Male gender increases sensitivity to renal injury in response to cholesterol loading

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Male gender increases sensitivity to renal injury in response to cholesterol loading. Am J Physiol Renal Physiol 284: F718–F726, 2003. First published December 17, 2002; 10.1152/ajprenal.00009.2002.—Males are at greater risk for renal injury than females. This may relate to nitric oxide (NO) availability, because female rats have higher renal endothelial NO synthase (NOS) levels. Previously, our laboratory found susceptibility to proteinuria induced by NOS inhibition in male compared with female rats. Dyslipidemia and hypercholesterolemia dose dependently decreased renal NOS activity and caused renal injury in female rats. We hypothesized that exposure of male rats to hypercholesterolemia would lead to more renal injury in male than in female rats due to an a priori lower renal NO system. Female and male rats were fed no, low-dose, or high-dose cholesterol for 24 wk. Cholesterol feeding dose dependently increased proteinuria in both female and male rats, but male rats developed more proteinuria at similar plasma cholesterol (P < 0.001). Control males had lower renal NOS activity than control females (4.44 ± 0.18 vs. 7.46 ± 0.37 pmol·min−1·mg protein−1; P < 0.05), and cholesterol feeding decreased renal NOS activity in males and in females (P < 0.05). Cholesterol-fed males developed significantly more vascular, glomerular, and tubulointerstitial monocyte/macrophage influx and injury than females. Thus under baseline conditions, male rats have lower renal NOS activity than female rats. This may explain why male rats are more sensitive to renal injury by factors that decrease NO availability, such as hypercholesterolemia; proteinuria; renal nitric oxide synthase activity.

THE OCCURRENCE OF CARDIOVASCULAR injury is related to gender. Males are at greater risk for cardiovascular disease. In females, the rate of cardiovascular disease increases in early middle age (37). Whether this is due to decreased estrogen levels is debated because of variable effects of estrogen replacement on cardiovascular outcome (40). Similar effects of gender on renal injury have been recognized (10). Aging men have a more rapid rate of progression to end-stage renal failure than women (21). Similarly, aging male rats develop spontaneous proteinuria and glomerulosclerosis, whereas females seem to be resistant to renal injury (6). Furthermore, male rats developed more renal injury in response to mild chronic nitric oxide (NO) synthase (NOS) inhibition (42). Females might be protected by an enhanced endothelial NO availability. Indeed, whole-body NO synthesis was higher in women compared with men (14). Furthermore, estrogen supplementation increased circulating levels of nitrate and nitrite in postmenopausal women (33). Gender differences in the renal NO system have also been observed. Renal endothelial NOS (eNOS) mRNA and protein levels were higher in female rats compared with male rats (30). However, little is known about the difference in renal NOS activity between males and females. Thus the question arose of whether male rats have lower renal NOS activity than female rats.

In a previous study, our laboratory found that cholesterol loading decreased renal NOS activity in female rats (4). Low- and high-dose cholesterol loading caused dyslipidemia and hypercholesterolemia, respectively. Dyslipidemia was defined by no significant increase in total cholesterol but marked increases in VLDL cholesterol and intermediate density lipoprotein (IDL) cholesterol. Dyslipidemia decreased renal NOS activity in female rats in the absence of proteinuria, whereas hypercholesterolemia caused proteinuria and renal injury. We hypothesized that exposure of male rats to hypercholesterolemia would lead to more renal injury in male than in female rats due to an a priori lower renal NO system.

METHODS

Animals. Female and male Sprague-Dawley rats (150–175 g; Harlan-Olac, Blackthorn, United Kingdom) were exposed to a 12:12-h light-dark cycle, ambient temperature of 22°C, and humidity of 60%. Sentinel animals, which were monitored regularly for infection by nematodes and pathogenic bacteria, as well as for antibodies for a large number of rodent viral pathogens (International Council for Laboratory Animal Welfare, Rm. F03.226, Univ. Medical Ctr., Heidelberglaan 100, PO Box 85500, 3508 GA Utrecht, The Netherlands (E-mail: J.A.Joles@med.uu.nl)).

Address for correspondence: J. A. Joles, Dept. of Nephrology and Hypertension, Rm. F03.226, Univ. Medical Ctr., Heidelberglaan 100, PO Box 85500, 3508 GA Utrecht, The Netherlands (E-mail: J.A.Joles@med.uu.nl).

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Animal Science, Nijmegen, The Netherlands), consistently tested negative for infection throughout the experiment. The Utrecht University Board for studies in experimental animals approved the studies.

Experimental protocol. Six groups of rats (n = 5–8 rats/group) were studied. Groups 1 and 2 were control females and control males, respectively. Groups 3 and 4 were females and males, respectively, on low-dose cholesterol; group 3 was fed 0.5% cholesterol + 0.125% cholate and group 4 was fed 0.25% cholesterol + 0.0625% cholate. Groups 5 and 6 were females and males, respectively, on high-dose cholesterol: group 5 was fed 1% cholesterol + 0.25% cholate and group 6 was fed 0.5% cholesterol + 0.125% cholate. These different regimens were applied because it has been reported that on the same diet male rats developed higher plasma cholesterol levels than female rats, even though food intake corrected for body weight was identical (38). Our purpose was that male rats would achieve comparable cholesterol levels at lower dietary cholesterol concentrations than females. Cholesterol + cholate were mixed through chow (RMH-TM, Cholesterol, 0.25% cholesterol and 0.125% cholate), consistently.

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At the end of the experimental protocol, the kidneys were removed and cut transversely into three slices. The poles were frozen in liquid nitrogen and stored at −80°C until being processed for NOS activity and NOS immunolocalization. The middle slice was immersion-fixed in PBS formaldehyde (4%, pH 7.35) and embedded in paraffin for morphological studies.

Food intake, plasma lipids, renal function, blood pressure, body weight, and proteinuria. Food intake was determined every 6 wk. At weeks 0, 6, and 18, blood samples were taken from the tail vein for determination of plasma creatinine, cholesterol, and triglycerides. At the end of the experiment (week 24), the animals were anesthetized with 60 mg/kg pentobarbital sodium ip to collect blood from the vena cava for determination of plasma lipids, lipoproteins, and creatinine. Plasma cholesterol and triglycerides were determined enzymatically (Roche Diagnostics, Mannheim, Germany). Plasma creatinine levels were determined colorimetrically (Sigma, St. Louis, MO). Systolic blood pressure was measured every 6 wk in the conscious rats, starting 1 wk before the start of treatment (week 0) by the tail-cuff method (IITC, San Diego, CA). Urine was collected every 6 wk starting at week 0 for determination of urinary protein and creatinine excretion. The rats were weighed and placed in metabolic cages for 24 h, with free access to food and water. Urinary protein levels were determined with Coomassie blue.

Lipoprotein isolation by density-gradient ultracentrifugation. Lipoproteins were separated in terminal plasma samples by density-gradient ultracentrifugation (41) into five fractions (chylomicrons and VLDLs, D < 1.006 g/ml; LDL, D = 1.006–1.019 g/ml; HDL, D = 1.019–1.063 g/ml; HDL, D = 1.063–1.21 g/ml). Lipoprotein cholesterol was measured as described above.

Renal NOS activity. NOS activity was measured by determining the formation of L-[3H]citrulline from L-[3H]arginine. Using an Ultrasorx, an aliquot of ~300 mg kidney tissue was homogenized in 1.5 ml of ice-cooled homogenization buffer, pH 7.4, consisting of 50 mmol/l Tris buffer, 320 mmol/l sucrose, 1 mmol/l EDTA, 1 mmol/l dithiothreitol, 2 mg/l apro tinin, and 100 mg/l phenylmethylsulfonyl fluoride. An aliquot of 50 μl of homogenate was incubated in a final volume of 100 μl at 37°C for 30 min in the presence of 1 mmol/l L-citrulline, 0.3 mmol/l tetrahydrobiopterin, 300 U/ml calmodulin, 0.5 mmol/l NADPH, 1 mmol/l CaCl2, 0.01 mmol/l L-arginine, and 3.7 kBeq l-(2,3,4,5)-arginine (Amersham Pharmacia Biotech, Buckinghamshire, UK) in 50 mmol/l KH2PO4 phosphate buffer, pH 7.2. In an additional tube, the NADPH was substituted by 100 mmol/l L-NAME to determine nonspecific activity. The reaction was stopped by the placement of the tubes on ice and addition of 20 mmol/l ice-cold HEPES buffer, pH 5.5, followed by separation of arginine and citrulline on Dowex 50X8–200 (Na+ form). [3H]citrulline was detected by scintillation counting. All measurements were performed in duplicate, and the results are expressed as picomoles per minute per milligram of protein.

NOS immunolocalization. Frozen tissue sections (5 μm) of rat kidneys were fixed with 4% paraformaldehyde and rinsed twice with PBS containing 0.1% Triton X-100 (PBST; Tween). Endogenous peroxidase reactions were blocked with 30% H2O2 in a phosphate-citrate buffer, pH 5.8. Tissue sections were incubated for 1 h at room temperature with eNOS or inducible NOS (iNOS) antibody (1:5,000 in 10% PBS; Transduction Laboratories) and then rinsed twice with PBST and fixed with formalin for 10 min. After a rinsing with PBST, sections were incubated for 30 min at room temperature with goat anti-rabbit PowerVision (polymerized-horseradish peroxidase-goat-anti-rabbit, Immunologic, Duiven, The Netherlands) and rinsed for 10 min with PBS. For eNOS, detection slides were rinsed for 5 min with acetate buffer (100 mmol/l, pH 4.8) followed by color development with 3-amino-9-ethylcarbazole substrate (Sigma). For iNOS, detection slides were rinsed for 5 min with phosphate-citrate buffer (100 mmol/l, pH 5.8) followed by color development with diaminobenzidine. After counterstaining with hematoxylin, sections were covered with paragon. The stained area was quantified morphometrically with Optimas software in 20 glomeruli/ kidney at ×400 magnification and expressed as the percentage of the total glomerular area.

Monocyte/macrophage localization. Paraffin sections (3 μm) of formaldehyde-fixed kidney were deparaffinized and rehydrated. Incubation with the ED-1 mouse monoclonal antibody (kindly provided by Ed Dub, Department of Cell Biology, Free University, Amsterdam, The Netherlands) demonstrated monocytes/macrophages. After application of ED-1 (dilution 1:2,500 in PBS containing 5% BSA and 0.4% sodium azide) to the slides at 22°C for 1 h, bound antibody was detected by the DAKO EnVision + System (preluted peroxidase-dextran-conjugated goat anti-mouse antibody and diaminobenzidine color reaction). The number of ED-1-antigen-positive monocytes/macrophages was determined with ×400 magnification in all arteries, 50 randomly distributed glomeruli, and 20 microscopic tubulointerstitial fields, for determination of monocytes/macrophages infiltration. An average score per glomerulus or per field was calculated.

ED-1 and iNOS double staining. Tissue sections prepared as described for NOS staining were preincubated for 15 min with 10% normal goat serum in PBS and then incubated with rabbit anti-iNOS antibody (dilution 1:1,000 in 10% normal goat serum, kindly provided by Dr. H. van Goor, Groningen, Netherlands) at 4°C overnight. Next, sections were rinsed with PBST and incubated for 30 min at room temperature with goat anti-rabbit PowerVision followed by color develop-
ment in 3-amino-9-ethylcarbazole substrate and counter-stain with hematoxylin. After blocking of endogenous biotin (biotin blocking kit, Vector Laboratories, Burlingame, CA), the iNOS-stained slides were incubated with biotinylated ED-1 antibody (60 min, room temperature), followed by streptavidin–FITC (dilution 1:100 in 1% normal rat serum in PBS, Vector Laboratories) for 30 min, and then rinsed in PBST, incubated with FITC-anti-streptavidin (dilution 1:100 in 1% normal rat serum in PBS, Vector Laboratories) for 30 min, and rinsed in PBS. Slides were covered with Pertex.

**Morphological studies.** Light microscopy was done on 3-μm paraffin sections of the formaldehyde-fixed kidney stained with hematoxylin–eosin. The sections were numbered. The investigators (D. M. Attia and M. A. Attia) were blinded to their identity. Glomerular injury (aneurysms and glomerular fibrosis) was assessed in 50 glomeruli semiquantitatively with a 0–4 scale: 0 = absent, 1 = slight, 2 = mild, 3 = moderate, and 4 = marked. Glomerular protein droplets were assessed by calculating the percentage of affected glomeruli. Tubulointerstitial damage (tubular dilatation, casts, flattened tubular epithelium, and tubular epithelial cell degeneration/necrosis) and cytoplasmic protein droplets in tubular epithelium were semiquantitatively graded in 20 fields in the same way as glomerular injury. A total glomerular and tubulointerstitial injury score was determined by summing (1 × score 1) + (2 × score 2) + (3 × score 3) + (4 × score 4).

**Statistical analyses.** Results are expressed as mean ± SE. To assess the influence of gender on cholesterol feeding, data were tested by two-way ANOVA followed by the Student-Newman-Keuls test for multiple comparison. Skewed data sets were either log converted (proteinuria) or ranked (morphological data) before statistical analysis. Analysis of covariance was used to analyze whether differences were present between regression coefficients of proteinuria against plasma cholesterol in male and female rats.

**RESULTS**

**Cholesterol intake.** Food intake was higher in male than in female rats (Table 1). However, when corrected for the gender-related differences in body weight, food intake was actually lower in the males (4.3 ± 0.2 vs. 5.4 ± 0.3 g/100 g body wt). Because of the differences in cholesterol content of the experimental diets administered to the male and female rats (see METHODS), 0.5% cholesterol in the chow was the only concentration where cholesterol intake could be directly compared. At this concentration, cholesterol intake, corrected for body weight, was slightly lower in male than in female rats. The data from week 18 are presented. They are representative of the whole experiment (Table 1).

<table>
<thead>
<tr>
<th>Food Intake, g</th>
<th>BW, g</th>
<th>Cholesterol in Chow, %</th>
<th>Cholesterol Intake, mg</th>
<th>Cholesterol Intake/100 g BW, mg/100 g BW</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Female</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>15.3 ± 0.9</td>
<td>258 ± 7</td>
<td>0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>Dyslipidemic</td>
<td>14.4 ± 1.0</td>
<td>280 ± 5</td>
<td>0.5</td>
<td>72 ± 5</td>
</tr>
<tr>
<td>Hypercholesterolemic</td>
<td>14.0 ± 1.2</td>
<td>278 ± 7</td>
<td>1</td>
<td>140 ± 12</td>
</tr>
<tr>
<td><strong>Male</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>18.2 ± 1.6</td>
<td>452 ± 7</td>
<td>0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>Dyslipidemic</td>
<td>19.4 ± 1.1</td>
<td>422 ± 8</td>
<td>0.25</td>
<td>48 ± 3</td>
</tr>
<tr>
<td>Hypercholesterolemic</td>
<td>18.7 ± 0.4</td>
<td>438 ± 12</td>
<td>0.5</td>
<td>93 ± 2</td>
</tr>
</tbody>
</table>

Values are means ± SE. BW, body weight.

**Plasma lipids, renal function, blood pressure, and body weight.** Both male and female rats fed high-dose cholesterol had similarly increased plasma cholesterol. Note that we achieved our goal, namely, that different cholesterol levels in chow resulted in comparable plasma cholesterol levels. Total plasma cholesterol levels were not increased in rats fed low-dose cholesterol (Fig. 1). However, these rats were dyslipidemic. Both VLDL cholesterol and IDL cholesterol levels were increased. The VLDL levels were increased more in dyslipidemic male rats than in dyslipidemic female rats. LDL cholesterol levels were unchanged in dyslipidemic male rats but decreased in dyslipidemic female rats. HDL levels remained unchanged. In hypercholesterolemic male and female rats, the changes in VLDL and IDL cholesterol content were even more pronounced. In hypercholesterolemic male rats, LDL and HDL cholesterol were decreased, whereas in hypercholesterolemic female rats LDL and HDL cholesterol levels remained unchanged (Table 2). Cholesterol feeding had no effects on body weight (Table 1), plasma triglycerides, plasma creatinine, and blood pressure (data not shown). Irrespective of diet, plasma creatinine levels and creatinine clearances were both significantly higher in males than in females (group means at 24 wk: 50 ± 2 vs. 41 ± 2 μmol/l, and 3.2 ± 0.1 vs. 2.3 ± 0.1 ml/min, respectively).

**Proteinuria.** Control male rats spontaneously developed proteinuria in contrast to control females. Dyslip-
idemic rats showed no increase in proteinuria compared with controls. Hypercholesterolemic male and female rats developed significantly more proteinuria than controls, and this was particularly pronounced in males (Fig. 2). Correcting proteinuria for creatinine clearance did not introduce significant changes in this pattern (data not shown). The increase in proteinuria was significantly correlated with the increase in plasma cholesterol levels both in male and in female rats. However, at weeks 18 and 24 male rats developed significantly more proteinuria at similar plasma cholesterol levels than did female rats (P < 0.001, analysis of covariance; Fig. 3). The data shown in Fig. 3 are from week 24 of the study. Male cholesterol-fed rats developed more proteinuria at identical plasma cholesterol levels during the entire experiment.

**Urinary TBARS.** Urinary TBARS were similar in control male and female rats. Dyslipidemic male rats had significantly increased urinary TBARS compared with control male rats and dyslipidemic female rats. Urinary TBARS were significantly increased in both hypercholesterolemic male and female rats (Fig. 4). Correcting urinary TBARS for creatinine clearance did not introduce significant changes in this pattern (data not shown).

**Renal NOS activity.** In control male rats, renal NOS activity was significantly lower than in control female rats (4.44 ± 0.18 in control male rats vs. 7.46 ± 0.37 pmol·min⁻¹·mg protein⁻¹ in control female rats; P < 0.05). Dietary cholesterol dose dependently decreased renal NOS activity in female rats. In dyslipidemic male rats, renal NOS activity was markedly decreased and even more so than in female dyslipidemic rats, but renal NOS activity was at control levels in hypercholesterolemic male rats (Fig. 5). To explore this remarkable finding, we semiquantitatively analyzed NOS isoform expression by immunohistochemistry (see below).

**NOS immunolocalization.** Glomerular eNOS immunolocalization was unchanged in hypercholesterolemic males and females compared with control males and females. However, hypercholesterolemic male rats had significantly increased glomerular iNOS-positive glomerular surface area compared with control male rats and hypercholesterolemic female rats (Fig. 6). An increase in tubulointerstitial iNOS was also observed in some nephrons in this group (Fig. 6), but this focal effect was not readily quantifiable.

**ED-1 and iNOS double staining.** Double staining for ED-1 and iNOS in hypercholesterolemic male rats (Fig. 7) revealed hardly any detectable iNOS expression in infiltrating monocytes/macrophages. The very limited colocalization of the two antibodies, in combination with glomerular and tubular iNOS staining, suggests that ED-1-positive cells provide a negligible contribution to the observed increase in renal NOS activity in hypercholesterolemic male rats.

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**Table 2. Cholesterol concentrations in lipoprotein fractions in female and male rats fed increasing concentrations of cholesterol**

<table>
<thead>
<tr>
<th></th>
<th>VLDL</th>
<th>IDL</th>
<th>LDL</th>
<th>HDL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female Control</td>
<td>0.04 ± 0.01</td>
<td>0.13 ± 0.04</td>
<td>0.83 ± 0.06</td>
<td>1.95 ± 0.13</td>
</tr>
<tr>
<td>Dyslipidemic</td>
<td>0.45 ± 0.08*</td>
<td>0.44 ± 0.09*</td>
<td>0.44 ± 0.04*</td>
<td>1.71 ± 0.17</td>
</tr>
<tr>
<td>Hypercholesterolemic</td>
<td>1.53 ± 0.16*</td>
<td>1.09 ± 0.13*</td>
<td>0.73 ± 0.09</td>
<td>1.72 ± 0.25</td>
</tr>
<tr>
<td>Male Control</td>
<td>0.10 ± 0.02</td>
<td>0.08 ± 0.03</td>
<td>1.07 ± 0.11</td>
<td>1.55 ± 0.27</td>
</tr>
<tr>
<td>Dyslipidemic</td>
<td>1.19 ± 0.28*</td>
<td>0.56 ± 0.05*</td>
<td>0.90 ± 0.10</td>
<td>1.12 ± 0.10</td>
</tr>
<tr>
<td>Hypercholesterolemic</td>
<td>1.90 ± 0.27*</td>
<td>0.68 ± 0.06*</td>
<td>0.65 ± 0.03*</td>
<td>0.85 ± 0.04*</td>
</tr>
</tbody>
</table>

Values are means ± SE. IDL, intermediate density lipoprotein. *P < 0.05 vs. female control. †P < 0.05 vs. male control. ‡P < 0.05 vs. dyslipidemic females. §P < 0.05 vs. hypercholesterolemic females.

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**Fig. 2.** Proteinuria in female (●) and male (▲) control rats, in female (●) and male (▲) dyslipidemic rats, and in female (●) and male (▲) hypercholesterolemic rats. *P < 0.05 vs. female control. †P < 0.05 vs. male control. ‡P < 0.05 vs. dyslipidemic females. §P < 0.05 vs. hypercholesterolemic females.

**Fig. 3.** Linear regression between plasma cholesterol levels and proteinuria in female (●; r = 0.8124, P < 0.01) and male (▲; r = 0.9019, P < 0.01) control and cholesterol-fed rats at week 24 of the study.
activity was decreased in hypercholesterolemic female rats, it remained unchanged in hypercholesterolemic males possibly because of increased expression of iNOS in proximal tubules.

It has been shown that renal levels of eNOS mRNA and protein are higher in females than in males (30). However, the present study is, to our knowledge, the first study to show that male rats have lower renal NOS activity. In a previous study, our laboratory showed that both estrogen and androgen contribute to the differences in sensitivity in response to mild chronic NOS inhibition between female and male rats (42). Several studies have suggested that NOS might be regulated by sex hormones. Estradiol increased renal NOS activity (43). Furthermore, uterine NOS activity was increased during pregnancy, when concentrations of estrogen and progesterone are high (43). A study on endothelial cells has shown that estrogen, by binding to its receptor in the caveolae, promotes the association between heat shock protein 90 and eNOS, which increases eNOS activity (36). Furthermore, estrogen has been shown to downregulate AT1 receptor expression (29). Testosterone, on the other hand, up-regulates renal ANG II (12), and it has been shown that inhibition of the renin-angiotensin system increased NOS activity in endothelial cells (18). Thus both estrogen and androgen may contribute to the lower renal NOS activity in male rats. Note that the in vitro NOS activity assay is conducted in the presence of excess cofactors and substrate. This may not be the case in vivo.

Previously, we found that dyslipidemia and hypercholesterolemia decreased renal NOS activity in female rats (4). In the present study, we have shown that renal NOS activity was also decreased in dyslipidemic male rats. We also recently found that hypercholesterolemia decreased renal NOS activity by upregulating renal caveolin-1 protein abundance via an ANG II-sensitive mechanism (3). Interestingly, renal NOS ac-

Vascular and glomerular morphology. The number of ED-1-positive cells attached to the endothelium and infiltrated into the intima and media of arteries was increased only in hypercholesterolemic male rats (Table 3). Cytoplasmic protein droplets in glomerular epithelium were increased in hypercholesterolemic female rats. In cholesterol-fed male rats, glomerular protein droplets were dose dependently increased. However, hypercholesterolemic male rats had more glomerular protein droplets than hypercholesterolemic female or dyslipidemic male rats. Dyslipidemic male and female rats had increased glomerular monocyte/macrophage infiltration. Hypercholesterolemic male rats had significantly more glomerular monocyte/macrophage infiltration than dyslipidemic male rats or hypercholesterolemic female rats. Glomerular injury dose-dependently increased in cholesterol-fed males but not in cholesterol-fed females (Table 3).

Tubulointerstitial morphology. Cytoplasmic protein droplets in tubular epithelium were only increased in hypercholesterolemic males. Dyslipidemic male rats had increased tubulointerstitial monocyte/macrophage influx, whereas dyslipidemic female rats had no tubulointerstitial monocyte/macrophage influx. Hypercholesterolemic male rats had significantly more tubulointerstitial monocyte/macrophage infiltration than dyslipidemic male rats or hypercholesterolemic female rats. Tubulointerstitial injury dose-dependently increased in cholesterol-fed males but only in the presence of hypercholesterolemia in females (Table 4).

DISCUSSION

The present study shows that male rats have lower renal NOS activity than female rats. Furthermore, dyslipidemia decreased renal NOS activity in both male and female rats and caused renal injury in males, whereas females were protected. Hypercholesterolemic male rats developed more extensive renal injury than hypercholesterolemic female rats. Although renal NOS activity was decreased in hypercholesterolemic female rats, it remained unchanged in hypercholesterolemic males possibly because of increased expression of iNOS in proximal tubules.

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The present study shows that male rats have lower renal NOS activity than female rats. Furthermore, dyslipidemia decreased renal NOS activity in both male and female rats and caused renal injury in males, whereas females were protected. Hypercholesterolemic male rats developed more extensive renal injury than hypercholesterolemic female rats. Although renal NOS activity was decreased in hypercholesterolemic female rats, it remained unchanged in hypercholesterolemic males possibly because of increased expression of iNOS in proximal tubules.

It has been shown that renal levels of eNOS mRNA and protein are higher in females than in males (30). However, the present study is, to our knowledge, the first study to show that male rats have lower renal NOS activity. In a previous study, our laboratory showed that both estrogen and androgen contribute to the differences in sensitivity in response to mild chronic NOS inhibition between female and male rats (42). Several studies have suggested that NOS might be regulated by sex hormones. Estradiol increased renal NOS activity (43). Furthermore, uterine NOS activity was increased during pregnancy, when concentrations of estrogen and progesterone are high (43). A study on endothelial cells has shown that estrogen, by binding to its receptor in the caveolae, promotes the association between heat shock protein 90 and eNOS, which increases eNOS activity (36). Furthermore, estrogen has been shown to downregulate AT1 receptor expression (29). Testosterone, on the other hand, up-regulates renal ANG II (12), and it has been shown that inhibition of the renin-angiotensin system increased NOS activity in endothelial cells (18). Thus both estrogen and androgen may contribute to the lower renal NOS activity in male rats. Note that the in vitro NOS activity assay is conducted in the presence of excess cofactors and substrate. This may not be the case in vivo.

Previously, we found that dyslipidemia and hypercholesterolemia decreased renal NOS activity in female rats (4). In the present study, we have shown that renal NOS activity was also decreased in dyslipidemic male rats. We also recently found that hypercholesterolemia decreased renal NOS activity by upregulating renal caveolin-1 protein abundance via an ANG II-sensitive mechanism (3). Interestingly, renal NOS ac-
Activity in hypercholesterolemic male rats was at control levels. In these rats, the iNOS isoform was upregulated, which may be related to the fact that they had large amounts of cytoplasmic protein droplets in the glomerular and tubular epithelium. In the setting of severe proteinuria, glomerular and tubular epithelial cells are no longer able to process filtered protein (31). The question remains whether the source of increased iNOS is infiltrating monocytes/macrophages. Hypercholesterolemic male rats that showed the strongest increase in arterial, glomerular, and tubulointerstitial monocyte and macrophage influx also exhibited increased iNOS staining. However, hypercholesterolemic female rats also showed significantly increased glomerular and tubulointerstitial monocyte and macrophage influx but showed no increase in iNOS expression. Expression of iNOS also occurs in tubular epithelial cells (39). Using double staining, we observed a clear separation of tubular iNOS staining from macrophage iNOS. Hence, we suggest that iNOS expression may be increased in tubular epithelial cells by protein reabsorption. In general, the beneficial effects of NO are...
attributed to NO synthesized by eNOS (7, 22), whereas the excessive amounts of NO produced by iNOS are thought to generate the damage via peroxynitrite (19). Examples include apoE-iNOS double knockout mice fed a Western-type diet, in which the atherosclerotic lesions and the plasma levels of lipoperoxides were lower compared with apoE knockout mice fed the same diet (27), and cyclosporine nephropathy, in which glomerular and tubular iNOS expression and activity were increased and correlated with the extent of renal injury (32). Therefore, normal total renal NOS activity in hypercholesterolemic male rats may be due to a reduction of eNOS activity caused by hypercholesterolemia on the one hand and a secondary increase of iNOS due to protein resorption on the other hand. In control and dyslipidemic male rats, tubular protein droplets were not significantly increased, indicating that the tubular epithelial cells were able to adequately process the filtered proteins in the lysosomes. Previously, we found that renal injury could be prevented by exogenous NO administration, suggesting that renal injury is NO dependent (4). Thus male rats have lower baseline renal NOS activity, which is further decreased in the initial phase of dietary cholesterol loading. This may explain why, in general, male rats are more sensitive to induction of renal injury than female rats.

Gender dependence of renal injury varies according to the model used. Spontaneously hypercholesterolemic male Imai rats were shown to be more susceptible to developing proteinuria and glomerulosclerosis than female rats (35). Administration of estrogen to these rats improved renal injury. Thus male gender may interact with hypercholesterolemia to accelerate renal injury. However, in rat models where hypertriglyceridemia is the prominent disorder, such as in analbuminemic (25) and obese Zucker (16) rats, female gender and estrogen treatment promoted the development of glomerulosclerosis, whereas ovariectomy retarded it (24).

Male cholesterol-fed rats appear to be especially sensitive to glomerular injury, whereas cholesterol-fed female rats developed no glomerular injury. Estradiol may limit the progression of glomerular injury by reducing extracellular matrix production and accumulation (28). In female rats, estrogen may have played a lipid-lowering role. In the present study, female rats had to be fed more cholesterol than male rats to achieve equal plasma cholesterol levels, despite the fact that when factored for body weight, chow intake was even higher in the females (Table 1). This suggests that female rats were protected from dietary cholesterol loading. It has been shown that when fed a similar commercial diet enriched with cholesterol, male and female rats had similar food intake when corrected for body weight. However, at a comparable chow cholesterol content, males developed higher plasma cholesterol levels (38). In our study, these gender-related differences in cholesterol metabolism were even more striking because, at similar plasma cholesterol concentrations, cholesterol intake, both in absolute terms and factored for body weight, was higher in female rats. Estrogens may cause more efficient cholesterol metab-

Table 3. Vascular and glomerular morphology in female and male rats fed increasing concentrations of cholesterol

<table>
<thead>
<tr>
<th></th>
<th>Arterial Monocytes, No./Artery</th>
<th>Glomerular Protein Droplets, %Glomeruli</th>
<th>Glomerular Monocyte/Macrophage Influx, No./Glomerulus</th>
<th>Glomerular Injury Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.10 ± 0.05</td>
<td>3 ± 2</td>
<td>1.0 ± 0.3</td>
<td>1.7 ± 0.5</td>
</tr>
<tr>
<td>Dyslipidemic</td>
<td>0.03 ± 0.02</td>
<td>5 ± 2</td>
<td>2.5 ± 0.3</td>
<td>1.0 ± 0.7</td>
</tr>
<tr>
<td>Hypercholesterolic</td>
<td>0.14 ± 0.04</td>
<td>23 ± 4c</td>
<td>3.6 ± 0.3c</td>
<td>4.3 ± 0.5c</td>
</tr>
<tr>
<td>Male</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.07 ± 0.03</td>
<td>6 ± 3c</td>
<td>0.8 ± 0.3</td>
<td>3.4 ± 0.5c</td>
</tr>
<tr>
<td>Dyslipidemic</td>
<td>0.25 ± 0.06</td>
<td>20 ± 2c</td>
<td>2.0 ± 0.3c</td>
<td>8.3 ± 1.2c</td>
</tr>
<tr>
<td>Hypercholesterolic</td>
<td>1.87 ± 0.63b,d,e</td>
<td>59 ± 12b,d,e</td>
<td>4.7 ± 0.3b,d,e</td>
<td>11.7 ± 1.3b,d,e</td>
</tr>
</tbody>
</table>

Values are means ± SE. aP < 0.05 vs. female control. bP < 0.05 vs. male control. cP < 0.05 vs. dyslipidemic females. dP < 0.05 vs. hypercholesterolic females. eP < 0.05 vs. dyslipidemic males.

Table 4. Tubulointerstitial morphology in female and male rats fed increasing concentrations of cholesterol

<table>
<thead>
<tr>
<th></th>
<th>Tubulointerstitial Protein Droplet Score</th>
<th>Tubulointerstitial Monocyte/Macrophage Influx, No./Field</th>
<th>Tubulointerstitial Injury Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>4 ± 2</td>
<td>4.4 ± 0.4</td>
<td>4 ± 2</td>
</tr>
<tr>
<td>Dyslipidemic</td>
<td>5 ± 2</td>
<td>4.8 ± 0.5</td>
<td>4 ± 2</td>
</tr>
<tr>
<td>Hypercholesterolic</td>
<td>10 ± 3</td>
<td>8.7 ± 0.7b,c</td>
<td>18 ± 3b,c</td>
</tr>
<tr>
<td>Male</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>2 ± 1</td>
<td>2.4 ± 0.5</td>
<td>8 ± 1b,c</td>
</tr>
<tr>
<td>Dyslipidemic</td>
<td>9 ± 1</td>
<td>5.3 ± 0.4b</td>
<td>21 ± 3b,c</td>
</tr>
<tr>
<td>Hypercholesterolic</td>
<td>20 ± 5b,d,e</td>
<td>12.9 ± 0.5b,d,e</td>
<td>32 ± 5b,d,e</td>
</tr>
</tbody>
</table>

Values are means ± SE. aP < 0.05 vs. female control. bP < 0.05 vs. male control. cP < 0.05 vs. dyslipidemic females. dP < 0.05 vs. hypercholesterolic females. eP < 0.05 vs. dyslipidemic males.
olism in females. Indeed, it has been shown that estrogen increases the catabolism and clearance of LDL (8) and VLDL by increasing activities of hepatic lipase and lipoprotein lipase (9). Furthermore, in cholesterol-fed ovariectomized rabbits, estrogen replacement attenuated aortic accumulation of cholesterol (20). In postmenopausal women, the levels of LDL increase and those of HDL decrease. Estrogen replacement therapy reversed postmenopausal alterations in serum lipoproteins (26). In contrast to LDL, HDL is cardioprotective (34) and testosterone administration decreases HDL cholesterol (15). In the present study, hypercholesterolemic female rats had unchanged HDL levels, whereas HDL levels were decreased in hypercholesterolemic male rats, suggesting that maintenance of HDL levels in female rats in response to dietary cholesterol renders them less prone to develop renal injury.

Renal protection provided by estrogen is not only due to its lipid-lowering role. As discussed above, estrogen might be responsible for enhancing renal NO activity in females, and NO is a potent oxygen radical scavenger. Gender-related differences in lipid peroxidation might thus contribute to differences in the progression of renal injury (2). Indeed, male dyslipidemic rats had increased lipid peroxidation (as measured by urinary TBARS), whereas urinary TBARS levels in dyslipidemic females remained unchanged. Thus in the present study, lipid peroxidation was only present in association with interstitial injury and monocyte/macrophage infiltration.

In summary, male rats have lower renal NO activity than female rats. Furthermore, dietary cholesterol loading decreases renal NO activity in male and female rats but only causes renal injury in male rats. This suggests that a priori lower activity of the renal NO system in male rats, in combination with an increased susceptibility to iNOS induction, determines their sensitivity to renal injury.

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