Renal NOS activity, expression, and localization in male and female spontaneously hypertensive rats

Jennifer C. Sullivan,1,2 Jennifer L. Pardieck,1 Kelly A. Hyndman,1 and Jennifer S. Pollock1,2

1Vascular Biology Center and 2Department of Pharmacology, Medical College of Georgia, Augusta, Georgia

Submitted 21 August 2009; accepted in final form 30 October 2009

Sullivan JC, Pardieck JL, Hyndman KA, Pollock JS. Renal NOS activity, expression, and localization in male and female spontaneously hypertensive rats. Am J Physiol Regul Integr Comp Physiol 298: R61–R69, 2010. First published November 4, 2009; doi:10.1152/ajpregu.00526.2009.—The goal of this study was to examine the status of the renal nitric oxide (NO) system by determining NO synthase (NOS) isoform activity and expression within the three regions of the kidney in 14-wk-old male and female spontaneously hypertensive rats (SHR). NOS activity, and NOS1 and NOS3 protein expressions and localization were comparable in the renal cortex and outer medulla of male and female SHR. In contrast, male SHR had significantly less NOS1 and NOS3 enzymatic activity (0 ± 5 and 53 ± 7 pmol·mg⁻¹·30 min⁻¹, respectively) compared with female SHR (37 ± 16 and 172 ± 40 pmol·mg⁻¹·30 min⁻¹, respectively). Lower levels of inner medullary NOS1 activity in male SHR were associated with less NOS1 protein expression [45 ± 7 relative densitometric units (RDU)] and fewer NOS1-positive cells in the renal inner medulla compared with female SHR (79 ± 12 RDU). Phosphorylation of NOS3 is an important determinant of NOS activity. Male SHR had significantly greater phosphorylation of NOS3 on threonine 495 in the renal cortex compared with females (0.25 ± 0.05 vs. 0.15 ± 0.06 RDU). NOS3 phosphorylation was comparable in males and females in the other regions of the kidney. cGMP levels were measured as an indirect index of NO production. cGMP levels were significantly lower in the renal cortex (0.08 ± 0.01 pmol/mg) and inner medulla (0.43 ± 0.02 pmol/mg) of male SHR compared with females (cortex: 0.14 ± 0.02 pmol/mg; inner medulla: 0.56 ± 0.02 pmol/mg). Our data suggest that the effect of the sex of the animal on NOS activity and expression is different in the three regions of the SHR kidney and supports the hypothesis that male SHR have lower NO bioavailability compared with females.

NITRIC OXIDE (NO) is an important regulator of blood pressure and kidney function (3, 15, 43). Therefore, it is not surprising that NO deficiencies have been linked with hypertension and the progression of chronic renal disease in both patients and experimental animals (2, 3, 45). All three NO synthase (NOS) isoforms have been localized to the kidney (1), and intrarenal inhibition of NO has been shown to increase blood pressure (32). NOS1 (neuronal NOS) is predominantly localized in the macula densa, neurons, Bowman’s capsule, and collecting duct, where it participates in the control of glomerular hemodynamics, renin release, and sodium excretion (1, 24, 46). Expression of NOS2 (inducible NOS) protein has been difficult to reproducibly detect in the normal kidney; however, there appears to be NOS2 mRNA in the medullary thick ascending limb (34). NOS3 (endothelial NOS) is localized in vascular endothelial cells and tubules, where it is important in the maintenance of glomerular filtration rate, vascular tone, and renal blood flow (1, 24, 46).

There are pronounced sex differences in the development of hypertension and renal injury with males developing a more severe pathology faster than age-matched females (22, 35, 41, 42, 51). Whole body NO levels have been reported to be greater in females compared with males (13, 16, 29, 36), and a sex difference in the NO pathway may contribute to sexual dimorphisms in cardiovascular and renal pathologies. Under normotensive conditions female rats have been shown to have greater renal NOS1 immunoreactivity and NOS3 protein expression (22, 36, 40, 55). In hypertension, female rats have been shown to have greater NOS1 immunoreactivity (55) or no sex differences in NOS1 and NOS3 protein (12, 22). While a majority of both the clinical and experimental data tends to support the hypothesis that NO levels are greater in females compared with males, the molecular mechanism responsible remains unclear. In particular, there is very little in the literature regarding how the sex of the animal influences NO expression, enzymatic activity, phosphorylation, and/or localization in the three regions of the kidney. We hypothesize that male spontaneously hypertensive rats (SHR) have a compromised renal NOS system relative to female SHR. The kidney is critical in the long-term regulation of blood pressure, and SHR were studied since there are known sex differences in blood pressure. The aim of this study was to determine which aspect of the NOS system in the renal cortex, outer medulla, and inner medulla of the male kidney is compromised relative to female SHR.

METHODS

Animals. Male and female SHR (Harlan Laboratories, Indianapolis, IN) were studied. All experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and approved and monitored by the Medical College of Georgia Institutional Animal Care and Use Committee. Rats were housed in temperature- and humidity-controlled, light-cycled quarters and maintained on standard rat chow (Harlan Teklad). A subset of female SHR were ovarioctomized at 10 wk of age as previously described (8). Following ovarioectomy, half of the females received a subcutaneous 17β-estradiol pellet (1.5 mg/pellet; Innovative Research of America, Sarasota, FL). At 14 wk of age, rats were anesthetized with pentobarbital sodium (Nembutal, 65 mg/kg ip; Abbott Laboratories, North Chicago, IL), a terminal blood sample was taken, and kidneys were then removed, separated into cortex, outer medulla, and inner medulla, and sections were snap-frozen in liquid nitrogen for biochemical analyses.

Tissue homogenization for NOS activity and Western blot analysis. The homogenization protocol was optimized in the three regions of the kidney to allow for the detection of NOS1 and the phosphorylated forms of NOS3. The renal cortex was homogenized as previously described (51), incubated with 20 mM 3-(1-(3-cholamidopropyl)di-methylammonio)-1-propanesulfonate (CHAPS) at 4°C for 20 min,
and centrifuged at 20,000 g for 30 min. Total NOS protein was partially purified from the supernatant fraction using 2.5% ADP Sephase as previously described (39). The partially purified NOS protein was then used in the NOS activity and Western blot protocols. The renal outer medulla was homogenized as previously described (51), incubated with 20 mM CHAPS at 4°C for 20 min, and centrifuged at 20,000 g for 30 min. The CHAPS soluble fraction was then used in the NOS activity and Western blot protocols. The renal inner medulla was homogenized as previously described (51), and the whole homogenate was then used in the NOS activity and Western blot protocols.

**Measurement of NOS activity.** Total NOS activity was determined based on the rate of L-[3H]citrulline formation from L-[3H]arginine and defined as L-[3H]arginine to L-[3H]citrulline conversion inhibited by the nonselective NOS inhibitor N^6^-nitro-L-arginine (L-NNA; 1 mM/mg). The remainder of the assay was done as previously described (48). Isoform-specific activity was determined using the NOS1-specific inhibitor N^6^-((1-imino-3-butenyl)-L-ornithine (VNIO; 1 μmol/l) and the NOS2-specific inhibitor 1400W-dihydrochloride (1400W; 100 mM/mg). NOS1 and -2 isoform activity were calculated using the following formula: NOS1 = total NOS activity – (pmol of NOS activity in the presence of NOS inhibitor). NOS3-specific activity was estimated using the following formula: NOS3 = total NOS activity – (NOS1-specific activity + NOS2-specific activity). NOS activity was normalized to milligrams of protein and expressed as picomoles per milligram protein per 30 min.

**Western blot analysis.** Western blotting was performed as previously described (51) using 10 μg of partially purified NOS protein from the renal cortex per lane, 200 μg CHAPS soluble protein from the outer medulla per lane, and 100 μg total protein from the renal inner medulla per lane. Briefly, following transfer of protein onto PVDF, membranes were blocked in Odyssey blocking buffer (LI-COR Biosciences, Lincoln, NE). Two-color immunoblots were performed using monoclonal primary antibodies to NOS1, NOS3, and NOS3 phosphorylated on serine residues 1177 (BD Biosciences, San Jose, CA) and 635 and threonine residue 495 (Upstate, Lake Placid, NY). In the renal cortex, densitometric results for NOS1, NOS3, and phospho-NOS31177 are reported normalized to protein loaded per lane. In the phospho-NOS1635 and phospho-NOS3495 blots are reported normalized to total NOS3. In the renal outer and inner medulla, NOS1, NOS3, and phospho-NOS31177, and phospho-NOS3635 and phospho-NOS3495 blots were normalized to total NOS expression. The phospho-NOS31177 antibody interferes with the NOS3 antibody precluding normalization to total NOS3 expression for this phosphorylation site. Specific bands were detected using the Odyssey Infrared Imagier (LI-COR Biosciences). Protein concentrations were determined by standard Bradford assay (Bio-Rad, Hercules, CA) by using bovine serum albumin as the standard.

**Immunocytochemical analysis.** Kidneys were processed as previously described (51). Briefly, kidneys were perfusion fixed with 10% neutral buffered formalin, for 10 min, excised from the animal, and further fixed in 10% neutral buffered formalin for 24 h at room temperature. Following this, tissue was paraffin embedded and sectioned at a thickness of 4 μm onto Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA). Slides were incubated in the absence or presence of primary antibodies to NOS1 (monoclonal; Zymed) or NOS3 (monoclonal; BD Transduction) in humidity chambers overnight at 4°C, followed by incubation with horseradish peroxidase-conjugated Polymer (BioCare, Concord, CA) for 30 min at room temperature. Staining was detected with diaminobenzamidine (Dako-Cytomation), counterstained with Mayer's hematoxylin, and coverslipped. Renal structures were identified based upon morphology. Images were taken with an Olympus BX40 compound microscope affixed with an Olympus DP70 digital camera. Brightness and contrast were adjusted with Adobe Photoshop CS3 (Adobe, San Jose, CA). Due to sex differences in inner medullary NOS1 activity and expression, NOS1 immunoreactivity was quantified by counting the number of NOS1-positive cells and NOS1-positive tubules in five randomly selected 200-μm² sections of the renal inner medulla of male and female SHR in a blinded manner (n = 3).

**Assays and chemicals.** cGMP was extracted as previously described (6) and quantitated by radioimmunoassay. Urinary NOx was measured by chemiluminescence as previously described (52). VNIO and 1400W were purchased from Cayman Chemicals (Ann Arbor, MI). [3H]arginine was from Amersham (Pittsburg, PA). All other chemicals were purchased from Sigma (St. Louis, MO).

**Statistical analysis.** All data are expressed as means ± SE. All male/female data were compared using a Student’s t-test (GraphPad Prism 4; LaJolla, CA). Data in Table 1 were compared using an ANOVA. For all comparisons, P < 0.05 was considered statistically significant.

**RESULTS**

**Cortical NOS activity and expression.** Total NOS protein was partially purified from the renal cortex of male and female SHR to ensure a sufficient amount of NOS protein was available to detect NOS1 and the phosphorylation status of NOS3. There were no sex differences in either total NOS activity or in isofrom-specific NOS activity in SHR (Fig. 1A). Furthermore, total NOS1 and total NOS3 protein expressions were comparable in the renal cortex of male and female SHR (Fig. 1B and C). There were two NOS1 immunoreactive bands in all three regions of the kidney, which likely represent NOS1 splice variants (33, 37, 47). We examined three phosphorylation sites that have been linked to altered NOS3 activity and NO production: threonine residue 495 (Thr495) and serine residues 635 (Ser635) and 1177 (Ser1177). There was significantly less phosphorylated NOS3-Thr495 in the renal cortex of female SHR compared with males, and increased phosphorylation on this site has been shown to be associated with a decrease in NO production in vitro (11) (Fig. 1D). NOS3 phosphorylation on Ser635 and Ser1177 were not different between the sexes (see the supplement for this article available online at the Am J Physiol Renal Integr Comp Physiol website.).

**Outer medullary NOS activity and expression.** NOS enzymatic activity and expression were quantitated in the renal outer medulla of male and female SHR following homogenization with CHAPS to optimize NOS protein expression. NOS enzymatic activity and protein expression were comparable in the outer medulla of male and female SHR (Fig. 2). In addition, sex of the animal did not influence the phosphorylation status of NOS3 on Thr495, Ser635, or Ser1177 (see online supplement).

**Inner medullary NOS activity and expression.** NOS enzymatic activity and expression were quantitated in the renal inner medulla of male and female SHR. Table 1 shows the differences in NOS activity and expression between the groups. There were no significant differences between the groups as compared by ANOVA.

<table>
<thead>
<tr>
<th>Group</th>
<th>Total NOS Activity</th>
<th>NOS1 Activity</th>
<th>NOS2 Activity</th>
<th>NOS3 Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>9</td>
<td>212±20</td>
<td>37±16</td>
<td>3±13</td>
</tr>
<tr>
<td>OVX</td>
<td>11</td>
<td>158±26</td>
<td>29±18</td>
<td>4±13</td>
</tr>
<tr>
<td>OVX + E</td>
<td>9</td>
<td>192±41</td>
<td>34±17</td>
<td>0±12</td>
</tr>
</tbody>
</table>

Values are means ± SE (in pmol·mg⁻¹·30 min⁻¹). NOS activity was measured in the renal inner medulla of intact female spontaneously hypertensive rats (SHR), ovarioctomized female SHR (OVX), and in ovarioctomized female SHR supplemented with estradiol (OVX + E). There were no significant differences between the groups as compared by ANOVA.
inner medulla of male and female SHR following homogenization. Male SHR had significantly less total NOS enzymatic activity in the renal inner medulla compared with female SHR. This was due to greater NOS1 and NOS3 isoform-specific activity in females (Fig. 3A). Male SHR also had less NOS1 protein expression, although NOS3 expression and the phosphorylation status of NOS3 was comparable between the sexes (Fig. 3, B and C; see online supplement for NOS3 phosphorylation). To assess whether sex differences in inner medullary NOS were mediated by female sex hormones, NOS activity was measured in the renal inner medulla of ovariectomized female rats and in ovariectomized female rats supplemented with estradiol. Manipulation of female sex hormones did not alter NOS activity, suggesting that the observed sex difference in NOS is not mediated by estradiol (Table 1).

**NOS localization.** To determine whether sex of the animal influences cellular distribution and expression of NOS1 and NOS3, immunohistochemical analyses were performed in kidney slices from male and female SHR. NOS1 was expressed in the macula densa (Fig. 4, A and B), vasa recta of the medulla (Fig. 4, E and F), and inner medullary collecting ducts (Fig. 4, G and H) in both male and female SHR. Although there were no apparent sex differences in renal NOS1 distribution and localization, the number of NOS1-positive cells and NOS1-positive tubules were counted in the renal inner medulla of male and female SHR to assess whether there was a sex difference in NOS1 expression. Consistent with inner medullary activity and Western blot data, there were significantly fewer NOS1-positive cells and tubules in the renal inner medulla of male SHR (38 ± 7 cells, 10 ± 3 tubules) compared with females (56 ± 3 cells, P = 0.05; 18 ± 1 tubules, P = 0.05) (Fig. 4, G and H). NOS3 was expressed in arterioles and glomeruli (Fig. 5, A and B), endothelium of the intrarenal arteries (Fig. 5, C and D), and vasa recta of the medulla (Fig. 5, E–H). There were no apparent differences in renal NOS3 localization between the sexes. Negative controls where the primary antibodies were omitted revealed an absence of specific immunoreactivity (data not shown).

**Nitrite/nitrate and renal cGMP.** NO status was assessed by measuring nitrite/nitrate (NOx) in plasma and urine and cGMP in the kidney. Plasma NOx levels were comparable in male (16.4 ± 1.2 μmol) and female (15.7 ± 1.0 μmol) SHR. In contrast, NOx urinary excretion rates were greater in male SHR (15.1 ± 1.7 μmol/day) compared with female SHR (10.3 ± 1.2 μmol/day, P < 0.05). Renal NO signaling was indirectly measured by determining tissue cGMP levels in three regions of the kidney. cGMP levels were lower in the renal cortex and inner medulla of male SHR compared with females (Fig. 6). In contrast, consistent with a lack of a sex effect on NOS activity and expression, outer medullary cGMP was comparable in male and female SHR.
DISCUSSION

Experimental and epidemiological studies have reported blunted NO production in males relative to females (13, 16, 29). The results of this study provide mechanistic support for the hypothesis that renal NO levels are lower in male SHR compared with females. There are three primary novel findings from this study. First, inner medullary NOS1 and NOS3 activity, NOS1 protein expression, and cGMP levels are less in male SHR compared with females. Moreover, sex differences

Fig. 2. NOS enzymatic activity and expression in the renal outer medulla (OM). NOS activities (A). NOS1 (individual and combined bands; B) and NOS3 (C) protein expression were comparable in the renal outer medulla of male and female SHR (n = 13).

Fig. 3. NOS enzymatic activity and expression in the renal inner medulla. A: total NOS activity, NOS1-specific activity, and NOS3-specific activity were significantly less in the renal inner medulla of male SHR compared with females. Total NOS1 protein expression was less in male SHR (individual and combined bands; B), although total NOS3 protein expression was comparable (C). *Significant difference from male (P < 0.05, n = 9–13).
in NOS activity in the renal inner medulla are not mediated by female sex hormones. Second, although total NOS activity and total NOS protein expression are comparable in the renal cortex of male and female SHR, male SHR have greater NOS3 phosphorylated on Thr495 and lower cGMP levels in the cortex, both of which support the hypothesis that NO levels are less in males. Third, sex of the animal does not modulate outer medullary NOS.

The effect of sex of the animal on NOS expression in medullary tissue separated into the outer and inner medullary regions has largely been unexplored. It is interesting to note that compared with the other regions of the kidney, the renal inner medulla possesses the greatest amount of NOS enzymatic activity and protein (57). As a result, dissecting the kidney into the three distinct regions allowed us to detect sex differences in NOS expression and activity that may have been obscured in previous studies. In the whole medulla, NOS1 and NOS3 protein expressions have been reported to be either comparable in young male and female Sprague-Dawley (SD) rats (12) or greater in females compared with males (36). In contrast,
NOS1 expression was shown to be greater in normotensive male sham rats compared with females with comparable NOS3 expression (22). However, in response to renal wrap hypertension, male rats have a decrease in medullary NOS1 expression (no change in females), resulting in an abolishment of the sex difference observed under normotensive conditions (22). While outer medullary NOS, particularly in the thick ascending limb of the loop of Henle, is known to play an important role in the regulation of salt and water reabsorption, we did not detect any sex differences in the outer medullary NOS system (38). This does not rule out a potential role for outer medullary NOS to contribute to other pathologies in SHR, just not in a sex-dependent manner. The most novel findings of our study were the pronounced sex differences in inner medullary NOS. This is especially interesting in light of the fact that NOS activity has been found to be 26 times higher in the renal inner medulla and four times higher in the outer medulla than in the cortex of male Sprague-Dawley rats (57).

Estrogen replacement therapy in postmenopausal women has been shown to increase plasma NO metabolite levels (4, 9, 38).
and in experimental animals, estradiol increases vascular NO release (19, 20, 28) and enhances NO-mediated blood pressure regulation (7). However, manipulation of female sex hormones using ovariectomy and estradiol replacement did not alter NOS enzymatic activity in the renal inner medulla. This is consistent with our previous findings that manipulation of female sex hormones did not alter renal cortical NOS activity, expression, or phosphorylation status in female SHR (49). In contrast to our findings, medullary NOS expression has been shown to be altered by ovariectomy in both the young female SD rats and the aged Dahl rats (30, 36), suggesting a strain-specific effect of female sex hormones on NOS.

Most studies in the literature agree with our findings on renal cortical NOS expression between the sexes. NOS1 protein expression is similar in the renal cortex of young (10–12 wk of age) male and female SD rats and in 17-wk-old male and female sham and renal wrap hypertensive rats (12, 22, 36). Interestingly, immunohistochemical analysis revealed that kidneys from male SD rats were found to have more intense NOS1 staining in tubules, with female SD having more NOS1 in glomerular vessels (55). However, in our studies in SHR, sex did not effect NOS localization in the renal cortex. With regard to renal cortical NOS3, expression is either comparable in young male and female normotensive SD rats (12, 36) or more highly expressed in sham females compared with males (22). Moreover, female SD rats have greater whole kidney NOS3 expression compared with males (40). In contrast, it has been reported that under hypertensive conditions, both in our study and in renal wrap hypertension, that NOS3 expression levels are comparable in males and females (22).

NOS3 phosphorylation status is a critical determinant of enzyme activity and NO production. There is a scarcity of data in the literature regarding the effect of sex on the animal on the phosphorylation status of NOS3, and we found significantly more NOS3 phosphorylated on Thr495 in the renal cortex of male SHR compared with female SHR, although the phosphorylation status of NOS3 in the medulla was comparable in male and female SHR. In vitro studies show that phosphorylation on Ser1177 and Ser635 and dephosphorylation on Thr495 increase NOS3 activity and NO production (5, 11). It should be noted that the higher level of phosphorylation on Thr495 in the renal cortex of male SHR was not accompanied by a decrease in inner medullary NOS3 enzymatic activity. There are a few potential explanations for this. The NOS activity assay was performed using purified NOS protein at optimal concentrations of cofactors, which may not be the case in vivo. During the purification process, stimulatory or inhibitory proteins may be lost, thereby altering activity. Alternatively, studies characterizing the effects of NOS3 phosphorylation on activity have been performed in cultured and transfected cells; it is conceivable that protein isolated from intact tissue may behave differently.

Male SHR are thought to be a model of reduced NO compared with the normotensive Wistar-Kyoto (WKY) rats; however, much less is known regarding the NOS system in female SHR. Although numerous studies suggest that the NO levels are impaired in male SHR compared with WKY rats, the mechanism for the decrease is controversial. At the whole kidney levels, NOS1 and NOS3 protein expressions have been reported to be either less in kidneys of male SHR vs. WKY rats (25) or greater in SHR (53). Welch et al. (56) reported that, despite male SHR having greater NOS1 and NOS3 protein expressions in the renal cortex, functional responses to microperfusion of a selective NOS1 inhibitor into the macula densa to assess tubuloglomerular feedback revealed that SHR were unresponsive, while WKY rats exhibited an increase in tubuloglomerular feedback responses. Moreover, compared with normotensive male WKY rats, male SHR have impaired synthesis of tetrahydrobiopterin (BH4) and chronic treatment with BH4 lowers blood pressure in SHR. This result may, however, be more related to a decrease in serum testosterone than an alteration in the NOS system (14, 18). In females, evidence suggests that young SHR have an organ-specific NO deficiency due to decreased arginine availability compared with WKY rats, which is evident in the kidney as early as 2 wk of age but not evident in the heart (23). In this same study, Koeners et al. (23) reported that young, prehypertensive male and female SHR had a defect in the arginine-citrulline pathway contributing to decreased arginine availability and decreased renal NO content compared with WKY rats as measured by electron paramagnetic resonance spectroscopy (23). To test this hypothesis, SHR dams and offspring were supplemented with citrulline from day 7 of gestation until 6 wk of age. Citrulline supplementation resulted in a persistent reduction in blood pressure in female SHR still evident at 8 mo of age. In contrast, citrulline supplementation suppressed blood pressure in male SHR; however, this effect was lost by 24-wk of age. These data suggest that supporting and maintaining NO levels in SHR, particularly in females, may be important in modulating the development of hypertension.

Lower renal cortical and inner medullary cGMP levels in male SHR supports the hypothesis that NO bioavailability is less in male SHR compared with females. Less cGMP in the renal cortex of male SHR compared with females may either support a functional consequence of differential NOS3 phosphorylation on Thr495 or reflect differences in scavenging of NO by superoxide. We have recently published that superoxide levels are greater in the renal cortex of male SHR compared with females (51) and hypothesize that this is the primary mechanism by which NO levels are differentially regulated in the renal cortex between the sexes in SHR. In contrast, we expect that lower levels of NO bioavailability in the renal inner medulla of male SHR compared with females is related to both a lower level of NO production resulting from less NOS activity and expression as well as higher levels of inner medullary oxidative stress (50). In addition, asymmetric dimethylarginine (ADMA) and dimethylarginine dimethylamino hydrodrolases (DDAHs) levels have been shown to determine NO bioavailability (10, 54). ADMA is an endogenous NOS

Fig. 6. cGMP levels were greater in control female SHR compared with males. *Significant difference from male SHR (n = 14–15).
inhibitor, and ADMA is metabolized by DDAH. Interestingly, ADMA levels have been shown to be higher in male SHR compared with male WKY rats in plasma and isolated hearts, indicating an increase with hypertension (26, 27) higher in men than in women (21). It is plausible, therefore, to speculate that sex differences in ADMA and DDAH may contribute to sex differences in cGMP levels in the kidney. Inner medullary NOS3 enzymatic activity was significantly less in male SHR compared with female SHR. This lower level of activity, however, was not associated with either a difference in total NOS3 expression or in the phosphorylation status of NOS3. Although not examined in this study, future experiments will be designed to assess the levels of ADMA and DDAH in the kidneys of male and female SHR.

**Perspectives and Significance**

It was very interesting to note the male SHR had significantly less NOS1 and NOS3 isoform-specific activities and less NOS1 protein expression compared with female SHR. Medullary NOS is an important regulator of blood pressure. Mattson et al. (32) reported that chronic medullary infusion of the nonspecific NOS inhibitor l-NAME resulted in hypertension in male Sprague-Dawley rats maintained on a normal salt diet, while Mattson and Bellehumeur (31) reported that selective NOS1 inhibition using either medullary interstitial infusion of a NOS1 antisense oligonucleotide or a pharmacological inhibitor of NOS1 resulted in an increase in blood pressure in male SD rats maintained on a high-salt diet. Specific NOS3 inhibition in the medulla has not been explored to determine the influence of medullary NOS3 on blood pressure status, and similar studies have not been performed in female experimental animals or in male SHR. Additional studies are needed to address the functional consequences of our findings; however, on the basis of the prominent role played by medullary NOS in regulating blood pressure, we speculate that the sex difference in inner medullary NOS1 and NOS3 expression and activity contributes to sex differences in blood pressure observed in SHR.

**ACKNOWLEDGMENTS**

The authors acknowledge the excellent technical expertise of Heather Smith and Janet Hobbs.

**GRANTS**

The authors acknowledge funding from National Heart, Lung, and Blood Institute Grants HL-60653 and HL-69999 (to J. S. Pollock) and the American Heart Association Scientists Development Grant (to J. C. Sullivan). J. S. Pollock is an Established Investigator of the American Heart Association.

**DISCLOSURES**

No conflicts of interest are declared by the author(s).

**REFERENCES**


22. Li D, Xia K, Li NS, Luo D, Wang S, Jiang DJ, Deng HW, Li YJ. Reduction of asymmetric dimethylarginine involved in the cardioprotec-


