Male gender increases sensitivity to proteinuria induced by mild NOS inhibition in rats: role of sex hormones

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Verhagen, A. Marjan G., Diana M. A. Attia, Hein A. Koomans, and Jaap A. Joles. Male gender increases sensitivity to proteinuria induced by mild NOS inhibition in rats: role of sex hormones. Am J Physiol Renal Physiol 279: F664–F670, 2000.—Men are at greater risk for renal injury than women. We studied whether male rats are more sensitive to the hypertensive and proteinuric effects of chronic nitric oxide synthase (NOS) inhibition than female rats. In addition, we studied whether androgens or estrogens are responsible for differences in sensitivity to proteinuria induced by chronic NOS inhibition. Females and males were treated with 10, 20, 30, and 100 mg/l L-nitro-L-arginine (L-NNA) during 24 wk. Systolic blood pressure (SBP) and proteinuria were measured regularly and compared with time-control measurements in control females and males. In females and males treatment with 10 mg/l L-NNA had no effect on SBP or proteinuria. Treatment with 20, 30, and 100 mg/l L-NNA resulted in a dose-dependent increase in SBP that was similar in males and females. However, females treated with 20 and 30 mg/l L-NNA were resistant to the development of proteinuria: maximum values were 16 ± 7 and 46 ± 21, respectively, vs. 16 ± 3 mg/day in controls, whereas males treated with those doses showed an increase in proteinuria [139 ± 35 (P < 0.05) and 318 ± 82 (P < 0.01), respectively, vs. 55 ± 11 mg/day in controls]. Treatment with 100 mg/l L-NNA increased proteinuria similarly in both females and males. To study the role of sex hormones in differences in sensitivity to proteinuria induced by mild chronic NOS inhibition, treatment with 20 mg/l L-NNA was repeated in ovariectomized (Ovx) and orchidectomized rats. Ovariectomy did not affect the increase in SBP caused by 20 mg/l L-NNA, but, in contrast to intact females, this dose of L-NNA did cause Ovx rats to develop proteinuria (51 ± 16 vs. 16 ± 7 mg/day in control Ovx rats; P < 0.05). Orchidectomy completely prevented the increased SBP as well as proteinuria induced by 20 mg/l L-NNA in male rats. In conclusion, male rats are more resistant than female rats to develop proteinuria induced by mild chronic NOS inhibition. Estrogens provide some protection in females, whereas androgens are responsible for the increased sensitivity of male rats to proteinuria induced by mild chronic NOS inhibition. Risk factors associated with a compromised nitric oxide system may be more detrimental to the kidney in men than in women.
enhanced endothelium-dependent relaxations in older men (19). Thus estrogens as well as androgens may contribute to differences in NO availability between females and males.

It is conceivable that NO can be regarded as a safety factor, because chronic impairment of NO synthesis results in hypertension and renal injury (4, 35, 41). Indeed, cardiovascular risk factors such as diabetes (20, 26), hyperlipidemia (11), smoking (7), hypertension (31), and aging (38) are all associated with a decreased availability of NO. In the present study, chronic NOS inhibition was regarded as a universal risk factor. We studied whether male rats are more sensitive to the hypertensive and proteinuric effects of chronic NOS inhibition than female rats. In addition it was studied whether androgens or estrogens are responsible for differences in sensitivity to proteinuria induced by mild chronic NOS inhibition.

MATERIALS AND METHODS

Animals. Female and male Sprague-Dawley rats (125–150 g at the start of the protocol; Harlan-Olac, Blackthorn, UK) were housed under conventional conditions and received a standard natural diet (RMH-TM: protein 22.2%, fat 4.8%, potassium 0.85%, sodium 0.4%; Hope Farms, Woerden, The Netherlands). Sentinel animals, which were monitored regularly for infection by nematodes and pathogenic bacteria, as well as antibodies to a large number of rodent viral pathogens (International Council for Laboratory Animal Science, Nijmegen, The Netherlands), tested negative for infection throughout the course of the experiment. The protocol was approved by the Utrecht University Board for studies in experimental animals.

Protocol I: Differences in sensitivity to chronic NOS inhibition between males and females. Female and male rats received the NOS inhibitor N\textsuperscript{\textdegree}nitro-L-arginine (L-NNA) (Sigma) dissolved in drinking water at 10, 20, 30, and 100 mg/l during 24 wk (n = 6–8 rats/group). Untreated females and males served as controls. Systolic blood pressure (SBP) was measured in the awake rats by the tail-cuff method (IITC, San Diego, CA). The rats were weighed and placed in metabolism cages for 24 h for determination of water intake and urinary protein excretion (U\textsubscript{p}V). Food and water were not withheld during this period. Urinary protein concentrations were determined by the Bradford method (6). SBP and U\textsubscript{p}V were measured once every 3 wk (30 and 100 mg/l L-NNA) or once every 6 wk (0, 10, and 20 mg/l L-NNA). When rats died before the end of the experiment, their last measured SBP and U\textsubscript{p}V were included to calculate the average SBP and U\textsubscript{p}V at later time points. After 24 wk, the maximum measured value of SBP and U\textsubscript{p}V were determined for every rat.

Protocol II: Roles of androgens and estrogens in differences in sensitivity to chronic NOS inhibition. To study the role of sex hormones in the observed differences in sensitivity for proteinuria due to mild chronic NOS inhibition between female and male rats, four additional groups of rats were studied. Female rats (n = 16), 4 wk old, were anesthetized with 0.19 mg/kg fentanyl citrate + 6 mg/kg fluanisone subcutaneously (Hynporn; Janssen Pharmaceutica, Beerse, Belgium) and midazolam (2.5 mg/kg ip; Dormicum, Roche, Netherlands) and underwent bilateral orariectomy. Eight Ovx rats served as the control, and eight Ovx rats received L-NNA (20 mg/l). Male rats (n = 15), 4 wk old, were similarly anesthetized and underwent bilateral orariectomy. Seven Orchx rats served as controls and eight Orchx rats received L-NNA (20 mg/l). This concentration of L-NNA was chosen, because in protocol I it was shown that treatment with this concentration did not result in proteinuria in female rats, whereas treatment of male rats with this concentration did result in proteinuria. SBP and U\textsubscript{p}V were measured every 6 wk for 24 wk. When rats died before the end of the experiment, their last measured SBP and U\textsubscript{p}V were included to calculate the average SBP and U\textsubscript{p}V at later time points. After 24 wk, the maximum measured value of SBP and U\textsubscript{p}V were determined for every rat.

RESULTS

Protocol I: Differences in sensitivity to chronic NOS inhibition between males and females. SBP was ~11 mmHg higher in untreated males than in untreated females (P < 0.01), and that this difference did not change over time (Fig. 1). U\textsubscript{p}V was also higher in untreated males than in untreated females from week 3 onward (P < 0.001). Moreover, the difference in U\textsubscript{p}V between males and females increased over time (Fig. 2). In addition, data sets were incomplete at 30 and 100 mg/l L-NNA because of early mortality (see below). Hence for further statistical analysis we evaluated dose-gender interaction at each time point. Treatment with 10, 20, 30, and 100 mg/l L-NNA dissolved in drinking water resulted in a dose-dependent increase in L-NNA intakes in female (0.8 ± 0.2, 1.5 ± 0.2, 2.9 ± 0.4, and 15.6 ± 3.5 mg·kg\textsuperscript{-1}·day\textsuperscript{-1}, respectively) as well as male rats (0.7 ± 0.1, 1.2 ± 0.04, 2.5 ± 0.2, and 12.8 ± 5.7 mg·kg\textsuperscript{-1}·day\textsuperscript{-1}, respectively). Increases in body weight were similar in untreated females and in females treated with 10 and 20 mg/l L-NNA (final body wt: 281 ± 15, 288 ± 11, and 295 ± 4 g, respectively). Similarly, males treated with 0, 10, and 20 g/l L-NNA showed no differences in body weight increment (448 ± 26, 455 ± 23, and 478 ± 16 g, respectively). Body weight in the groups treated with the higher doses, 30 and 100 mg/l, could only be compared at 3 wk. At this time point 0, 30, and 100 g/l L-NNA had no effect on body weight, in either females (221 ± 5, 212 ± 5, and 241 ± 4 g, respectively) or males (276 ± 6, 273 ± 1, and 277 ± 6 g, respectively).

In comparison to untreated rats, treatment with the lowest dose 1-L-NNA, 10 mg/l, during 24 wk did not result in an increase in SBP (Fig. 1) and U\textsubscript{p}V (Fig. 2) in female or male rats. Treatment with 20 mg/l L-NNA resulted in an increase in SBP after 6 wk in females as well as males. With the exception of the value found at
as males, but increases in males were stronger for both variables. All rats treated with 100 mg/l L-NNA died before the end of the experiment. Median survival at this dose was 7 wk in males and 9.5 wk in females (NS). Maximum $U_pV$ was $140 \pm 39 \text{ mg/day in females (} P < 0.01 \text{ vs. untreated females)}$ and $136 \pm 39 \text{ mg/day in males (NS vs. untreated males).}$

**Protocol II: Roles of androgens and estrogens in differences in sensitivity to chronic NOS inhibition.** Treatment with 20 mg/l L-NNA during 24 wk resulted in a significant difference in the development of proteinuria between females and males: although females were protected, males showed a marked and progressive increase in proteinuria. To investigate the role of sex hormones in these differences in sensitivity for the development of proteinuria due to chronic NOS inhibition between female and male rats, treatment with L-NNA (20 mg/l was repeated in Ovx rats and Orchx rats during 24 wk). Ovariectomy as well as orchidectomy had no effect on L-NNA intake ($1.4 \pm 0.1$ and $1.2 \pm 0.1 \text{ mg \cdot kg}^{-1} \cdot \text{day}^{-1}$, respectively). Final body weight was similar in untreated Ovx rats and in Ovx rats treated with 20 mg/l L-NNA ($336 \pm 10$ and $345 \pm 12 \text{ g, respectively)$, although higher than in intact fe-

6 wk, which was higher in males, this increase was not significantly different. However, females treated with 20 mg/l L-NNA were resistant against proteinuria, whereas, compared with untreated males, males treated with this dose showed a progressive increase in $U_pV$ after 12 wk of treatment. This resulted in a maximum $U_pV$ that was significantly increased in males ($139 \pm 35 \text{ vs. } 55 \pm 11 \text{ mg/day in untreated males, } P < 0.05$) but not in females ($16 \pm 7 \text{ vs. } 16 \pm 3 \text{ mg/day in untreated females$). Treatment with 30 mg/l L-NNA resulted in increases in SBP in both female and male rats. However, from 6 wk onward the increase in males was stronger (Fig. 1). Treatment of females with this concentration resulted in a slight increase in $U_pV$ after 12 wk, whereas males already showed a marked increase in $U_pV$ after 9 wk. Maximum $U_pV$ was $318 \pm 82 \text{ mg/day in males (} P < 0.01 \text{ vs. untreated males)}$ and $46 \pm 21 \text{ in females [not significant (NS) vs. untreated females]. Five males and two females treated with 30 mg/l L-NNA died before the end of the experiment. Treatment with the highest dose L-NNA, 100 mg/l, increased SBP and $U_pV$ within 3 wk in females as well
males treated with 0 and 20 mg/l L-NNA (final body wt: 281 ± 15 and 295 ± 4 g, respectively). Similarly, final body weight was similar in untreated Orchx rats and in Orchx rats treated with 20 mg/l L-NNA males treated with 0 and 20 g/l L-NNA (397 ± 11 and 394 ± 7 g, respectively) although lower than in intact males treated with 0 and 20 mg/l L-NNA (final body wt: 448 ± 26 and 478 ± 16 g, respectively).

Untreated Ovx rats showed no change in blood pressure. Treatment of Ovx rats with 20 mg/L L-NNA resulted in an increase in SBP (Fig. 3). Although there was a difference in baseline blood pressure, the increase induced by L-NNA was numerically similar to that found in intact female rats. Maximum SBP was 173 ± 12 vs. 127 ± 2 mmHg in untreated Ovx females (P < 0.05). Untreated Ovx rats showed very little change in UₚV. In contrast to treated intact female rats, treated Ovx rats showed a significant increase in UₚV (Fig. 3). Maximum UₚV was 51 ± 16 vs. 16 ± 7 mg/day in untreated Ovx females (P < 0.05). Two proteinuric Ovx rats treated with L-NNA died before the end of the experiment. Unexpectedly, initial SBP was quite high in Orchx rats (~157 mmHg) and showed a time-dependent decrease. In contrast to the response in intact males, treatment of Orchx rats with 20 mg/l L-NNA had absolutely no effect on SBP (Fig. 3). As a result SBP was significantly higher in L-NNA-treated intact males than in L-NNA-treated Orchx rats at 18 and 24 wk. Orchx rats showed a mild time-dependent increase in proteinuria that was absolutely not affected by L-NNA. As a result UₚV was significantly higher in L-NNA-treated intact males than in L-NNA-treated Orchx rats from 6 wk onward (Fig. 3).

DISCUSSION

Males are at greater risk for renal injury than females, but the mechanisms behind these gender differences in renal disease are unknown. It is conceivable that an increased sensitivity to the deleterious effects of cardiovascular risk factors may contribute to an increased risk for renal injury. Because well-known risk factors for renal injury, such as diabetes (20, 26), hypertension (31), and aging (38) are associated with a decreased availability of NO, we regarded chronic NOS inhibition as a universal risk factor. The present study shows that the sensitivity to develop proteinuria during chronic NOS inhibition is increased in male rats compared with female rats. The results with Ovx females indicated that estrogens provide some protection against proteinuria induced by mild chronic NOS inhibition. The results with Orchx males indicated that androgens have a permissive effect for hypertension as well as proteinuria associated with mild chronic NOS inhibition in intact males.

Blood pressure was slightly higher in male than in female rats. Moreover, at the higher L-NNA doses the hypertensive effect of chronic NOS inhibition was somewhat more outspoken in the males. This does not agree with data showing that blood pressure was not different between untreated male and female rats and that treatment with nitro-L-arginine methyl ester for 2 wk increased blood pressure to similar levels in males and females (33). However, in the latter study blood pressure was only measured under anaesthesia at one time point, whereas we performed multiple repeated measurements in conscious animals. Nevertheless, treatment with 20 mg/l L-NNA increased proteinuria in males but not in females whereas the increase in blood pressure was quite similar. In the general population, under 50 yr of age, men have higher diastolic blood pressure than women and overall have a higher prevalence of hypertension (2). Thus male gender is associated with an increased risk for both renal disease and hypertension. Whether these risk patterns are directly associated is a matter of debate (37).

In the second part of this study it was explored whether sex hormones are responsible for the observed differences in sensitivity to hypertension and proteinuria induced by mild chronic NOS inhibition. As expected, blood pressure in untreated Ovx females was not different from untreated intact females, whereas treatment with L-NNA resulted in a comparable increase in blood pressure in these groups. Surprisingly, although treatment of intact males with L-NNA resulted in an increase in blood pressure, blood pressure in L-NNA-treated Orchx males was not different from control Orchx males. Similarly, in spontaneously hypertensive rats it has been observed that development of hypertension is androgen dependent (16, 34). Testosterone may contribute to hypertension by reducing pressure-natriuresis (34) and activating the renin-
angiotensin system. Male rats have higher angiotensinogen levels than females, whereas testosterone treatment increases and orchidectomy decreases angiotensinogen mRNA and plasma renin (8, 14). However, this cannot explain why blood pressure in L-NNA-treated Orchx males was even lower than in L-NNA-treated intact females. In the absence of sex hormones, the hypertensive as well as the proteinuric effects of chronic NOS inhibition were not equal in males and females. This shows either that other factors are also involved in differences in sensitivity to hypertension and renal injury between males and females or that sex hormones contributed to differences in sensitivity before they were removed at the age of 4 wk.

The effects on proteinuria and mortality in Ovx rats treated with 20 mg/l L-NNA were comparable to those observed in intact females treated with 30 mg/l L-NNA. However, proteinuria was not increased to levels similar to those observed in intact males treated with 20 mg/l L-NNA. This suggests that although estrogens provide some protection against proteinuria induced by mild chronic NOS inhibition, they cannot be solely responsible for the observed differences in sensitivity to proteinuria between males and females. Orchidectomy could completely prevent the hypertension and proteinuria that were observed in intact males treated with 20 mg/l L-NNA. Thus testosterone was responsible for the increased sensitivity in males for proteinuria induced by mild chronic NOS inhibition. It should be pointed out that it is unlikely that orchidectomy would convey such full protection at a higher dose of L-NNA. How do sex hormones influence sensitivity to proteinuria induced by mild chronic NOS inhibition? It is conceivable that endothelium-derived NO can be regarded as a defense mechanism against injury induced by risk factors. NO is not only an important vasodilator (40) but also prevents platelet aggregation (32), leukocyte adhesion (23), as well as vascular smooth muscle proliferation (10), and controls endothelial permeability (22), processes that are important in the pathogenesis of atherosclerosis and glomerulosclerosis. Indeed, it has been shown that estrogens stimulate NO production (18, 21) and decrease inactivation of NO by oxygen radicals because of their antioxidant properties (1). In addition, androgen deprivation in humans is associated with enhanced endothelium-dependent relaxation (19), and long-term estrogen therapy improved vascular function in male-to-female transsexuals. Although these differences could be due to increased estrogen levels as well as decreased testosterone levels, testosterone was a better predictor of flow-mediated vasodilation than estrogen (29). It has also been shown that androgen exposure increased human monocyte adhesion to endothelial cells (25), a process that can be inhibited by NO. Not much is known about differences in renal NO availability between males and females. It has been shown that renal levels (33) and renal medullary levels (28) of eNOS mRNA and protein are higher in kidneys from females than from males. In addition, ovariectomy reduced renal medullary eNOS to levels observed in males (28). However, in the latter study NOS activity was not measured, and no differences were observed between males and females in urinary excretion of nitrite and nitrate (33). Although it has been shown that renal NOS activity was increased after treatment of guinea pigs with large doses of estradiol, testosterone treatment had no effect (42). It is possible that testosterone decreases NO effects by stimulating renal angiotensin II (14), which can be regarded as an antagonist of NO, because of its vasoconstrictive and proliferative properties. Further studies are needed to determine whether NO availability is actually increased in kidneys in females compared with males and whether this increased NO availability contributes to gender-related differences in sensitivity to renal injury.

The present study shows that male as well as female sex hormones play a role in gender-related differences in sensitivity to develop proteinuria. In addition to effects of sex hormones on NO availability, it has been shown that estrogens as well as testosterone influence many other processes involved in progression of renal disease, including mesangial cell proliferation and matrix accumulation, as well as the synthesis and release of cytokines, vasoactive agents, and growth factors (37). This suggests that testosterone as well as ovariectomy disturb the balance between vasodilating, antiproliferative and vasoconstrictive, proliferative factors that are necessary to maintain renal function, making the kidney more vulnerable to a further disturbance of this balance induced by chronic NOS inhibition. In addition, differences in kidney structure and function can contribute to differences in sensitivity for renal injury between males and females. Glomerular volume is larger in males than in females (5, 15, 30), and this difference is eliminated by castration (5, 15). If these differences in glomerular volume are a reflection of filtration surface area, a similar increase in the sieving coefficient for albumin in males, females, and castrated males would cause a greater filtered load in the males than in the other groups. However, ovariectomy does not increase glomerular volume compared with age-matched intact females (5), so this cannot explain all our findings. Increases in proteinuria induced by chronic NOS inhibition are probably glomerular, because changes in proteinuria and albuminuria in this model are practically identical (13). Higher afferent and efferent arteriolar resistances have been reported in female rats compared with males in the absence of differences in arterial and glomerular pressure (27). This increased renal vascular resistance in female rats may protect their glomeruli from hyperfiltration-induced injury secondary to an increase in systemic blood pressure, whereas a comparable increase in blood pressure may result in renal injury in male rats (3). These inherent differences may also have contributed to the disparity between the genders in changes in proteinuria observed after chronic NOS inhibition.

In conclusion, female rats were more resistant to proteinuria induced by chronic inhibition of the NO system than are male rats. Thus risk factors for renal
disease that are associated with a compromised NO system such as diabetes, hypertension, and aging may be more detrimental in males than in females. Both estrogens and androgens contribute to the differences between female and male rats in sensitivity to proteinuria induced by mild chronic NO inhibition. Because NO can be regarded as a safety factor, therapeutic strategies aimed at improving NO availability may reduce the risk of renal injury when risk factors are present.

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