Sex differences in renal injury and nitric oxide production in renal wrap hypertension

Hong Ji,1,3 Carlo Pesce,4 Wei Zheng,1,3 James Kim,1,3 Yinghua Zhang,1,2 Stefano Menini,4 Joseph R. Haywood,3 and Kathryn Sandberg1,2

1Center for the Study of Sex Differences, Departments of 2Medicine and 3Physiology and Biophysics, Georgetown University, Washington, DC; 4Dipartimento di Scienze e Tecnologie Biofisiche, Mediche ed Odontostomatologiche, University of Genova, Genova, Italy; and 5Department of Pharmacology, Michigan State University, East Lansing, Michigan

Submitted 24 June 2004; accepted in final form 18 August 2004

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The effect of prolonged RW hypertension on the kidney has not been previously investigated. Using radiotelemetry transmitters to monitor MAP continuously over 3 mo, we previously showed that the level of hypertension produced in the 1-kidney, figure-8 renal wrap (RW) model of experimental hypertension is markedly attenuated in female Sprague-Dawley (SD) rats maintained on a high-sodium diet compared with their male counterparts (7). On a normal sodium diet, however, the hypertension was indistinguishable between males and females 2 wk after RW. The effect of prolonged RW hypertension on the kidney has not previously been investigated.

Clinical studies indicate that chronic renal failure (CRF) is associated with dysfunction of the nitric oxide (NO) system (20, 21). Animal studies support these clinical observations and suggest NO deficiency contributes to CRF (4, 22). In this study, we investigated the effect of RW hypertension on renal damage, renal function, and renal NO synthase (NOS) expression in male and female rats. To address whether or not sex is an independent contributing factor in RW-induced renal injury, we investigated renal damage and renal function in RW animals maintained under conditions in which minimal differences in hypertension were observed between the sexes (i.e., a normal salt diet). To address whether or not the sex of the animal influences the effect of RW hypertension on the NO pathway, we compared endothelial NOS (eNOS) and neuronal NOS (nNOS) protein expression in the renal cortex and medulla of male and female sham-operated and RW animals.

A META-ANALYSIS of the development and progression of four causes of chronic renal failure (CRF), including nondiabetic renal disease, polycystic kidney disease, membranous nephropathy, and IgA nephropathy, showed that men exhibit a more rapid decline in renal function than women (14). Sex differences have also been reported in experimental animal models of progressive renal disease. Compared with females, male rats are more vulnerable to the development of renal injury after subtotal nephrectomy (10) and two-kidney, one-clip (16).

It is well known that hypertension is a major risk factor for progressive renal disease (8), and clinical studies indicate that the incidence of hypertension is greater in men compared with women until women reach their seventh decade (19). Sex differences in blood pressure control have also been shown in several animal models of hypertension with females having lower resting mean arterial pressure (MAP) than males (2, 3, 9). It remains unclear how much sex differences in blood pressure control contribute to the sex differences observed in progressive renal disease.

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Experimental Procedures

Animal maintenance. SD male and female rats (7–8 wk old) were purchased from Harlan (Madison, WI) and maintained on a phytoestrogen-free normal salt (NS, 0.4% NaCl) diet.

Animal grouping and body weights. All animal procedures were approved by Georgetown University Animal Care and Use Committee (GUACUC). With the rat under isoanesthesia, the right kidney was removed while the contralateral kidney was tied using 2.0 silk (GUACUC). With the rat under isoflurane anesthesia, the right kidney was removed and frozen at −80°C for future studies.

Blood pressure measurements and blood sampling. Rats were anesthetized with Inactin (100 mg/kg) (Sigma) and placed on a heated table to maintain body temperature at 37°C. A tracheotomy was performed to allow spontaneous breathing. A catheter was placed in the carotid artery for blood pressure measurements using a blood pressure analyzer (Digi-Med; Louisville, KY) and for blood sampling. At the time of euthanasia, blood was collected in ice-cold Vacutainer tubes (Becton Dickinson) containing heparin sulfate. Samples were centrifuged at 2,000 g for 10 min, and the plasma (supernatant) was removed and frozen at −20°C for future studies.

Histology preparation. After 9 wk, rats were anesthetized with Inactin (100 mg/kg). Kidneys were fixed in 10% formaldehyde and embedded in 2-hydroxyethyl-methacrylate (Technovit 7100, Kulzer; Wehrheim, Germany) to minimize tissue distortion associated with paraffin embedding. Renal tissue was cut into 2-μm sections and stained with hematoxylin and eosin (for general morphological examination), Periodic acid Schiff’s (PAS) (for assessment of basement membrane changes), or Masson’s trichrome stain (for demonstration of collagen deposition). PAS-stained sections were examined using a Nikon Eclipse E600 light microscope.

Renal pathology. All specimens were examined by a pathologist (C. Pesce) blinded to the group assignment of the experimental animals. One hundred glomeruli per section were assessed, and the degree of glomerular sclerosis was graded on a scale of 0–4 [grade 0, (normal); grade 1, sclerotic area up to 1–25% (minimal sclerosis); grade 2, sclerotic area 26–50% (moderate sclerosis); grade 3, sclerotic area 51–75% (moderate-severe sclerosis); and grade 4, sclerotic area 76–100% (severe sclerosis)]. The glomerulosclerotic index (GSI) index was calculated using the following formula: GSI = (1 x n1 + (2 x n2) + (3 x n3) + (4 x n4))/n0 + n1 + n2 + n3 + n4, where n0 is the number of glomeruli in each grade of glomerulosclerosis (GS). The degree of tubulointerstitial fibrosis was defined as tubular atrophy or dilatation, deposition of extracellular matrix (ECM), and interstitial cell proliferation and was assessed in Masson’s trichrome-stained sections.

Morphological analysis. A custom-made C language macro was written to measure the area of glomerular tuft profiles with the Optimas 6.5 image analysis system (MediaCybernetics; Silver Spring, MD). The areas of at least 100 glomerular tuft profiles per kidney were measured. The glomerular tufts considered were subsequent unselected occurrences falling in the observation field of the operator who moved the stage in a serpentine fashion from the outer to the juxtamedullary cortex. The mean glomerular volume (MGV) was estimated from the harmonic mean of the profile areas as previously described (12).

Urine protein excretion. Rats were placed in metabolic cages for determination of 24-h urine protein excretion rates using the Bio-Rad protein assay method.

Glomerular filtration rate. Serum and urinary creatinine levels were measured with a creatinine autoanalyzer (Creatinine Analyzer 2, Beckmann), and creatinine clearance per 100 g body wt was calculated on the basis of 24-h urine collection. Plasma standards were run to ensure chromagen interference was minimal in these samples.

eNOS and nNOS protein expression. eNOS and nNOS protein expression were determined by Western blot analysis as previously described using monoclonal anti-eNOS and anti-nNOS antibodies (BD Biosciences Pharmingen) at 1:3,000 and 1:2,000 dilution of the primary antibody, respectively, and 1:10,000 and 1:7,000 dilution of the secondary antibody, respectively (1, 18). Rat fetal brain protein was used for the positive controls.

Results

Mean arterial pressure. Nine weeks after surgery, there were no differences in MAP between the sexes in sham-operated animals (Table 2). RW increased MAP in both male and female animals to hypertensive levels (MAP > 135 mmHg), but there were no sex differences observed in the degree of hypertension.

Body weight. At the onset of the study, male (264 ± 6.0 g) and female (223 ± 6.9 g) rats were of equivalent weight. Although body weights increased in both male (Sham, 53%; RW, 58%) and female (Sham, 24%; RW, 24%) rats during the 9-wk experiment, the increase in body weight was greater in the males (Table 2). There was no effect of RW on either female or male body weights (Table 2).

Renal damage. Whereas renal lesions developed in both male and female animals 9 wk after RW, there were striking sex differences in the incidence and severity of renal damage (Fig. 1, Table 1). The pathology was exacerbated in the male RW kidneys. Many glomeruli were enlarged and frequently showed PAS-positive deposits in the mesangium, mesangial expansion, and areas of segmental necrosis (Fig. 1D, Table 1).

Table 1. Effect of RW hypertension on renal pathology

<table>
<thead>
<tr>
<th>Group</th>
<th>Diffuse GS</th>
<th>Nodular GS</th>
<th>GSI</th>
<th>Tubules</th>
</tr>
</thead>
<tbody>
<tr>
<td>F-Sham</td>
<td>0</td>
<td>0</td>
<td>0.40±0.08</td>
<td>0</td>
</tr>
<tr>
<td>M-Sham</td>
<td>0</td>
<td>0</td>
<td>0.43±0.09</td>
<td>0</td>
</tr>
<tr>
<td>F-RW</td>
<td>+</td>
<td>−</td>
<td>0.70±0.10</td>
<td>1†</td>
</tr>
<tr>
<td>M-RW</td>
<td>++</td>
<td>+</td>
<td>2.2±0.60</td>
<td>2 (++)</td>
</tr>
</tbody>
</table>

Values are means ± SE. Kidneys were processed as in Fig. 1. Diffuse and nodular glomerulosclerosis (GS), glomerulosclerosis index (GSI), and tubular damage were assessed 9 wk after surgeries in female and male sham-operated (F-Sham, n = 10; M-Sham, n = 7) and renal wrap (RW) animals (F-RW, n = 14; M-RW, n = 13). *Mainly polar, synechiae, capsular fibrosis. †Few casts. ‡Proteinaceous casts, damaged tubular cells, interstitial fibrosis.
**Proteinuria.** Sex differences in proteinuria were observed (Fig. 3). Urine protein levels were 2.9-fold higher in sham-operated males compared with sham-operated females and 1.8-fold higher in RW males compared with RW females. Proteinuria increased markedly after RW in both male and female animals.

**GFR.** GFR markedly decreased after RW in both male (by 35%) and female (by 39%) animals; however, no sex differences were observed between the levels or in the magnitude of the effect of RW on GFR (Table 2).

**Renal eNOS protein expression.** In the sham-operated female, the levels of eNOS protein expression were 2.8-fold greater in the renal cortex compared with the sham-operated male and did not change after RW hypertension (Fig. 4A). In contrast, eNOS levels increased by 3.2-fold 9 wk after RW hypertension in the male renal cortex. Although no sex differences in eNOS protein expression were observed in the renal medulla of sham-operated animals, after RW hypertension, eNOS protein expression increased by 2.2-fold in the male, whereas no changes were observed in the female medulla (Fig. 4B).

**Renal nNOS protein expression.** In the renal cortex, no sex differences were observed in nNOS protein expression in sham-operated animals and RW hypertension had no effect in either sex (Fig. 5A). In contrast, in the medulla, nNOS levels were twofold greater in sham males compared with sham females and 9 wk after RW hypertension, nNOS levels decreased by 57% in males but remained unchanged in females (Fig. 5B).

**DISCUSSION**

In this study, the first question we addressed was the following: Is sex an independent contributing factor in RW-induced renal injury? To address this question, we maintained all of the animal groups on a normal salt diet, because previously, using telemetry in conscious animals, we found no sex differences in the degree of hypertension 2 wk after RW in animals maintained on a normal salt diet (7). Our current findings support this earlier study. Although the MAP increased by ~25% 9 wk after RW, no differences in MAP were observed between male and female RW animals.

RW males exhibited greater GS and tubular damage and greater MGV than RW females. Proteinuria was also greater in RW males compared with RW females. The finding that sex differences in vulnerability to renal injury were observed even though no sex differences in MAP were detected suggests that the sex of the animal is an independent contributing factor in determining the degree of renal damage. These findings support observations in the subtotal nephrectomy model of GS in which disparate degrees of proteinuria and GS between males and females were found despite similar degrees of systemic arterial hypertension and GFR (10). Nonetheless, we cannot rule out the possibility that small differences in blood pressure between males and females may also have contributed to the sex differences in renal damage. Whereas changes in blood pressure differences were observed between male and female sham-operated animals and RW hypertension had no effect in females, the effect of RW on GFR (Table 2).

**Table 2. Effect of RW hypertension on MAP, body weight, and GFR**

<table>
<thead>
<tr>
<th>Group</th>
<th>MAP, mmHg</th>
<th>Body Weight, g</th>
<th>GFR, ml/min/100 g body wt⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>F-Sham</td>
<td>117±5.9</td>
<td>277±6.8</td>
<td>0.36±0.04</td>
</tr>
<tr>
<td>M-Sham</td>
<td>109±8.1</td>
<td>428±12.5</td>
<td>0.55±0.04</td>
</tr>
<tr>
<td>F-RW</td>
<td>146±8.2*</td>
<td>276±7.2</td>
<td>0.22±0.06*</td>
</tr>
<tr>
<td>M-RW</td>
<td>139±8.6*</td>
<td>404±7.0</td>
<td>0.36±0.05*</td>
</tr>
</tbody>
</table>

Values are means ± SE. MAP, mean arterial pressure; GFR, glomerular filtration rate. *P < 0.05 compared with same sex sham control.
pressure observed under Inactin anesthesia often reflect changes occurring in conscious animals, MAP measurements under anesthesia are, in general, less sensitive to perturbations than in the conscious animal (6, 15). Furthermore, differences in renal vascular resistance per glomeruli between males and females may also contribute to the observed sex differences in renal pathology. Studies show that while the number of glomeruli are the same between males and females, renal vascular resistance is much higher in females and thus the male kidney is vasodilated relative to the female (13).

The second question we addressed was the following: Does the sex of the animal influence the effect of RW hypertension on the NO pathway? Sex differences were observed in eNOS and nNOS protein expression in sham-operated animals. When compared with the male, eNOS was 2.8-fold higher in the female renal cortex, whereas nNOS was 49% lower in the renal medulla. These observations in sham-operated animals extend previous studies showing that sex differences exist in NO production in the kidney. In this regard, Reckelhoff et al. (18) showed that eNOS protein and mRNA expression were higher in the female kidney compared with males. Second, we found that RW hypertension had no effect on eNOS and nNOS protein expression in either the renal cortex or medulla of the female rat. In stark contrast to the female, eNOS protein expression was upregulated in both the renal cortex and medulla while nNOS protein expression was markedly reduced in the medulla of the male kidney. These observations suggest that males are more susceptible to changes in NO production than females in RW hypertension.

Clinical studies suggest that CRF is a condition of NO deficiency. Total NO production measured by urinary nitrates (NOx) is low in patients with chronic renal disease (20) and in patients with end-stage renal disease on peritoneal dialysis (21). Animal studies support the clinical observations and suggest that NO deficiency contributes to CRF. Wistar Furth (WF) rats are less prone to developing proteinuria, severe kidney damage, and decreased renal function than SD rats after 5/6 renal ablation/infarction (A/I) (5). Resistance of the WF strain to developing CRF in the A/I model was associated with greater NO production compared with the more susceptible SD strain. Baseline levels of NO were elevated and total NO production was maintained in the WF rat despite a decrease in remnant kidney nNOS abundance. Moreover, low-dose inhibitors of NOS led to rapid progression of CRF in WF rats subjected to A/I (5).

The finding that RW hypertension in the male rat results in increased renal eNOS protein expression while reduced nNOS raises the possibility that in males, eNOS is upregulated in both the renal cortex and medulla to compensate for the loss in medullary nNOS. This interpretation is supported by studies showing that tubuloglomerular feedback (TGF) responsiveness is initially normal in male rats subjected to chronic nNOS inhibition, suggesting that other sources of NO production compensate for the loss in renal nNOS (17). Eventually, however, chronic systemic nNOS inhibition leads to hypertension with a fall in GFR (11).

Our studies also support the hypothesis that sex differences in NO regulation contribute to the increased risk men face for kidney disease compared with women and are consistent with previous studies showing that females are more resistant than male rats to developing proteinuria induced by chronic NOS inhibition; higher doses of N^o-nitro-L-arginine are needed to...
induce the same amount of proteinuria in females as males (22). Moreover, renal NOS activity declines with age in the SD male rat, whereas no decline is observed in the aging female (4). This aging study suggests that females are protected from age-dependent kidney damage by their ability to maintain NOS activity, whereas aging males suffer from renal NO deficiency with age, which contributes to greater age-dependent kidney damage. In summary, we found that sex differences exist in the effects of RW hypertension on the kidney under conditions in which minimal changes in blood pressure and GFR were observed. Male rats had more severe GS, tubular damage, glomerular hypertrophy, and proteinuria than female rats after RW. Sex differences were also observed in the NO system. In the male rat, eNOS expression was increased in the renal cortex and medulla, whereas nNOS expression was decreased in the medulla after RW hypertension. RW hypertension had no effect on eNOS and nNOS protein expression in the female kidney. Together, these studies strongly suggest that the sex of the animal is an independent contributing factor in the progression of renal damage in RW hypertension and that sex differences in renal NO production contributes to the mechanisms underlying the greater susceptibility of males to renal injury induced by RW hypertension.

ACKNOWLEDGMENTS

The authors thank Drs. William Welch, Tina Chabrashvilli, and Christine Maric for critical review of the manuscript.

GRANTS

This research was supported by an American Heart Association Beginning Grant-in-Aid 0060205U and a National Capital Area National Kidney Foundation Grant-in-Aid to H. Ji and a Genzyme Renal Innovations Program grant and National Institutes of Health Grants HL-57502 and AG-19291 to K. Sandberg.

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