AJH 1998;11:97–104

Gender Differences in the Renal Nitric Oxide (NO) System

Dissociation Between Expression of Endothelial NO Synthase and Renal Hemodynamic Response to NO Synthase Inhibition

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Many studies have shown that nitric oxide (NO) production is higher in the systemic vasculature of females than males and is stimulated during pregnancy, a high estrogen state. The present study was performed in rats to determine whether females had a greater expression of endothelial NO synthase (eNOS) in kidneys than did males; whether there were gender differences in the excretion of NO metabolites, nitrate/nitrite; and whether there were gender differences in the renal hemodynamic response to NO synthase inhibition. The renal levels of eNOS mRNA (as measured by ribonuclease protection assays) and protein (as measured by Western blot) were 80% higher in kidneys from females than from males ($P < .001$). Urinary excretion of NO metabolites, nitrate/nitrite, were not different between males and females. To inhibit eNOS, rats were treated with nitro-L-arginine methyl ester (L-NAME, 3 to 4 mg/kg/day) for 2 weeks. Although there were no differences in basal renal hemodynamics between males and females, when factored for kidney weight, chronic L-NAME increased renal vascular resistance by 130% in males but by only 60% in females, and decreased renal plasma flow by 40% in males but had no effect in females. These data show that although the renal levels of eNOS mRNA and protein are higher in females than in males, the renal vasculature of males is more responsive to NO synthase inhibition. The data suggest that the renal vasculature of males may be more dependent on NO than is the renal vasculature in females. Am J Hypertens 1998;11:97–104 © 1998 American Journal of Hypertension, Ltd.

KEY WORDS: **Renal vascular resistance, renal plasma flow, androgens, sexual dimorphism.**

Received January 6, 1997. Accepted July 14, 1997.

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These studies were supported by a grant from the American Heart Association, Mississippi Affiliate to JFR and by National Institutes of Health grant HL 51971.

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0895-7061/98/$19.00
PII S0895-7061(97)00360-9

Nitric oxide (NO), which is formed by NO synthase from L-arginine, is important in the control of renal vascular tone and renal hemodynamics.[1,2] Inhibition of NO synthase results in hypertension, increases in renal vascular resistance, and decreases in renal plasma flow.[3,4]

Many studies have now shown that estrogen stimulates NO production.[5,6] NO synthase activity and NO release response to acetylcholine infusion have been shown to be higher in females than males.[7,8] Furthermore, serum, renal, and uterine NO production is also increased during pregnancy, a high estrogen condition, when compared with that of the nonpregnant state.[9,10]

Despite the interest in the role of NO in controlling renal hemodynamics and the stimulation of NO production caused by estrogen, there have been no studies to date in which the renal level of NO synthase mRNA and protein have been correlated with the renal vascular responses to chronic NO synthase inhibition in females. Nor have there been studies that correlated possible gender differences in renal hemodynamic response to NO synthase inhibition with the levels of expression of endothelial NO synthase in the kidney.

Therefore, the present studies were performed to address the following questions: 1) are there gender differences in the urinary excretion of NO metabolites; 2) are there gender differences in the renal mRNA and protein levels of the endothelial isoform of NO synthase (eNOS); and 3) are there gender differences in the renal vascular response to NO synthase inhibition.

MATERIALS AND METHODS

Rats Protocols for all animal experiments were approved by the Animal Care and Use Committee of the University of Mississippi Medical Center and were performed according to the ''Guide for the Care and Use of Laboratory Animals'' from the National Institutes of Health. Male and female Sprague Dawley rats, aged 3 to 5 months, were purchased from Harlan SD (Indianapolis, IN). Rats were allowed to equilibrate for at least 1 week prior to study. All rats were maintained on standard rat diet (Harlan Teklad, Madison, WI) and tap water in a 12 h/12 h light/dark cycle.

Metabolism Cage Studies Rats were weighed and placed in metabolism cages that had been washed with propanol and allowed to dry. Rats were fasted during the 24 h collection period to minimize the effect of diet on nitrate/nitrite excretion. Urine samples were collected on ice during the 24 h period to prevent bacterial contamination. Urine was measured and protein concentration was assayed using the Bradford method,[11] and a commercially available dye reagent (Bio-Rad, Richmond, CA). Bovine serum albumin was used as the standard and the data are expressed as the mg protein excreted/24 h.

Urinary Nitrate/Nitrite Excretion Urinary nitrate/nitrite was determined using *Escherichia coli* as the source of nitrate reductase to convert nitrate to nitrite,[12] using sodium nitrate as the standard to verify that all nitrate was converted to nitrite. The concentration of nitrite was then measured colorimetrically using the Griess reagent.[13] Sodium nitrite was used as the standard and the data are expressed as μmol nitrate/nitrite excreted/24 h/kg body weight of the rat.

Protein/RNA Isolation Rats were weighed and were anesthetized with Inactin (110 mg/kg wt intraperitoneally). Then both kidneys were removed, quick-frozen in liquid nitrogen, and stored at −70°C. Each kidney was ground using a liquid nitrogen–chilled mortar and pestle and stored in a sterile tube at −70°C until use. For Western analyses, the kidney was homogenized 20% (w/v) in buffer containing 20 mmol/L HEPES, pH 7.5, 100 μmol/L pepstatin A, 100 μg/mL aprotinin, 10 mmol/L EDTA, 100 μg/mL leupeptin, 1 mmol/L phenanthroline, and 1 mmol/L E-64 (Sigma Chemical Company, St. Louis, MO). Total protein concentration was determined using the method described by Lowry et al.[14]

Total RNA was isolated by homogenizing the frozen ground tissue in a monophase solution of phenol/guanidinium thiocyanate followed by two chloroform extractions. The solutions were then isopropanol precipitated at room temperature and washed three times with 75% ethanol, centrifuged after each wash. The final pellet was resuspended in DEPC treated, RNAse free water, stored at −20°C, and used within 72 h. Total RNA concentration and purity were determined spectrophotometrically using A_{260} and $A_{260/280}$ ratio, respectively. Total RNA integrity was checked by using gel electrophoresis.

RNAse Protection Assays cDNA clones of rat endothelial NO synthase were obtained from Dr. Robert Star (The University of Texas Southwestern Medical Center, Dallas). Each partial clone in Bluescript (Stratagene, La Jolla, CA) was transfected into the *E. coli* strain XL1-Blue for amplification of the cDNA. *E. coli* colonies were incubated in LB Broth and plasmid DNA was extracted using the Wizard plasmid miniprep system (Fisher Scientific, Pittsburgh, PA). The cDNA was linearized (using PST I overnight at 37°C), proteinase K treated, and phenol/chloroform extracted. After isopropanol precipitation and three 75% ethanol washes, the DNA pellet was resuspended in water. The purity and concentration was checked spectrophotometrically as above. The linearized cDNA of each isoform was used to make labeled RNA

probes for ribonuclease protection assays (RPAs) using the MAXIscript Kit (Ambion, Austin, TX). Briefly, the cDNA was incubated with α-^{32}P-UTP, CTP, ATP, GTP, and RNA polymerase I for transcription at 37°C. Template DNA was removed using DNAse I and the RNA probe was gel purified and stored in 0.5 mmol/L ammonium acetate at −20°C and used within 1 week. A probe was also made with linearized glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA clone (Ambion) using the same protocol. Each probe was hybridized with an equivalent amount of total RNA overnight at 42°C. The solutions were treated with a mixture of RNAse A plus RNAse T1 to remove any single stranded RNA. The samples were precipitated and resuspended in gel loading buffer. After heating samples to 90°C ± 5°C for 5 min, each was loaded onto a denaturing gel of 8 mmol/L urea and 5% polyacrylamide. The gels were run at 240 V for an appropriate length of time, washed in a solution of 15% methanol:5% acetic acid to remove urea, and dried and exposed to a phosphorimager plate; concentrations were determined using a phosphorimager (Bio-Rad, Richmond, CA). Control experiments, in which the amount of added RNA was varied over a wide range, confirmed that the probe was in excess and that the response was linear over the range tested. Subsequently, the dried gels were subjected to autoradiography and the films were scanned by densitometer for quantification. Kidneys from two rats of each gender were subjected to RNase protection assays four times to verify findings.

Western Blots Protein samples were electrophoretically size separated using a Laemmli gel system[6] with a 7.5% polyacrylamide resolving gel. Equivalent amounts of total protein from each rat kidney tissue sample were loaded along with prestained transferable molecular weight markers (Bio-Rad). Following separation, the proteins were electrophoretically tranferred onto nitrocellulose. Identical gels were run and stained with Coomassie Brilliant Blue (CBB) to detect protein bands. Verification of loading amounts was made by densitometric scans of Coomassie stained gels. Efficiency of transfer was determined using Ponceau-S staining on the nitrocellulose and CBB staining on the remaining gel. The membranes were washed in 0.1% Triton-X 100 phosphate-buffered saline, blocked for 1 hour with 5% nonfat dry milk, and incubated overnight with a 1:1250 dilution of mouse monoclonal antibody against the endothelial isoforms of NO synthase (Transduction Laboratories, Lexington, KY) in 5% nonfat dry milk at 4°C. The membranes were washed in Tris buffered saline, blocked for 1 h, and then incubated with a 1:1000 dilution of horseradish peroxidase conjugated goat anti-mouse IgG (Amersham, Arlington Heights, IL) for 1 h at room temperature. The bound antibody was detected by chemiluminescence (using the ECL kit, Amersham) on X-Omat AR5 film (Kodak, Rochester, NY). Density of bands was determined by scanning densitometry (Bio-Rad). Kidney samples from 5 to 7 rats of each gender were subjected to Western analyses to verify differences between groups.

Chronic Inhibition of NO Synthase Male and female rats were divided into four groups: group 1, untreated male rats (n = 7); group 2, male rats treated with nitro-L-arginine methyl ester (L-NAME, 4 mg/kg/day) in drinking water (n = 6); group 3, untreated female rats (n = 7); and group 4, female rats treated with L-NAME (4 mg/kg/day) in drinking water (n = 7). The water consumed by the rats each day was measured to calculate the doses of L-NAME received. Rats were maintained on L-NAME for 14 to 19 days before renal hemodynamics were measured.

Measurement of Renal Hemodynamics On the day of study, rats were anesthetized with Inactin (100 to 110 mg/kg body weight intraperitoneally; A. Lockwood, Sturtevant, WI). Rats were placed on a temperature regulated surgery table to maintain rectal temperature at 36° to 38°C. Catheters were placed in the femoral artery, for continuous monitoring of blood pressure and for blood sampling, and in the femoral vein for infusion of isoncotic artificial rat plasma (2.5 g/dL bovine immunoglobulin, 2.5 g/dL bovine serum albumin (Sigma, St. Louis, MO) in Ringer's solution) at 12.5 mL/kg/h for 45 min during the preparatory surgery and thereafter at 1.5 mL/kg/h throughout the experimental period to maintain a euvolemic preparation.[4,15] A catheter was placed in the left jugular vein for infusion of 0.9% saline or ^{3}H-inulin (15 to 20 μCi/mL 0.9% saline; New England Nuclear, Wilmington, DE) at 1 mL/h. A tracheotomy was performed. A midline abdominal incision was then made, and a catheter was placed in the left ureter for collections of urine samples into oil in graduated glass tubes. The left renal vein was cannulated in the retrograde position with a 23-gauge needle connected to PE-50 tubing to be used for blood sampling.

Following a 40 min equilibration period for ^{3}H-inulin infusion, two 30 min urine collections were made, and midpoint arterial and renal venous blood samples were also taken. After the experiment the left kidney was removed and weighed.

Analytical Methods Samples of urine (1 μL) and of femoral arterial and renal venous plasma (5 μL) were counted by liquid scintillation counting (Tracor Model 6881, TmAnalytic, Elk Grove, IL).

Calculations Glomerular filtration rate was measured by the clearance of ^{3}H-inulin and renal plasma flow was measured by extraction of ^{3}H-inulin across the

kidney. Renal vascular resistance was also calculated. All data values were expressed as means ± SEM and factored for kidney weights because the intact male rats were larger than the females.

Statistical Analyses Statistical differences between groups of densitometric scans of RPA or Western blots and renal hemodynamic measurements were determined by analyses of variance (ANOVA), with $P < .05$ defined as significant.

RESULTS

Gender Differences in the Urinary Excretion of Nitrate/Nitrite, NO Metabolites Urinary excretion of NO metabolites represents systemic NO production. There were no significant differences in the urinary excretion of NO metabolites, nitrate/nitrite, between male and female rats (males, 12.5 ± 1.6; females, 10.8 ± 2.2 μmol/kg/24 h).

Gender Differences in the Renal Expression of Endothelial NO Synthase As shown in Figure 1, the protein levels of endothelial NO synthase (eNOS) in whole kidney were 80% higher in female rats than in males, as determined by densitometry scanning ($P < .001$). Ribonuclease protection assays (Figure 2) verified that mRNA for eNOS was also higher in females than in males.

Gender Differences in Renal Hemodynamic Response to NO Synthase Inhibition As shown in Table 1, body weights and kidney weights were lower in females than in age matched males. For this reason, all renal functional data were factored for left kidney weight. Hematocrits were also lower in females than in males, as is typical for hematocrits in males and females in a euvolemic surgical preparation (untreated males, 49.7 ± 0.6; L-NAME males, 49.8 ± 0.9; untreated females, 42.1 ± 1.3; L-NAME females, 44.8 ± 1.6, $P < .01$ compared with treated or untreated males).

Mean arterial pressure was not different between untreated male and female rats. With L-NAME treatment, mean arterial pressure increased to similar levels in males and females. Renal vascular resistance when factored for kidney weight was not different between untreated males and females. However, with L-NAME treatment, renal vascular resistance increased by 130% in males, but was blunted to 60% in females.

Glomerular filtration rate (GFR) as shown in Table 1, when factored for left kidney weight, was not different between untreated males and females, and chronic L-NAME treatment for 2 weeks had no effect on GFR in either group. This is consistent with findings in previous studies from our laboratory.[4] Renal plasma flow was similar in untreated males and females. With chronic L-NAME treatment, renal plasma flow decreased by 40% in males but was not affected significantly in female rats.

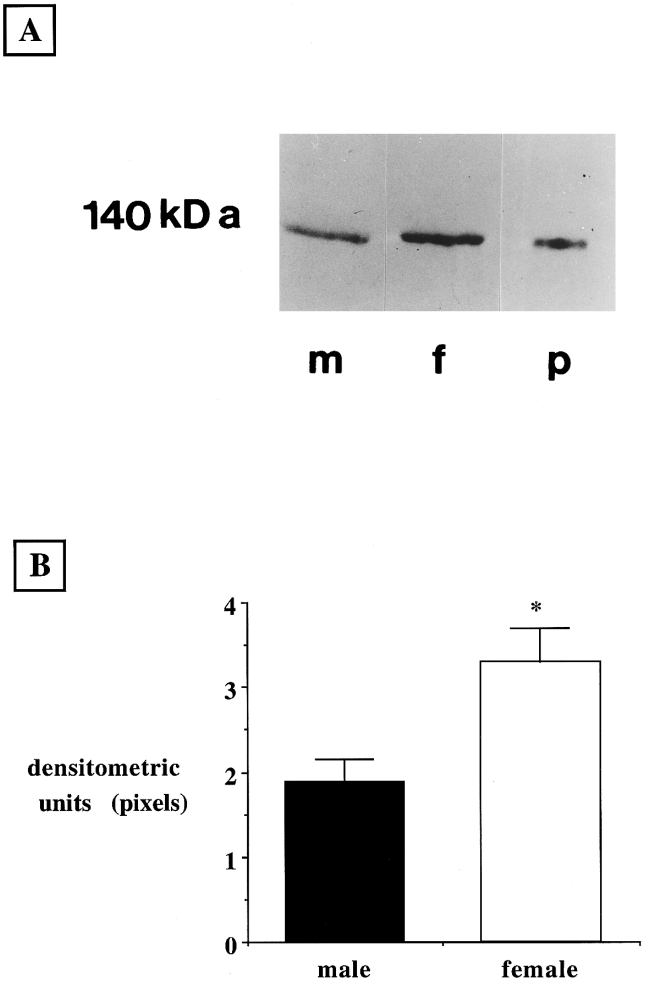

FIGURE 1. *Levels of endothelial NO synthase protein in kidneys of male and female rats. Equal amounts of kidney homogenate protein (100 μg) from kidneys of male and female rats were separated by electrophoresis and subjected to Western blot analyses using an anti-eNOS antibody. Data are expressed as mean ± SEM for densitometric scans of the Western blots.* *P < .001, *for females compared with males.* **Panel A:** *representative Western blot from male and female rats: m, male; f, female; p, positive eNOS control.* **Panel B:** *densitometric scans of Western blots from four males and four females; assays repeated three times.*

Taken together these data show that despite higher eNOS expression in the kidneys of females compared with males, the urinary excretion of NO metabolites was similar between the two groups. Despite similar changes in mean arterial pressure with L-NAME, the renal hemodynamic response to L-NAME inhibition was greater in males than in females.

DISCUSSION

In this study we have made three novel observations: 1) the urinary excretion of NO metabolites is similar in

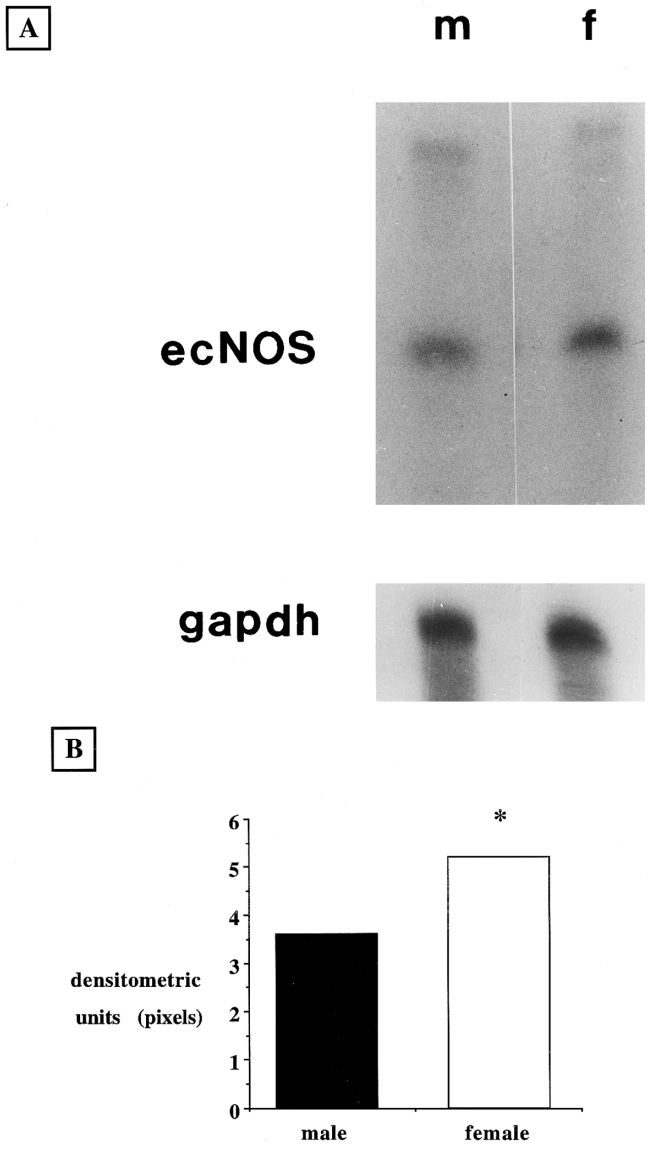

FIGURE 2. *Levels of endothelial NO synthase mRNA in kidneys of male and female rats. Equal amounts of total RNA from kidneys of male and female rats were subjected to ribonuclease protection assays (RPA). Data are expressed as mean values from densitometric scans of phosphorimages.* **Panel A:** *representative RPA from male and female rats. GAPDH was used as the contol.* **Panel B:** *average densitometric scans from phosphorimager of RPAs from two male and two female rats. Assays were performed on kidney tissues from two males and two females. RPAs on each kidney sample were performed four times.*

males and females; 2) the levels of eNOS mRNA and protein are higher in kidneys of female rats than of male rats; 3) in contrast to the molecular levels of eNOS, NO synthase inhibition results in greater renal hemodynamic responses in males than in females.

The urinary excretion of nitrate/nitrite is generally thought to reflect the systemic production of NO. However, the amount contributed by the kidneys to the total urinary nitrate/nitrite excretion has not been determined as yet. Because the rats in the present study were fasted during the urine collections, the contribution of the diet to the urinary nitrate/nitrite was minimized. The nitrate/nitrite excretion thus should not be artificially higher due to the larger dietary intake of males compared with that of females. Therefore, the dietary consumption of nitrate/nitrite cannot explain the similar nitrate/nitrite excretion rates in males and females despite significantly lower renal eNOS mRNA and protein in males.

This is the first demonstration that eNOS protein and mRNA levels in the kidney are higher in females than in males. Despite the differences in eNOS enzyme, the functional response to NO synthase inhibition was more pronounced in males than in females. On one hand, the molecular data suggests that the eNOS activity and thus the NO production is higher in kidneys from females than from males; on the other hand, however, the blunted response to NO synthase inhibition suggests one of at least four possibilities: that 1) there is more NO production in the kidneys of males than of females, perhaps due to differences in levels of other isoforms of NO synthase, such as the neuronal NO synthase, rather than eNOS; 2) the vasculature of the male kidney is more sensitive to the L-NAME inhibitor; 3) the kidneys of males are more dependent on the amount of NO produced even if the amount is less than in the female kidneys; and 4) removal of the vasodilator NO uncovers the presence of a more potent vasoconstrictor(s) in males than in females.

Gender differences in the NO system have been described in various cardiovascular systems, and there appear to be differences in the production of NO and/or in the response to NO synthase inhibition depending on the vascular system studied. For example, in the present study, as shown in Figure 3, with L-NAME inhibition and similar increases in mean arterial pressure there were greater increases in renal vascular resistance in males than in females. There were also greater L-NAME mediated reductions in renal plasma flow in males. Similarly, Jilma and colleagues reported that men had higher concentrations of exhaled NO than did women even when factored for body weight.[16] In contrast, Kauser and Rubanyi found that aortic rings isolated from female rats had a greater endothelial mediated relaxation response to acetylcholine (a response in part mediated by NO) than did rings from males rats, regardless of whether the rats were normotensive or hypertensive.[7,8] Moreover, these investigators also found that inhibition of the NO system in the isolated aortae with nitro-L-arginine resulted in greater attenuation of the acetylcholine response in females than in males.

Both male and female sex steroids have been shown

TABLE 1. BODY AND LEFT KIDNEY WEIGHTS, AND SYSTEMIC AND RENAL HEMODYNAMICS IN MALE AND FEMALE SPRAGUE DAWLEY RATS, AGED 3 TO 4 MONTHS, UNTREATED OR TREATED WITH L-NAME (4 mg/kg/day) FOR 2 WEEKS

	BW (g)	LKW (g)	MAP (mm Hg)	GFR (mL/min/g)	RPF (mL/min/g)	RVR (mm Hg/min/mL/g)
Male (n = 6)	379.0 ± 5.6	1.28 ± 0.04	103 ± 3	0.996 ± 0.074	3.943 ± 0.670	14.01 ± 1.32
+L-NAME (n = 6)	376.7 ± 11.6	1.26 ± 0.05	140 ± 4†	0.909 ± 0.079	2.411 ± 0.271†	31.69 ± 3.84†
Female (n = 7)	227.0 ± 2.9†	0.81 ± 0.02†	99 ± 4	1.032 ± 0.057	3.651 ± 0.245	15.19 ± 0.97
+L-NAME (n = 7)	247.4 ± 5.1†	0.80 ± 0.03†	126 ± 7†	1.026 ± 0.054	2.919 ± 0.260*†	23.92 ± 1.81*†

Values are mean ± SEM. All renal hemodynamic data are factored for left kidney weight. BW, body weight; LKW, left kidney weight; MAP, mean arterial pressure; GFR, glomerular filtration rate; RPF, renal plasma flow; RVR, renal vascular resistance.

** P < .05 compared with untreated rats of the same sex;*

† P < .05, compared with males with same treatment.

to have an effect on NO production. Treatment with estradiol, and less so testosterone, has been shown to increase NO production.[5,17,18] Weiner and colleagues found that chronic estradiol therapy (for 5 days) in intact female guinea pigs caused increases in NO synthase activity in heart, kidney, skeletal muscle, and cerebellum.[5] In contrast, testosterone treatment in intact females resulted in increases in NO synthase activity in cerebellum only.[5] In male guinea pigs estradiol caused increases in NO synthase activity in heart, kidney, and skeletal muscle, although the animals required 10 days of treatment for a difference to be detected.[5] Pregnancy, a high estrogen state, has also been shown to be associated with increased NO and NO synthase activity.[9,10,19] In contrast to estrogen, progesterone has been shown to inhibit NO production in macrophages.[20] Androgens have also been shown to increase NO synthase activity in tissues of the male reproductive tract.[21]

Reports on whether the female reproductive cycle has an effect on NO production are controversial. Morris and colleagues reported that there was no effect of menstrual cycle on exhaled NO or on the excretion of nitrate/nitrite.[22] In contrast, Kharitonov and colleagues reported that women had higher exhalation of NO at midcycle than during menstruation.[23] In the present study the phases of the estrous cycle of the female rats were not determined, although the rats were chosen randomly (that is, they were not from the same cages) and so should have been cycling randomly. Thus it is doubtful that differences in stages of the estrous cycle adversely affected the present studies.

Although we measured the protein and mRNA for eNOS, we did not measure endothelial NO synthase activity in the kidneys of the male and female rats. The kidney has very low NO synthase activity in comparison with other tissues, such as the cerebellum.[24] Thus the citrulline assay that is commonly used in cerebellar assays is not sensitive enough in the kidney to measure anything but very large differences in NO synthase activity (as in comparing the basal activity of inducible NO synthase (iNOS) with the level of stimulated iNOS activity, which generally increases by at least 10-fold in the kidney) (Reckelhoff, unpublished observations). Thus it is possible that, despite the increased message and protein of eNOS in female kidneys, the activity of the enzyme is higher in the kidneys of males than of females, and that this explains the greater renal hemodynamic response to L-NAME inhibition in males. This does not seem to be a very plausible explanation, however.

It is well known that men and male animals are at greater risk for renal injury than are females, but the mechanism(s) for the gender differences in renal disease are unknown. It is also not clear whether NO plays a role in the gender differences in renal disease and injury. Ito and colleagues demonstrated that the isolated afferent arteriole has a greater acetylcholine response than does the efferent arteriole, suggesting that there is more NO produced in the afferent arteriole.[25] Micropuncture studies have shown that afferent arteriolar resistance is lower in males than in females.[26,27] Taken together the data suggest that males may have higher afferent NO production than do females. The data in the present study do not support this hypothesis, however, as the eNOS mRNA and protein were higher in females than in males. In contrast, the male rats had a greater vascular response to NO synthase inhibition, suggesting that, despite the lower levels of NO, the male vasculature may be more dependent on NO than is the vasculature of the female. One possible explanation that could reconcile both the parameters found in this study is that local regional differences in NO production may be present that were masked by the fact that the mRNA and protein levels for eNOS were measured in whole kidney. Future studies will be necessary to determine whether there are gender differences in regional NO production that could account for the lower afferent

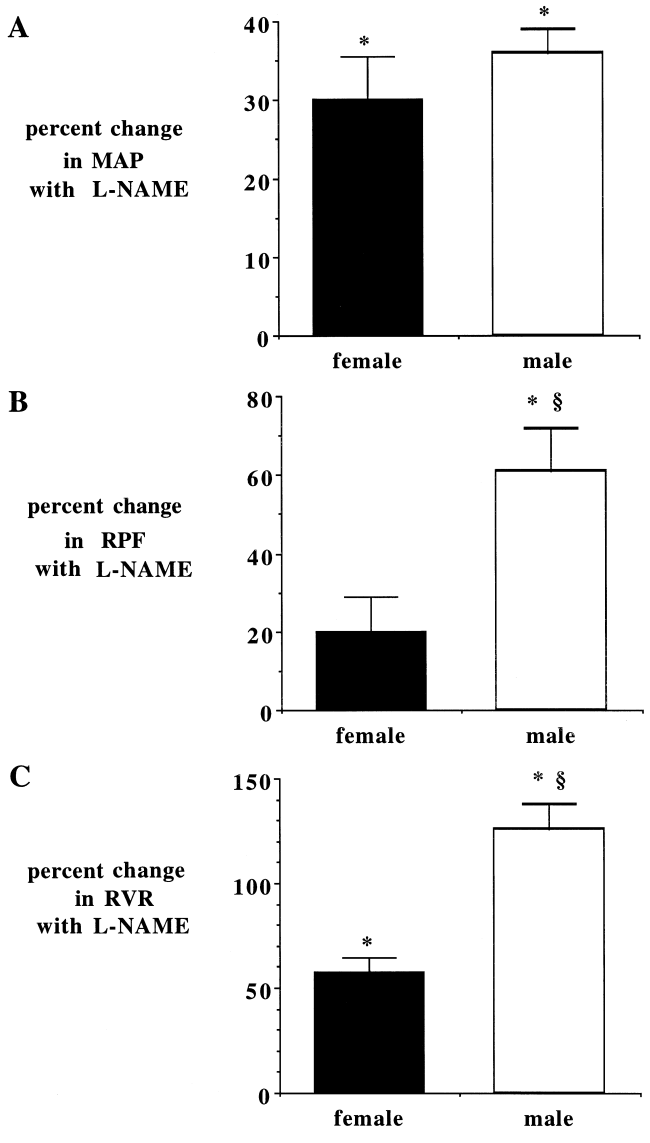

FIGURE 3. *Gender differences in the effects of NO synthase inhibition with chronic L-NAME on systemic and renal hemodynamics. Data are expressed as the percentage change with L-NAME in systemic and renal hemodynamics from control (untreated rats). MAP, mean arterial blood pressure; RPF, renal plasma flow; RVR, renal vascular resistance.* *$P < .05$, *compared with untreated rats of same gender;* §$P < .05$, *compared with L-NAME-treated males.*

arteriolar resistance in the male and the impact that the lower afferent resistance may play in promoting glomerular injury in males.

In summary, despite there being no gender difference in urinary nitrate/nitrite excretion, the mRNA and protein of eNOS is higher in kidneys from female than from male rats. In contrast, NO synthase inhibition with L-NAME results in greater increases in mean arterial pressure and renal vascular resistance, and in greater decreases in renal plasma flow in males than in females.

ACKNOWLEDGMENTS

The technical assistance of Joseph Kellum Jr, is gratefully acknowledged.

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