rad AS-100 Auto Sampler, Hitachi L-7100 Gradient Pump, Waters (15 cm×3.9 mm, 5μ) column, Waters 474 Fluorescent Detector (excitation, 338A; emission, 425A), and a Hitachi D-2500 Integrator. Validation experiments demonstrated a clean separation of a standard cocktail consisting of 17 amino acids. With six repeated injections, the coefficient of variation (CV) for the arginine peak was 0.3% for retention time, 3.3% for peak area, and 1.65% for peak height. Peak area and height were linear for any given amino acid over the range of 30 nmol/L to 10 μmol/L (r²=0.99 for arginine peak height and r²=0.99 for arginine peak area). A plasma pool injected 23 times on 6 different days yielded a mean of 109 μmol/L for arginine (CV, 4.9%) and 74 μmol/L for citrulline (CV, 5.3%).

**Protocol 2: Influence of Intravenous Aminoguanidine Infusion on NOS Activity**

A separate group of animals was infused intravenously with aminoguanidine (10 mg·kg⁻¹·h⁻¹, n=6) or saline vehicle (n=6) for 3 days. After the third day of infusion, the rats were euthanized with an overdose of sodium pentobarbital, and the renal medulla was rapidly removed and frozen on dry ice. The whole tissue was homogenized using a Potter-Elvehjem tissue grinder at 3000 rpm in a solution containing 250 mmol/L sucrose, 1 mmol/L EDTA, 0.1 mmol/L PMSF, and 5 mmol/L potassium phosphate, pH 7.7. All chemicals were purchased from Sigma, unless otherwise noted. The homogenate was centrifuged at low speed (15,000 g, 4°C, 20 minutes), and the protein concentration of the supernatant was determined by use of a Coomassie blue protein assay (Pierce), with albumin as a standard. The NOS enzymatic activity of the tissue was determined with the protocol described below. We attempted but were unable to quantify enzyme activity in the renal cortex and the aorta of these animals because the NOS activity in these tissues is below the detection level of our present assay.

The NOS enzyme assay is based on previously described methods.19,20 The total tissue homogenate was incubated with 2 mmol/L CaCl₂, 1 mmol/L NADPH, 25 μmol/L FAD, 1.25 μg/mL calmodulin, 10 μmol/L tetrahydrobiopterin, and H-arginine (approximately 300,000 cpm, S.A. 68 Ci/mmol) in 20 mmol/L HEPES buffer, pH 7.2, at 37°C for 5 minutes. Calcium-independent activity was measured after the addition of 0.5 mmol/L EDTA and the elimination of calcium/calmodulin from the assay. Chromatograms obtained from spleen of lipopolysaccharide-treated rats (a tissue that contains primarily calcium-independent iNOS) and cerebellum of normal rats (a tissue that contains predominantly calcium-dependent constitutive NOS) incubated with and without calcium are illustrated in Fig 1. As shown in the left panel, incubation of spleen or cerebellum with radiolabeled arginine produced a marked citrulline peak (the cerebellar sample converted all of the arginine to citrulline). However, while elimination of calcium-calmodulin and addition of 0.5 mmol/L EDTA had no influence on citrulline formation by the spleen protein homogenerate, these conditions eliminated citrulline formation by the cerebellum. These experimental conditions were thus used to obtain an index of inducible and constitutive NOS activity in the renal medullary tissue homogenates. The arginine and converted citrulline were separated by isocratic reverse-phase HPLC with a Supelco LC-18-DB column (mobile phase 11.5% methanol, 11.5% acetonitrile, 0.1 mol/L KH₂PO₄, pH 5.9). The amount of converted citrulline and the total counts were quantified by radiochemical detection (Packard). With use of these experimental conditions, the concentration of radiolabeled citrulline must exceed 0.2 mmol/L in the reaction vial to be distinguished from the background, for most tissues.

**Protocol 3: Acute Effects of Aminoguanidine on Blood Pressure, Renal Cortical and Medullary Blood Flow, and Sodium and Water Excretion in Anesthetized Rats**

Sprague-Dawley rats (n=7) were anesthetized with Inactin (100 mg/kg IP) and placed on a heated table to maintain body temperature at 37°C. Cannulas were placed in the femoral artery for measurement of arterial pressure, in the femoral vein for intravenous infusion, and in the ureter for urine collection. The trachea was intubated to facilitate respiration. The kidney was placed in a stainless steel holder as previously described, with optical fibers implanted into the renal cortex (1.5 mm deep) and to the outer/inner medullary border (4 to 5 mm) to monitor blood flow by laser-Doppler flowmetry.24 Surgical fluid losses were replaced by continuous intravenous infusion of 1% bovine serum albumin (fraction V) in a 0.9% sodium chloride solution at a rate of 1.0 mL/h per 100 g body weight throughout the experiment. After a 1-hour postsurgical equilibration period, the experimental protocol was begun. Data were obtained during two 20-minute control periods (C1 and C2) and during two 20-minute periods (A1 and A2) in which aminoguanidine was infused intravenously (60 mg·kg⁻¹·h⁻¹). During each experimental period, mean arterial pressure and the laser-Doppler signal from optical fibers implanted in the renal cortex and medulla were continually recorded. In addition, urine was collected for determination of urinary flow rate and sodium excretion.
Urine volume was determined gravimetrically; the urinary sodium concentration was determined by flame photometry.

Statistical Methods

Data are expressed as the mean ± SE. The within-group changes in protocols 1 and 3 were evaluated using a one-way ANOVA for repeated measures, with a Duncan post hoc test. Comparison of raw data in protocol 2 was performed using an unpaired two-tail t test. A level of P < .05 was considered significant.

Results

Protocol 1: Influence of Chronic Intravenous Infusion of Aminoguanidine on Blood Pressure in Conscious Rats

As illustrated in Fig 2, mean arterial pressure averaged 104 ± 2 mm Hg on the initial control day in rats (n = 7) maintained on a high sodium diet, was increased to 114 ± 3 mm Hg by the second day of aminoguanidine infusion, and was maintained at that level throughout the 6-day infusion period, with an average value of 118 ± 3 mm Hg on the sixth day. When the aminoguanidine infusion was stopped, mean arterial pressure decreased to a value not significantly different from that of the control on the first postcontrol day and averaged 108 ± 2 mm Hg on the second postcontrol day. The heart rate in this group of rats was unaltered from the control value of 371 ± 9 bpm throughout the experiment. The body weight of the rats in this group averaged 355 ± 9 g in the control period, was significantly increased to 370 ± 8 g on the second experimental day, and was maintained at this level throughout the experimental and postcontrol period.

Pretreatment of a second group of rats with oral L-arginine (2% wt/vol, n = 5) prevented the increase in mean arterial pressure after chronic aminoguanidine infusion. Control mean arterial pressure averaged 111 ± 3 mm Hg and was 116 ± 2 mm Hg after 6 days of aminoguanidine infusion. The change in mean arterial pressure in the groups of rats treated with aminoguanidine and those pretreated with L-arginine and then administered aminoguanidine is illustrated in Fig 3. Heart rate averaged 368 ± 10 bpm and body weight averaged 357 ± 10 g in the control period; they were not altered throughout the protocol. Daily water intake ranged from 60 to 80 mL/d throughout the protocol. Comparison of plasma amino acids of the control group (n = 6) versus rats given oral L-arginine in drinking water (n = 5) demonstrated that plasma arginine was significantly increased from 118 ± 6 to 21 ± 22 μmol/L by this diet. Plasma citrulline and ornithine levels were unaltered from the control values of 75 ± 5 and 58 ± 9 μmol/L, respectively.

Protocol 2: Influence of Chronic Intravenous Aminoguanidine Infusion on NOS Activity

There were no significant differences in calcium-dependent NOS activity in the medulla of the aminoguanidine-treated rats when compared with vehicle-treated animals (Fig 4). In contrast, calcium-independent NOS activity in whole renal medullary tissue homogenate was significantly decreased by 49% in the aminoguanidine-treated group. The total calcium-dependent counts converted to citrulline in the renal medullary homogenate averaged 39 979 ± 3463 and 32 217 ± 2992 cpm in the control and treated groups, respectively. The counts converted to citrulline by the renal medullary homog-
enated under calcium-free conditions were significantly decreased in the aminoguanidine group, averaging 12,399±2320 cpm in the control group and 6269±1190 cpm in the drug-treated group.

**Protocol 3: Acute Effects of Aminoguanidine on Blood Pressure, Renal Cortical and Medullary Blood Flow, and Sodium and Water Excretion in Anesthetized Rats**

During the acute experimental period, the hematocrit and plasma protein levels were unaltered from control levels of 44±2% and 4.6±0.3g/dL during the experimental infusion. The influence of acute aminoguanidine infusion on blood pressure and renal cortical and medullary blood flow is illustrated in Fig 5. Mean arterial pressure was not altered from the control value of 110±3 mm Hg during the experimental period. Cortical blood flow also was not altered from the arbitrary flow signal value of 1.95±0.13 V throughout the aminoguanidine infusion period. The flow signal from optical fibers implanted in the renal medulla was significantly increased by approximately 8% from a mean value of 0.51±0.05 V in the control period to 0.55±0.05 V during the second experimental period. The influence of aminoguanidine on sodium and water excretion is shown in Fig 6. Urinary flow significantly decreased from a control value of 14.2±2.7 to 10.4±2.3 µL/min per gram of kidney weight during the final aminoguanidine infusion period. Sodium excretion was decreased from a control value of 2.1±0.3 to 1.6±0.3 µEq/min per gram of kidney weight during the final aminoguanidine period, but it was not statistically significant from the control group.

**Discussion**

Numerous studies have described the renal vasoconstrictor, antinatriuretic, and hypertensive effects of the arginine analog NOS inhibitors.1–7 However, the arginine analogs are not isoform specific,25 so the functional role of the different NOS isoforms is not clear. In the present study, the effects of the iNOS selective enzyme inhibitor aminoguanidine on renal function and blood pressure were evaluated in normal Sprague-Dawley rats. Acute infusion of aminoguanidine to

anesthetized rats did not alter mean arterial pressure or renal cortical blood flow and slightly increased medullary blood flow. Despite the lack of vasoconstrictor effects, urine flow was decreased by 30% and sodium excretion was decreased by 24%.

In conscious rats, continuous aminoguanidine infusion for 6 days increased mean arterial pressure by 14 mm Hg. Pressure returned to control levels in the postcontrol period, demonstrating reversibility of these hypertensive effects. These experiments provide evidence supporting a role for iNOS in the control of renal function and blood pressure, possibly by effects on renal tubular function.

Previous experiments have shown that aminoguanidine is a selective iNOS inhibitor.25 In vitro, aminoguanidine has a K<sub>i</sub> value that is 32 to 52 times less for iNOS than for constitutive NOS.26,27 In situ, 0.1 to 1.0 mol/L aminoguanidine has no significant influence on the tone of aortic rings, whereas L-NAME and L-NMMA are potent constrictors in aortic rings from normal rats.26,28 In vivo, aminoguanidine is 40 times less potent than L-NMMA to acutely increase blood pressure in rats.29 These studies indicate that aminoguanidine can be used as a selective inhibitor of iNOS in vivo.

Biochemical and functional data provide evidence that aminoguanidine selectively inhibited iNOS in the present studies as well. Calcium-independent NOS activity in the renal medulla of rats chronically treated with aminoguanidine was significantly decreased by 49%. Calcium-dependent NOS activity in the renal medulla of aminoguanidine-treated rats tended to decrease but was not statistically different from the control values. These in vitro measurements indicate minimal effect of aminoguanidine on constitutive NOS but significant inhibitory action on iNOS in the present studies. Because of the low endogenous NOS activity in other portions of the body, which is below the detectable limit of our NOS assay, we were unable to document that aminoguanidine did not inhibit constitutive NOS activity in other tissues. Functional data, however, support iNOS selectivity in this study. Acute infusion of aminoguanidine at 6 times the chronic infusion rate did not increase arterial pressure or decrease renal blood flow,
indicating that these doses of aminoguanidine have a minimal influence on constitutive eNOS. Surprisingly, it was observed that acute aminoguanidine infusion led to a significant increase in renal medullary blood flow; the mechanism of this increase is presently unclear. Together, these data indicate that aminoguanidine selectively blocked iNOS in the present studies.

Despite the specificity of aminoguanidine to inhibit iNOS in the present studies, a number of nonspecific side-effects have been reported for this compound. Aminoguanidine has been reported to inhibit the formation of advanced glycation end products, cause aggregation of leukocytes in mesenteric venules, inhibit diamine oxidase and alter the renal response to insulin-like growth factor 1. Though these or other side-effects could indeed influence the biological effects of this compound, the prevention of the long-term effects of aminoguanidine on blood pressure by administering excess NOS substrate (2% L-arginine in drinking water) argues against nonspecific effects of this drug in the present studies.

The presence and distribution of iNOS in the kidney of normal rats has been described in a number of studies. On a whole-tissue basis, a large amount of iNOS immunoreactive protein has been detected in the renal inner and outer medulla of normal Sprague-Dawley rats, but iNOS protein was not detected in the renal cortex in one study. Reverse transcription-polymerase chain reaction of microdissected renal tubules and microvessels has been used to demonstrate the presence of iNOS mRNA in arcuate and interlobular arteries, glomeruli, proximal tubules, thick ascending limbs, and collecting ducts. Because the present studies did not attempt to isolate the functional effects of aminoguanidine to any specific region of the kidney or body, the aminoguanidine infusion in the present studies could be acting at any or all of these sites in the kidney or other sites in the body. The observation that aminoguanidine infusion significantly decreased urinary flow rate in the absence of alterations in blood pressure or renal cortical blood flow indicates that the acute effects are at the level of the renal tubules. One possible site of action is the collecting ducts, where NO has been shown in isolated tubules to inhibit both sodium and water reabsorption. Because relatively large amounts of iNOS are found in the medulla and the iNOS message has been localized in the collecting ducts, the collecting ducts are a likely site of action of aminoguanidine. It is not possible, however, to clearly determine the site of action in the present study.

As an extension of the acute effects on renal function, chronic aminoguanidine administration led to a sustained increase in arterial pressure. The mechanism of hypertension is possibly due to decreased renal sodium and water excretion, which led to an expansion of extracellular volume in this study. This interpretation is supported by the significant increase in body weight during chronic aminoguanidine. It was also observed, however, that blood pressure returned to control levels during the postcontrol period, but body weight did not decrease. Because we have previously observed that changes in
body weight do not always reflect changes in daily sodium and water balance in rats, further studies are required to determine whether the hypertension that occurs during chronic aminoguanidine infusion is dependent on sodium and water retention.

In summary, the results of the present study indicate that iNOS participates in the regulation of renal function and arterial pressure. Systemic aminoguanidine infusion led to minimal acute hemodynamic alterations, reduced urinary flow rate, and the development of hypertension. Further studies need to be performed to elucidate the regulatory role of iNOS in the control of renal function and arterial pressure.

Acknowledgment
This work was partially supported by National Heart, Lung, and Blood Institute grant HL-29587.

References
Inducible Nitric Oxide Synthase and Blood Pressure
David L. Mattson, Celso Y. Maeda, Timothy D. Bachman and Allen W. Cowley, Jr

Hypertension. 1998;31:15-20
doi: 10.1161/01.HYP.31.1.15

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1998 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the
World Wide Web at:
http://hyper.ahajournals.org/content/31/1/15

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org/subscriptions/