Development of progressive albuminuria in male Munich Wistar Frömter rats is androgen dependent

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PREVIOUS EXPERIMENTAL and clinical studies indicate that sex differences are important for the development of cardiovascular and renal organ damage (3, 5, 8, 24, 26, 32, 38, 43). Systolic blood pressure tends to rise progressively in both men and women throughout adult life, while mean values for systolic blood pressure are lower in women than in men during early adulthood (8). Cardiovascular and renal organ damage seems also to be more prevalent and/or aggravated in men (24, 25). The prevalence of coronary artery disease in men is higher than that of age-matched premenopausal women (24). In a meta-analysis of 68 studies Neugarten and colleagues (25) found that sex status had an important effect on the progression of nondiabetic chronic renal disease with men showing a more rapid decline in renal function than women. Nevertheless, despite the controversial results obtained in clinical intervention studies (6), a sexual dimorphism in cardiovascular and renal function and the development of organ damage has been well documented in both animal models and humans (4, 7, 17, 30–33, 42).

Male spontaneously hypertensive rats (SHR) display a higher blood pressure than female SHR. Orchietomy (Ox) of male SHR reduced blood pressure and total body oxidative stress (39), whereas testosterone substitution of ovariecutomized female SHR resulted in an increase in blood pressure (27). Treatment with the androgen receptor antagonist flutamide caused a reduction in mean arterial pressure (MAP) in male SHR (28). In Dahl salt-sensitive rats a high-salt diet caused a progressive increase in blood pressure, protein and albumin excretion, and glomerular sclerosis in male rats that could be attenuated by castration (44). Testosterone replacement in castrated rats increased blood pressure, renal injury, and upregulated renal angiotensinogen (44). Normal male Munich Wistar rats develop glomerular damage and proteinuria, whereas male rats after Ox, normal females, and females after ovariecтомy are protected from renal damage, suggesting that androgens exhibit a detrimental effect on kidney injury (3). The inbred Munich Wistar Frömter (MWF) genetic rat model develops mild hypertension and inherits a similar nephron deficit in both male and female rats, while the development of progressive albuminuria exhibits a striking sexual dimorphism with more severe phenotypes in males (10, 21, 31, 33). However, previous genetic studies indicate that the genetic predisposition to develop albuminuria in MWF rats is pivotaly influenced by genetic variants on rat chromosomes (RNO) 6 and 8 in both sexes (32, 40). In the current study we therefore set out to test whether the more pronounced albuminuria phenotype in male MWF rats is influenced by testosterone through the androgen receptor.

METHODS

Animals

Male rats were obtained from our MWF/Rkb (laboratory code Rkb, http://dels.nas.edu/ilar/) and Wistar colonies at the Charité - Universitätsmedizin Berlin, Germany. Rats were grouped under conditions of regular 12 h diurnal cycles with an automated light switching device and climate-controlled conditions at a room temperature of 22°C. The rats were fed a normal diet containing 0.2% NaCl and had free access to food and water. All animal experiments were approved by a government...
committee in accordance with national animal protection guidelines (Landesamt für Gesundheit und Soziales, Berlin, Germany).

Pilot Study

In a pilot study we first analyzed the time course of urinary albumin excretion (UAE) in relation to testosterone serum concentrations in male MWF animals and evaluated the effect of Ox and testosterone substitution on UAE.

Testosterone supplementation after castration was tested by two different strategies to meet physiological serum levels. In one group testosterone was administered by intraperitoneal (i.p.) implantation of pellets (150 mg/pellet, release time: 90 days, Innovative Research of America, Sarasota, FL); in a second group testosterone pellets were applied subcutaneously (data not shown). It turned out that i.p. substitution of testosterone with pellets was feasible and led to physiological testosterone serum concentrations in MWF Ox animals.

To evaluate the effect of Ox and i.p. testosterone substitution on albuminuria in young male MWF rats, one set of MWF animals was sham-operated (Sham n = 11), one set was orchiectomized (Ox n = 12), and one set was orchiectomized and testosterone supplemented (OxT n = 5), and UAE was measured over time course (Fig. 1).

Main Study

Subsequently, we examined the role of Ox and the androgen receptor on progressive albuminuria development and renal injury in male MWF compared with Wistar rats with low-grade albuminuria. One set of male MWF and Wistar animals was sham-operated (Sham n = 11–12, respectively) and compared with a second set of orchiectomized animals (Ox n = 10–12, respectively). A third set of animals was orchiectomized, testosterone supplemented, and treated with the androgen receptor antagonist flutamide (OxT n = 7–10, respectively). Sham and Ox surgery was performed at 10 wk of age and groups were followed for 8 wk. Additional groups of rats after Ox with testosterone treatment only, i.e., OxT groups, were omitted, since we demonstrated the efficacy of testosterone substitution on UAE in MWF animals in the pilot study.

Experimental Protocols

In sham-operated rats both testes were exposed by a midline scrotal incision, but not removed (Sham). For castration both testes were exposed and bilaterally orchiectomized (Ox groups). Surgical procedures were performed during anesthesia with fentanyl-midazolam-medetomidin (0.005, 2.0, and 0.15 mg/kg, respectively).

Testosterone-supplemented rats received i.p. implants of testosterone propionate pellets (Innovative Research of America). Flutamide treatment was daily applied by subcutaneous injections (30 mg/kg/day sc; LKT Laboratories, St. Paul, MN) in accordance with previous studies (1, 2) between 10 and 18 wk of age. For applications 500 mg flutamide were dissolved in 10 ml absolute ethanol and blended with sesame oil as a carrier.

Laboratory Measurements

Direct blood pressure measurements via indwelling arterial catheters were carried out at 18 wk of age. Animals were anesthetized with fentanyl-midazolam-medetomidin (0.005, 2.0, and 0.15 mg/kg). A thin polyethylene catheter (PE-50; Reichelt Chemietechnik, Heidelberg, Germany) filled with heparinized saline solution was placed into the femoral artery, tunneled subcutaneously to the back of the neck, and fixed by a ligature as reported previously (20). For blood pressure measurements catheters were connected to a pressure transducer system (ADInstruments, Spechbach, Germany), and three repetitive blood pressure recordings were obtained in awake animals on 2 consecutive days, respectively; data were averaged to obtain individual blood pressure values for each rat.

Time-course analysis for UAE was performed between 12 and 18 wk of age. Animals were placed in metabolic cages for 2 days. The first day was used for adaptation, and urine was collected for the last 24 h for determination of UAE by a rat-specific ELISA technique (21). Blood samples for the determination of serum testosterone levels were obtained from the retrobulbar venous plexus at 12, 14, 16, and 18 wk of age. At 18 wk of age animals were killed under ketamin-xylazin anesthesia (87 and 13 mg/kg body wt, respectively) by excision of the heart. Kidneys were harvested for biochemical and histological analysis, including determination of glomerulosclerosis index (GSI) and tubulo-interstitial damage index (TDI) (34). Cystatin C levels in plasma and testosterone levels in serum were measured by routine laboratory methods (Labor 28, Berlin; Germany and SynLab, Labor Berlin, Berlin, Germany).

In addition, we measured gene expression of hepatitis A virus cellular receptor 1 [Havec1, Kim1 (kidney injury molecule 1), GenBank accession number NM_173149] and renal neutrophil gelatinase-associated lipocalin (NGAL, GenBank accession number NM_130741) as well-established molecular markers reflecting tubular injury as reported (Table 1) (19, 33). Furthermore, we analyzed gene expression of monoacylglycerol O-acyltransferase 1 (Mogat1, GenBank accession number NM_001108803), which was recently associated with elevated albuminuria, nephron deficit, and increased testosterone levels in mice (23). To normalize our expression data, we used Hmbs (hydroxymethylbilane synthase) as a reference gene (GenBank accession no. X06827) (35).

To evaluate oxidative stress in the kidney, we measured superoxide anion production in the kidney, as previously described (37). Briefly, tissue samples (100 mg) were homogenized in 300 μl 50 mmol/l Tris buffer containing 5 mmol/l EDTA and then centrifuged. Superoxide anion production was assessed by a lucigenin-enhanced chemiluminescence method (14). Differences between the values obtained before and after adding the samples to the buffer medium were calculated.
Overall characteristics of male MWF and Wistar rats at 18 wk of age

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Strain</th>
<th>Sham</th>
<th>Ox</th>
<th>OxTF</th>
<th>Overall</th>
<th>Sham vs. Ox</th>
<th>Sham vs. OxTF</th>
<th>Ox vs. OxTF</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW, g</td>
<td>Wistar</td>
<td>478.1 ± 14.8</td>
<td>479.6 ± 8.9</td>
<td>505.1 ± 7.8</td>
<td>0.23</td>
<td>1.00</td>
<td>0.36</td>
<td>0.42</td>
</tr>
<tr>
<td></td>
<td>MWF</td>
<td>333.8 ± 4.2*</td>
<td>338.9 ± 13.2*</td>
<td>346.1 ± 4.9*</td>
<td>0.49</td>
<td>1.00</td>
<td>0.71</td>
<td>1.00</td>
</tr>
<tr>
<td>LV weight/BW, mg/g</td>
<td>Wistar</td>
<td>1.69 ± 0.05</td>
<td>1.65 ± 0.04</td>
<td>1.56 ± 0.07</td>
<td>0.24</td>
<td>1.00</td>
<td>0.30</td>
<td>0.68</td>
</tr>
<tr>
<td></td>
<td>MWF</td>
<td>1.89 ± 0.03 #</td>
<td>1.91 ± 0.06 #</td>
<td>1.88 ± 0.03*</td>
<td>0.81</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Kidney weight/BW, mg/g</td>
<td>Wistar</td>
<td>5.65 ± 0.16</td>
<td>5.40 ± 0.15</td>
<td>5.08 ± 0.19</td>
<td>0.09</td>
<td>0.85</td>
<td>0.04</td>
<td>0.59</td>
</tr>
<tr>
<td></td>
<td>MWF</td>
<td>5.81 ± 0.16</td>
<td>4.80 ± 0.18 #</td>
<td>5.18 ± 0.11</td>
<td>0.0003</td>
<td>0.0003</td>
<td>0.02</td>
<td>0.31</td>
</tr>
<tr>
<td>Cystatin C, mg/l</td>
<td>Wistar</td>
<td>0.10 ± 0.009</td>
<td>0.09 ± 0.005</td>
<td>nd</td>
<td>0.25</td>
<td>—</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td></td>
<td>MWF</td>
<td>0.11 ± 0.005</td>
<td>0.10 ± 0.004</td>
<td>nd</td>
<td>0.16</td>
<td>—</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>MAP, mmHg</td>
<td>Wistar</td>
<td>123.1 ± 3.4</td>
<td>115.8 ± 2.4</td>
<td>114.3 ± 3.2</td>
<td>0.13</td>
<td>0.38</td>
<td>0.21</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>MWF</td>
<td>122.5 ± 4.8</td>
<td>120.9 ± 4.2</td>
<td>128.9 ± 3.9 #</td>
<td>0.46</td>
<td>1.00</td>
<td>0.96</td>
<td>0.75</td>
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</table>

Phenotype values are means ± SE. BW, body weight; LV, left ventricular; MAP, mean arterial pressure; MWF, Munich Wistar Frömter; nd, not determined; Ox, orchiectomized; OxTF, orchiectomized, testosterone-, and flutamide-treated; Sham, sham-operated. *P < 0.0005 vs. Wistar same group; #P < 0.03 vs. Wistar same group.
Sham group (but markedly and significantly lower compared with the MWF week 12 Sham between and 18 and decreased steadily, yet not significantly between week 12, 14 with Wistar Sham (Fig. 2 A2E) testosterone levels were significantly higher compared with MWF Sham compared with Wistar Sham, but was significantly lower compared with the corresponding Sham group during the observation period (P < 0.05, respectively; Fig. 2, A and C). MWF Ox animals (Fig. 2D) demonstrated low-range testosterone levels that were still significantly higher compared with the Wistar Ox group between week 12 and 16 (P < 0.02, respectively), but markedly and significantly lower compared with the MWF Sham group (P < 0.0001, respectively; Fig. 2, B–D). UAE increased significantly until week 18 in MWF Ox (P < 0.05) but was significantly lower compared with MWF Sham at all time points (P < 0.04; Fig. 2, B and D).

In testosterone + flutamide-treated Wistar OxTF rats (Fig. 2E) testosterone levels were significantly higher compared with Wistar Sham (Fig. 2A) in week 12, 14, and 18 (P < 0.05) and decreased steadily, yet not significantly between week 12 and 18. UAE remained unchanged compared with Wistar Sham between week 12 and 18 (Fig. 2, A and E). MWF OxTF animals (Fig. 2F) revealed similar testosterone levels compared with Wistar OxTF, but significantly higher levels compared with MWF Sham in week 12, 16, and 18 (P < 0.02, respectively; Fig. 2, B, E, and F). UAE in MWF OxTF demonstrated higher values compared with Wistar OxTF (P < 0.0001), but UAE remained significantly lower compared with MWF Sham at all time points (P < 0.04, respectively) and compared with MWF Ox in week 12, 16, and 18 (P < 0.04, respectively; Fig. 2, B, D, E, and F).

Additional renal phenotypes. Wistar and MWF Sham rats presented similar values for GSI (Fig. 3A) and TDI (Fig. 3B). Ox did not affect glomerulosclerosis in both strains; however, OxTF treatment significantly reduced GSI in Wistar OxTF and MWF OxTF (P < 0.03, Fig. 3A). Tubular damage tended to be higher in MWF Sham compared with Wistar Sham (Fig. 3B). Ox reduced TDI in both strains and OxTF treatment significantly decreased TDI in Wistar OxTF (P < 0.03, Fig. 3B). Representative histology pictures for showing the differences between Sham and OxTF animals for both MWF and Wistar animals are shown in Fig. 4.
The renal tubular injury markers Kim1 (Fig. 5A) and NGAL (Fig. 5B) demonstrated significantly and markedly increased expression levels in MWF Sham compared with Wistar Sham ($P < 0.0008$, respectively). Orchiectomy alone and testosterone + flutamide treatment in Ox animals did not affect Kim1 and NGAL gene expression patterns in Wistar Ox and OxTF rats (Fig. 5, A and B). In contrast, in MWF Ox animals Kim1 expression was significantly reduced compared with MWF Sham ($P < 0.01$, Fig. 5A), whereas NGAL exhibited a numerical but not significant decrease (Fig. 5B). Both markers showed a significantly decreased gene expression in MWF OxTF animals compared with MWF Sham and MWF Ox ($P < 0.05$, Fig. 5, A and B). Thus, in MWF animals the decrease of Kim1 and NGAL mRNA expression in MWF Ox and MWF OxTF paralleled the decrease in albumin excretion in these groups. In subsequently performed correlation analysis a significant correlation between albumin excretion levels and Kim1 ($r = 0.86$, $P < 0.0001$) and NGAL ($r = 0.64$, $P = 0.002$) mRNA expression was observed in MWF animals.

Mogat1 expression was significantly lower in MWF Sham compared with Wistar Sham ($P < 0.04$, Fig. 5C). In Wistar rats, Mogat1 showed significantly decreased gene expression in Ox and OxTF animals compared with Wistar Sham ($P < 0.05$, Fig. 5C). In contrast, Ox and OxTF did not affect gene expression in MWF animals (Fig. 5C).

Superoxide anion levels in the kidney were significantly elevated in all MWF groups compared with their corresponding Wistar groups ($P < 0.04$, respectively; Fig. 6). Although superoxide anion levels tended to decrease in the MWF strain in response to Ox and OxTF treatment, superoxide anion levels were not significantly different in MWF Ox and MWF OxTF compared with MWF Sham, respectively (Fig. 6).

**DISCUSSION**

In this study we show that testosterone contributes to the progression of albuminuria and kidney damage development in male MWF rats through the androgen receptor.
Earlier investigations had already indicated a role of androgens for the blood pressure sexual dimorphism observed in SHR (13). Moreover, treatment with the androgen receptor antagonist flutamide prevented kidney damage in transgenic mREN2 rats with an activated renin-angiotensin-system (2), and sex-dependent differences in the activation of this system have been implicated as an early marker of diabetic nephropathy (9).

In the current study, we confirmed the important role of the androgen receptor for the progressive albuminuria development in male MWF rats. We first developed a protocol based on i.p. testosterone substitution to achieve testosterone serum concentrations in orchiectomized MWF animals that were similar to levels in normal, i.e., Sham, MWF rats. The resulting testosterone-substituted male MWF OxT animals developed progressive albuminuria that was similar to MWF Sham rats indicating the critical role of testosterone for albuminuria development (Fig. 1). We then analyzed a group of MWF OxTF animals, in which additional treatment with the androgen receptor antagonist flutamide was performed on top of Ox and testosterone substitution. In these MWF OxTF animals albuminuria progression was clearly suppressed.

In orchiectomized animals little amounts of testosterone are still produced in other organs, e.g., the adrenals (22); therefore, we still measured marginal concentrations of testosterone in serum of Ox animals. Consequently, the remaining testosterone production in Ox animals could still promote albuminuria development in MWF and explain the fact that the blockade of the androgen receptor with flutamide resulted in a further suppression of UAE in OxTF animals compared with Ox animals. Interestingly, treatment with flutamide in OxTF led, in male animals at 18 wk of age, to albuminuria levels that were in the same range and statistically not significantly different from those observed in age-matched female MWF rats and male consomic MWF-6SHR animals (6.40 ± 1.19 mg/24 h, respectively) (33, 35). In contrast, male double-consomic MWF-6SHR8SHR animals demonstrated UAE levels (0.61 ± 0.06 mg/24 h) that were even lower compared with OxTF (P < 0.0001) (40). Taken together with previous findings (11, 18, 26, 29, 41) these data support the notion that androgens exhibit detrimental effects on kidney injury.

GSI and TDI did not differ significantly between MWF and Wistar. While the findings in MWF Sham are in agreement with previous findings (35) the observation in Wistar Sham animals appears somewhat counterintuitive. However, it is a well-known phenomenon that some normal rat strains develop structural kidney changes with normal aging including Wistar rats (15). Thus, the unexpected finding is the extent of glomerulosclerosis observed in the Wistar reference strain, while the
Tubulo-interstitial injury markers might be more sensitive and strain differences for these markers may precede the detection of differences in histologic kidney damage. In agreement with this notion, the mRNA expression of the tubulo-interstitial injury markers Kim1 (40) and NGAL (31) was strikingly increased in MWF Sham compared with Wistar Sham rats. Interestingly, however, while no significant strain differences between MWF Sham and Wistar Sham were observed for GSI and TDI, androgens appear to affect these parameters, since GSI values were significantly lower in OxTF animals of both strains compared with the corresponding Sham groups. The TDI values were also lower but only numerically in the MWF OxTF and significantly in the Wistar OxTF compared with the respective Sham group. Taken together the data in the Wistar strain indicate that the development of GSI and TDI and their modulation by androgens are independent from the development of albuminuria. In contrast, the elevated expression of Kim1 and NGAL in MWF Sham animals was significantly lowered by OxTF treatment, while MWF Ox animals exhibited intermediate levels. Moreover, Kim1 and NGAL expression levels correlated significantly with the albumin excretion levels in the MWF strain supporting a pathophysiological link between the parameters.

Interestingly, Long et al. (23) recently analyzed male and female animals from two mouse strains with naturally occurring differences in UAE levels and demonstrated that low numbers of glomeruli and testosterone are potential mechanisms leading to albuminuria in these mice. The authors performed microarray analysis in isolated glomeruli of these mouse strains and detected a correlation between albuminuria and mRNA expression of several genes (23). One of these genes, i.e., Mogat1, maps within a UA quantitative trait locus (QTL) on RNO9 previously identified in the MWF model (34). Mogat1 encodes a monoacylglycerol O-acethyltransferase that catalyzes the synthesis of diacylglycerol and triacylglycerol and is associated with early onset of Type 2 diabetes (16). Mogat1 was significantly upregulated in male mice of both strains and therefore associated with an increased UAE in male mice vs. the respective females in the study by Long et al. (23). However, in our experimental model Mogat1 expression was significantly lower in all MWF groups compared with Wistar Sham ($P < 0.04$, respectively; Fig. 5C). Thus, we could not confirm an association between Mogat1 mRNA expression and albuminuria development in the MWF model. Moreover, Mogat1 expression was not affected by Ox or flutamide treatment in the MWF strain.

Testosterone and the androgen receptor may contribute to albuminuria development by several mechanisms including effects on systemic blood pressure, glomerular hemodynamics, and by affecting the function and integrity of kidney cells including mesangial cells, proximal tubule cells, and podocytes (36, 41). The observed changes in albuminuria in MWF animals are not attributable to blood pressure alterations because no significant differences in blood pressure between MWF Sham, Ox, and OxTF groups were observed. This is in contrast to previous studies in rat models with more severe hypertension where a significant decrease of blood pressure in male rats in response to Ox or antiandrogen treatment was observed (2, 13, 27, 39, 44). In MWF rats physiological serum testosterone levels decrease with age; however, UAE and kidney damage progress continuously. This might be due to a vicious circle where glomerular endothelial cell injury leads to podocyte damage, while podocyte loss further exacerbates endothelial cell injury, promoting the development of albuminuria (12). Once this process is started, even a reduced testosterone serum concentration cannot stop UAE progression.

We have previously shown that oxidative stress as assessed by superoxide anion concentrations is increased in kidneys and aortas of MWF and that oxidative stress associates with endothelial dysfunction in this model (37). Androgens can stimulate the renin-angiotensin-aldosterone and endothelin systems that are associated with an increase in reactive oxygen species leading to impaired renal function and renal injury (26). In the current study we confirm the higher superoxide anion production in kidneys of MWF Sham compared with Wistar Sham rats. However, orchectomy and treatment with flutamide exhibited no significant effect on superoxide anion production in either Wistar or MWF animals, although a trend towards lower levels were observed in MWF animals. Consequently, the significant reduction in albuminuria observed in MWF Ox and MWF OxTF cannot be attributed to changes in oxidative stress in the kidney. Thus, the mechanisms by which androgens contribute to progressive albuminuria and renal injury remain to be elucidated.

We did not include an OxT group in our main study, since we demonstrated the efficacy of testosterone substitution in the pilot study. Moreover, additional groups of Sham or Ox animals treated with flutamide, respectively, were also not included. By lacking these groups we cannot determine the effects that are mediated by androgen receptor blockade alone. The latter represents a major limitation of the current study. Nevertheless, taken together our data can still address the main aim of the study by showing that albuminuria development in male MWF rats is clearly androgen dependent.

Nevertheless, prior to this study one had to realize a discrepancy related to the striking difference in albuminuria between male and female MWF rats in face of the fact that both sexes of MWF rats inherit a similar inborn nephron deficit (10) and that albuminuria development in both sexes is pivotally influenced by albuminuria QTL on rat chromosome 6 and 8 (31, 35). This problem is now resolved, at least in part, since we demonstrated that the sexual dimorphism in albuminuria development in the MWF model can be attributed to testosterone and the androgen receptor in male rats. In future studies it will be of interest to identify the causative genetic variants and molecular mechanisms of albuminuria in MWF and how they interact with testosterone to increase albuminuria.

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GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


