Neuronal Nitric Oxide Synthase-Dependent Afferent Arteriolar Function in Angiotensin II-Induced Hypertension

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Abstract—This study was designed to determine the influence of neuronal nitric oxide synthase (nNOS) in tubular flow-dependent regulation of afferent arteriolar diameter in hypertensive Sprague-Dawley rats that received 60 ng/min angiotensin II (Ang II) subcutaneously for 13 days. Systolic blood pressure of control and Ang II–infused rats averaged 122±2 (n=23) and 194±2 mm Hg (n=24). Afferent arteriolar responses to the nNOS inhibitor S-methyl-L-thiocitrulline (L-SMTC; 0.1 to 10 μmol/L) and the nonselective NOS inhibitor Nω-nitro-L-arginine (L-NNA; 1 to 100 μmol/L) were assessed in vitro using the blood-perfused juxtamedullary nephron preparation. At a perfusion pressure of 160 mm Hg, afferent arteriolar diameters from control and Ang II–infused rats averaged 18.7±1.1 μm (n=8) and 18.1±1.1 μm (n=9), respectively, and decreased by 19.9±1.5% and 11.8±1.1%, respectively, in response to 10 μmol/L L-SMTC. The L-SMTC–induced afferent arteriolar constriction was significantly greater in control than in Ang II–infused rats. In contrast, 100 μmol/L L-NNA constricted afferent arterioles similarly in both control (n=8) and Ang II–infused (n=7) rats. After transection of the loops of Henle to interrupt flow to the macula densa, the vasoconstrictor responses to L-SMTC but not to L-NNA were reversed. Increasing distal volume delivery by addition of 10 mmol/L acetazolamide to the blood perfusate significantly enhanced the afferent arteriolar constrictor responses to 10 μmol/L L-SMTC (34.5±4.8% vs 19.9±1.5%, n=7) in normotensive rats. In contrast, in Ang II–infused rats, acetazolamide treatment did not enhance the responses to L-SMTC (n=8). These results indicate that chronic Ang II infusion reduces the ability of nNOS-derived nitric oxide to counteract the afferent arteriolar response to increased distal tubular flow. (Hypertension. 1999;33[part II]:462-466.)

Key Words: rats • arterioles • macula densa • acetazolamide • papillectomy

Chronic infusion of subpressor doses of angiotensin II (Ang II) produces a hypertension that mimics 2-kidney, 1 clip (2K1C) Goldblatt hypertension.1 In this model of hypertension, renal Ang II levels are elevated, and afferent arteriolar responsiveness to Ang II is enhanced.2 However, renal blood flow and glomerular filtration rate are either not reduced or only slightly diminished,3–6 partially because nitric oxide (NO) production is maintained at the level of whole kidney6,7 and in afferent arterioles8 in this model of hypertension and counteracts the enhanced afferent arteriolar reactivity to Ang II.

NO is constitutively generated from endothelial NO synthase and neuronal NO synthase (nNOS). Recent studies have indicated that nNOS, which has been localized to macula densa cells, also contributes to the regulation of preglomerular and postglomerular microcirculation.9,10 In particular, when the activity of the tubuloglomerular feedback (TGF) mechanism is increased, the macula densa nNOS-derived NO influence increases to partially counteract the TGF-mediated preglomerular vasoconstriction.10,11 Recent immunohistochemical studies have demonstrated that NADPH diaphorase staining, which reflects NOS activity, decreased in macula densa cells in the nonclipped kidneys of 2K1C Goldblatt hypertensive rats.12 In addition, macula densa NADPH diaphorase staining and renal nNOS mRNA levels have been shown to be increased in angiotensinogen gene knockout mice13 and angiotensin type 1A receptor gene knockout mice14 compared with wild-type mice. These findings suggest that chronic elevations in Ang II levels and/or perfusion pressure can downregulate macula densa nNOS activity.

We hypothesized that nNOS activity may be selectively reduced in Ang II–infused hypertensive rats, leading to reduced influences on afferent arteriolar tone. To test this hypothesis, we used the in vitro blood-perfused juxtamedullary nephron preparation15,16; we determined the effects of nNOS inhibition on afferent arteriolar diameters under normal conditions and during increased distal nephron volume and sodium delivery, which was achieved by inhibition of proximal tubular reabsorption after addition of acetazolamide to the blood perfusate.10

Methods

Preparation of Animals

Male Sprague-Dawley rats (Charles River Laboratories, Wilmington, Mass) were housed in wire cages and maintained in a temperature-controlled room that was regulated on a 12/12-hour
light/dark cycle. Rats had free access to water and standard rat chow (Ralston-Purina). All experimental protocols were approved by the Tulane University Animal Care and Use Committee. Rats (175 to 200 g body weight) were anesthetized with sodium pentobarbital (50 mg/kg IP). An osmotic minipump (model 2002, Alza Corp) was implanted subcutaneously at the dorsum of the neck. Ang II–infused rats received Ang II (Novabiochem) at a rate of 60 ng/min for 13 days. Control rats were sham-operated. Systolic arterial pressure was measured every 3 days in conscious rats by tail-cuff plethysmography (model 52-0338, Harvard Apparatus).

Assessment of Afferent Arteriolar Responsiveness
Afferent arteriolar responsiveness was assessed on day 13 after minipump implantation using the in vitro blood-perfused juxtaglomerular nephron preparation combined with videomicroscopy, as previously described.2,8,10,13–17 Briefly, each experiment used 2 rats from the same treatment group, with 1 rat serving as the blood donor and the second rat as the kidney donor. Rats were anesthetized with sodium pentobarbital (50 mg/kg IP). Donor blood was collected into a heparinized (500 U) syringe and centrifuged to separate the plasma and cellular fractions. Plasma oncotic pressure was adjusted to 18 mm Hg by the addition of bovine serum albumin (Sigma Chemical Co). After processing of the plasma through filters, erythrocytes were added to achieve a hematocrit level of 33%. The reconstituted blood was stirred continuously in a closed reservoir that was pressurized with a 95% O2-5% CO2 gas mixture.

The right renal artery of the kidney donor was cannulated and perfused with Tyrode’s solution (pH 7.4) containing 5.1% bovine serum albumin and a mixture of L-amino acids (Sigma).16 The kidney was excised and prepared as previously described2,8,10,13–17 so that the vascularule of the juxtaglomerular nephrons could be visualized directly. The arterial supply of the exposed microvasculature was isolated by ligating the large branches of renal artery with fine suture (nylon black monofilament, 10-0; Vanguard Surgical System).

After the dissection was completed, the Tyrode’s perfusate was replaced with the reconstituted blood. Renal perfusion pressure (RPP) was monitored by a pressure cannula centered in the perfusion cannula and regulated by adjusting the rate of gas inflow into the blood reservoir. RPP was set at 100 mm Hg for the initial 5 minutes and then raised and maintained at 160 mm Hg during the experimental protocols. The perfusion chamber was warmed, and the inner cortical surface was continuously superfused with warmed (37°C) Tyrode’s solution containing 1% bovine serum albumin.

The tissue was transilluminated on the fixed stage of a Leitz Laborlux-12 microscope. Video images of the microvessels were obtained with a Newvicon camera (model NC-67 M, Dage-MTI) and recorded on videotape for later analysis (videocassette recorder HR-V181U, JVC). Afferent arteriole diameters were measured at 12-second intervals using a calibrated digital image-shearing monitor (Instrumentation for Physiology and Medicine) that yielded vascularule measurements reproducible within 0.5 μm. Afferent arteriolar diameters were measured at sites within 100 μm upstream from the glomerulus. A minimum 10-minute equilibration period was allowed before the initiation of each experimental procedure. The average diameter during the final 2 minutes of each 5-minute treatment period was used for statistical analysis of steady-state responses.

Afferent Arteriolar Response to nNOS Inhibition
To assess the degree of nNOS influence on afferent arteriolar diameters of control normotensive rats and Ang II–infused hypertensive rats, afferent arteriolar diameters were measured under control conditions and during exposure to increasing concentrations (0.1, 1, and 10 μmol/L) of the nNOS inhibitor S-methyl-L-thiocitrulline (L-SMTC; Alexis Co). Using another series of control normotensive rats and Ang II–infused hypertensive rats, we also examined the nNOS activity in afferent arterioles during increased distal nephron volume and sodium delivery, which was achieved by inhibition of proximal tubular reabsorption after acetazolamide treatment. After control measurements were made to determine baseline diameter, 10 mmol/L acetazolamide (Sigma) was added to the blood perfusate. Our previous study confirmed that this dose of acetazolamide significantly decreases afferent arteriolar diameters through a tubular flow-dependent mechanism.10 After a 5-minute stabilization period, afferent arteriolar responsiveness to the same concentrations of L-SMTC was determined.

Because juxtaglomerular nephrons have long loops of Henle reaching into the papilla,13 acute papillectomy interrupts the flow from proximal tubules to distal nephron segments.14 To confirm that the effects of L-SMTC and acetazolamide depend on intact distal tubular flow, at the end of protocols the papilla was transected near the corticomedullary junction, and afferent arteriolar diameters were measured.

Afferent Arteriolar Response to NOS Inhibition
To determine the total NOS influence in afferent arterioles of control normotensive rats and Ang II–infused hypertensive rats, afferent arteriolar diameters were assessed under control conditions and during exposure to increasing concentrations (1, 10, and 100 μmol/L) of the nonselective NOS inhibitor Nω-nitro-L-arginine (L-NNA; Aldrich Chemical Co). To examine whether the effect of L-NNA depends on intact distal tubular flow, acute papillectomy was also performed at the end of these protocols.

Statistical Analysis
One-way ANOVA for repeated measures combined with Newman-Keuls post hoc test was used for within-group analyses. Between-group analyses were performed with a 2-way ANOVA for repeated measures combined with Newman-Keuls post hoc test. A value of P<0.05 was considered significant. Data are presented as mean±SEM.

Results
On day 13, conscious systolic arterial pressures averaged 194±2 mm Hg in chronically Ang II–infused rats (n=24). This value was significantly greater than that determined in control rats (122±2 mm Hg, n=23). In control and Ang II–infused rats, afferent arteriolar diameters at an RPP of 100 mm Hg averaged 21.8±0.7 (n=23) and 21.6±0.9 (n=24), respectively, and decreased by 14.6±0.9% and 13.1±1.0% in response to elevating RPP to 160 mm Hg.

Afferent Arteriolar Response to nNOS Inhibition
Figure 1 describes afferent arteriolar responsiveness to nNOS inhibition with L-SMTC at an RPP of 160 mm Hg. In control rats, basal afferent arteriolar diameter averaged 18.7±1.1 μm (n=8) and significantly decreased by 8.4±1.2%, 12.9±1.8%, and 19.9±1.5% in response to 0.1, 1, and 10 μmol/L L-SMTC, respectively. In Ang II–infused hypertensive rats, basal afferent arteriolar diameter averaged 18.1±1.1 μm (n=9), and the same concentrations of L-SMTC significantly decreased afferent arteriolar diameters by 3.4±0.9%, 6.0±0.9%, and 11.8±1.1%, respectively. The decrease in diameter observed in hypertensive rats was significantly lower than that observed in control rats. In control and Ang II–infused rats, papillectomy reversed afferent arteriolar constriction responses to 10 μmol/L L-SMTC, and afferent arteriolar diameters after papillectomy averaged 19.8±1.3 and 19.0±1.0 μm, respectively. The inhibition of L-SMTC–induced afferent arteriolar constriction by papillectomy is consistent with our previous finding that L-SMTC at a level of up to 10 μmol/L does not affect afferent arteriolar diameters in papillectomized kidneys.10
Afferent Arteriolar Response to nNOS Inhibition During Increased Distal Nephron Volume Delivery

As shown in Figure 2, afferent arteriolar responsiveness to L-SMTC was also assessed at an RPP of 160 mm Hg when 10 mmol/L acetazolamide was added to the blood perfusate to increase distal nephron volume delivery. Acetazolamide treatment decreased afferent arteriolar diameters of control and Ang II–infused rats similarly by 14.0±0.9% and 11.3±2.1%, respectively, at an RPP of 160 mm Hg.

In the presence of acetazolamide, afferent arteriolar diameters of control rats averaged 15.2±0.7 μm (n=7) and significantly decreased by 8.7±0.5%, 16.8±2.6%, and 34.5±4.8% in response to 0.1, 1, and 10 μmol/L L-SMTC, respectively. The decrease in diameter in Ang II–infused rats was similar to that observed in the absence of acetazolamide (Figure 1) and was significantly lower than that determined in acetazolamide-treated kidneys of control rats. Thus, acetazolamide treatment significantly enhanced afferent arteriolar responsiveness to L-SMTC in control rats but did not influence the L-SMTC response in afferent arterioles of Ang II–infused hypertensive rats. In acetazolamide-treated kidneys of control rats and Ang II–infused rats, papillectomy reversed the afferent arteriolar vasoconstriction caused by 10 μmol/L L-SMTC and increased afferent arteriolar diameters to 17.3±0.7 and 20.8±1.6 μm, respectively. These values were similar to afferent arteriolar diameters before addition of acetazolamide. Thus, consistent with our previous findings,10 papillectomy significantly prevented the effect of acetazolamide on afferent arterioles.

Afferent Arteriolar Response to NOS Inhibition

Figure 3 demonstrates afferent arteriolar responsiveness to NOS inhibition with L-NNA at an RPP of 160 mm Hg. In control rats, basal afferent arteriolar diameter averaged 19.4±1.4 μm (n=8) and significantly decreased by 6.9±0.6%, 11.1±0.9%, and 16.6±1.3% in response to 1, 10, and 100 μmol/L L-NNA, respectively. In Ang II–infused hypertensive rats, basal afferent arteriolar diameter averaged 18.1±1.5 μm (n=7). Superfusion with 1, 10, and

Figure 1. Effects of L-SMTC on afferent arteriolar diameters of control rats (○, n=8) and Ang II–infused hypertensive rats (●, n=9). Papillectomy (PX) was performed at the end of the experiment. Data are expressed in micrometers (A) and percent changes (B). Experiments were performed at an RPP of 160 mm Hg. *P<0.05 vs control diameter; †P<0.05 vs control rats.

Figure 2. Effects of L-SMTC on afferent arteriolar diameters of control rats (○, n=8) and Ang II–infused hypertensive rats (●, n=8) during addition of 10 mmol/L acetazolamide to the blood perfusate. Papillectomy (PX) was performed at the end of experiment. Data are expressed in micrometers (A) and percent changes (B). Experiments were performed at an RPP of 160 mm Hg. *P<0.05 vs control diameter; †P<0.05 vs control rats; §P<0.05 vs response in the absence of acetazolamide.

Figure 3. Effects of L-NNA on afferent arteriolar diameters of control rats (○, n=8) and Ang II–infused hypertensive rats (●, n=7). Papillectomy (PX) was performed at the end of experiment. Data are expressed in micrometers (A) and percent changes (B). Experiments were performed at an RPP of 160 mm Hg. *P<0.05 vs control diameter.
100 μmol/L L-NNA significantly decreased afferent arteriolar diameters by 6.0±1.6%, 12.5±1.6%, and 17.5±1.3%, respectively, and the decrease was similar to that observed in control rats. Papillectomy did not significantly influence the afferent arteriolar constrictor response to 100 μmol/L L-NNA in either control rats or Ang II–infused rats.

Discussion
Renal constitutive NOS activity has been reported to be maintained or elevated in various models of hypertension including young and adult spontaneously hypertensive rats (SHR),19–21 stroke-prone SHR,22 early hypertensive 1-kidney, 1 clip rats,23 and chronically Ang II–infused hypertensive rats.6–8 Nevertheless, a recent study demonstrated that NOS activity and protein levels decrease in the macula densa cells of the nonclipped kidney of 2K1C rats.12 In normotensive rats, nNOS plays an important role in the counteracting modulation of TGF-mediated afferent arteriolar constriction,9,10 and thus it would be possible that a decreased nNOS activity partially accounts for the enhanced TGF activity observed in Ang II–dependent hypertension.24 The present experiments were performed to determine the contribution of nNOS activity to the tubular flow-dependent regulation of afferent arteriolar tone.

The present study evaluated the contributions of total NOS and nNOS activity to afferent arteriolar tone. Afferent arteriolar responses to L-NNA at an RPP of 160 mm Hg were similar in normotensive control and Ang II–infused hypertensive rats. We previously reported that L-NNA responses at an RPP of 100 mm Hg were greater in Ang II–infused hypertensive rats than in normotensive control rats,8 suggesting an overall increase in NOS contribution to afferent arteriolar tone. In contrast, the decrease in afferent arteriolar diameter in response to L-SMTC was significantly less in Ang II–infused hypertensive rats compared with that in normotensive control rats. These results suggest that nNOS activity is inappropriately reduced in Ang II–infused hypertensive rats, while total NOS activity is similar or increased in Ang II–infused hypertensive rats compared with that in normotensive rats.

We previously demonstrated that L-SMTC did not influence afferent arteriolar diameter of papillectomized kidneys in which distal tubular flow past the macula densa was interrupted,10 suggesting that renal nNOS activity depends on intact distal tubular flow. The present results are consistent with the previous report that papillectomy prevented the L-SMTC–induced afferent arteriolar constriction. However, the afferent arteriolar constrictor responses to L-NNA were not influenced by papillectomy. This result indicates that L-NNA–induced afferent arteriolar constriction does not depend on distal tubular flow past the macula densa segment and thus may be distinguished from the L-SMTC actions on afferent arterioles.

In normotensive control rats, nNOS influence on afferent arteriolar diameter was enhanced after procedures that increase distal nephron volume and sodium delivery, such as during inhibition of proximal tubular reabsorption rate with acetazolamide. Because papillectomy prevented the acetazolamide effect, the possibility that nonspecific effects of acetazolamide other than tubular flow-dependent effects might influence afferent arteriolar diameters was eliminated. The acetazolamide-induced enhancement of the afferent arteriolar response to L-SMTC in normotensive rats at an RPP of 160 mm Hg is similar to our previous results in experiments performed at an RPP of 100 mm Hg.10 In contrast, acetazolamide-induced increases in distal nephron delivery did not influence the nNOS contribution to afferent arteriolar diameter in Ang II–infused hypertensive rats. These results indicate that the ability of nNOS to counteract the afferent arteriolar constriction in response to increased volume and sodium chloride delivery past the macula densa is impaired in Ang II–infused hypertensive rats. This may be a physiological mechanism to allow maximum TGF sensitivity under sodium-depleted conditions, which would be the major stimulus activating the renin-angiotensin system.

Previous studies have shown that renal nNOS activity is regulated by changes in the renin-angiotensin system and blood pressure. Macula densa NADPH diaphorase staining, an indicator of nNOS activity, decreased in the nonclipped kidney of 2K1C Goldblatt hypertensive rats.12 Likewise, macula densa NADPH diaphorase staining and renal nNOS mRNA levels were elevated in angiotensinogen gene13 and angiotensin type 1A receptor gene knockout mice14 compared with levels in wild-type mice. In addition, the intensity of nNOS immunostaining in the glomeruli has been reported to decrease in hypertensive rats treated with deoxycorticosterone acetate salt that have low plasma levels of renin and Ang II.25 Therefore, Ang II and/or RPP play important roles in the regulation of the macula densa nNOS activity. The diminished nNOS activity after increased distal nephron delivery observed in Ang II–infused hypertensive rats may be the result of a decreased nNOS mRNA, protein, and/or enzyme activity. However, a recent study has shown that the number of nNOS immunostaining-positive glomeruli is increased in hypertensive rats infused with Ang II (40 ng/min, 14 days).25 One possible explanation for these contradictory findings could be that the enzyme activity, but not the protein level, of the macula densa nNOS may be specifically decreased in Ang II–infused hypertensive rats. Alternatively, afferent arterioles of Ang II–infused hypertensive rats may have a decreased sensitivity to NO derived from the macula densa nNOS compared with normotensive control rats.

The diminished contribution of nNOS in counteracting TGF-mediated afferent arteriolar constriction in Ang II–infused hypertensive rats could also be due to alterations in the signaling components of the macula densa cells. The macula densa senses distal sodium chloride or solute concentration and responds by releasing vasoconstrictor TGF mediators to the afferent arteriole. This feedback mechanism is thought to depend on cytosolic Ca2+26 and intracellular Ca2+ is also required for activation of constitutive NOS enzymes.27 In Ang II–infused hypertensive rats, increased renal Ang II levels1–2 may elevate cytosolic Ca2+ concentration. Thus, the macula densa cells of Ang II–infused hypertensive rats could be less sensitive to further changes in intracellular Ca2+ caused by increased volume and sodium chloride delivery.

The delivery of the NO substrate l-arginine to macula densa cells may also influence the amount of NO generated...
by nNOS. In salt-restricted rats, decreased NO generation at the macula densa may result from decreased distal tubular l-arginine delivery and cellular uptake.28 This report suggests that tubular supply of a NO substrate is one of the decisive factors in the ability of the macula densa nNOS to generate NO. Therefore, distal tubular l-arginine delivery and cellular uptake may decrease in Ang II–infused hypertensive rats, although this issue was not assessed in the present study.

The impaired ability of nNOS to counteract the afferent arteriolar response to increased distal tubular flow in Ang II–infused hypertensive rats could also be secondary to altered cyclooxygenase-2 (COX-2) levels at the macula densa. We recently demonstrated that during increased distal nephron volume and sodium delivery, increased levels of nNOS-derived NO stimulate COX-2, which is constitutively present in the macula densa segment of rat kidney29 and generates vasodilatory COX-2 products.30 Therefore, an impaired COX-2–mediated afferent arteriolar vasodilatory mechanism may be involved in the diminished responsiveness to L-SMTC observed in Ang II–infused hypertensive rats. In support of this concept, studies have shown that COX-2 mRNA levels are decreased in the nonclipped kidneys of 2K1C hypertensive rats30 and that conversion of prostaglandin H2 to prostaglandin I2 is impaired in aortic rings harvested from Ang II–infused hypertensive rats.31

In conclusion, afferent arteriolar constrictor responses to L-SMTC were decreased in Ang II–infused hypertensive rats compared with those in normotensive control rats, and the hypertensive rats exhibited an impaired ability to increase nNOS activity in response to acetylazolamide treatment. This was specific to nNOS-mediated actions because afferent arteriolar constrictor responses to L-NNa were similar in normotensive control and Ang II–infused hypertensive rats, suggesting that total NOS activity is maintained in kidneys of this model of hypertension. These results indicate that chronic elevations in renal Ang II levels and/or blood pressure selectively reduce the ability of the macula densa nNOS activity to counteract TGF-mediated afferent arteriolar constriction.

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