

**Regular Article**

**Testosterone and Mast Cell Interactions in the Development of Kidney Fibrosis after Unilateral Ureteral Obstruction in Rats**

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Mast cell and testosterone interactions involved in renal fibrosis in rats subjected to unilateral ureteral obstruction (UUO) were investigated. Orchietomized (ORX) and nonorchietomized Wistar rats were subjected to UUO, and a nonorchietomized group was sham-operated (control: SO). Animals from the UUO group were treated with salinar or sodium cromoglycate (CG). Some ORX rats from the saline or CG groups also received testosterone propionate replacement (TR). Kidneys and blood were collected 14 d after UUO or SO. Kidney sections were stained with toluidine blue to quantify mast cells, and picrosirius red was used for collagen analysis. Immunohistochemistry for α-smooth muscle actin (α-SMA) and proliferating cell nuclear antigen (PCNA) expression was also performed. Plasma testosterone levels (PTLs) were measured. ORX decreased and TR normalized PTLs. UUO increased mast cell density in the kidney pelvis, but not in the kidney parenchyma. UUO increased mast cell degranulation, and CG or ORX inhibited this effect. TR partially reversed the effect of ORX on mast cell degranulation, and CG partially inhibited that effect of TR. UUO increased the collagen areas of the renal parenchyma, whereas CG or ORX abolished that alteration; TR reversed the effects of ORX, and CG partially inhibited that effect of TR. UUO increased tubulointerstitial α-SMA expression and PCNA-positive cells, and these changes were sensitive to ORX or CG to the same degree, while TR again reversed the effect of ORX. Renal fibrosis after UUO appears to be determined by interactions between testosterone and mast cells.

**Key words** testosterone; mast cell; fibrosis; kidney

Testosterone was shown to increase renal injury induced by ureteral obstruction,\(^1,2\) and testosterone administration to oophorectomized female rats also increased tubulointerstitial fibrosis after unilateral ureteral obstruction (UUO); moreover, kidney collagen deposition was greater in male than in female rats in this model,\(^1,2\) and orchiectomy (ORX) can decrease renal fibrosis after UUO.\(^1,2\) Those data pointed to testosterone as a key hormone in the development of renal fibrosis under pathological conditions. However, the underlying cellular mechanisms of the profibrotic effects of testosterone are not well understood.

Mast cell activity is involved in fibroblast stimulation and extracellular matrix remodeling processes,\(^3,4\) and renal fibrosis appears to be closely related to kidney mast cell density.\(^5,6\) However, while some studies found profibrotic activity related to mast cells in murine models of UUO,\(^7,8\) others suggested that mast cells protect the kidney from fibrosis development in response to nephrotoxic agents and UUO.\(^9,10\) Although conflicting data have been published concerning mast cells and kidney fibrosis, they are not simple bystander cells in the context of renal damage associated with collagen deposition. Therefore, considering the profibrotic effects of testosterone and the potential modulating actions of mast cells on collagen deposition in the kidney, we evaluated possible interactions between testosterone and mast cells in determining renal fibrosis in rats subjected to UUO, focusing on the cellular mechanism involved.

**MATERIALS AND METHODS**

**Animals** Male Wistar rats (200–300 g) were subjected to UUO as previously described,\(^3\) with some modifications. Briefly, on day 0, all animals were subjected to sham operation (SO) or ORX, and on day 7, their left ureters were tied with cotton thread (UUO groups); one group of nonorchietomized rats, but subjected to SO for ORX, as described above (control group), also underwent SO for UUO (SO group). Testosterone replacement\(^11\) (TR group; testosterone propionate, 0.5 mg/kg; three times a week, subcutaneously (s.c.), dissolved in olive oil) started 1 d after ORX (i.e., on day 1), and sodium cromoglycate treatment\(^12\) (CG group, 50 mg/kg/day intraperitoneally (i.p.), dissolved in NaCl 0.9%) was initiated on day 5 (i.e., 2 d before UUO to ensure mast cell inhibition before the obstruction). Appropriate vehicles were also administered (i.e., saline as a control for CG treatment in the SO, UUO, UUO+ORX, and UUO+ORX+TR groups; olive oil as a control for TR in the SO, UUO, UUO+CG, UUO+ORX, and UUO+ORX+CG groups). In summary, the following groups were investigated: SO (n=6); UUO (n=5); UUO+CG (n=6); UUO+ORX (n=5); UUO+ORX+CG (n=6); UUO+ORX+TR (n=6); and UUO+ORX+TR+CG (n=5). Fourteen days after SO/UUO (day 21 of experimentation, always 30 min and 6 h after the administration of CG or saline and testosterone propionate or olive oil, respectively, the animals were killed to remove the left kidney and/or to collect a blood sample.

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by cardiac puncture. All surgical procedures and euthanasia were conducted in anesthetized rats (ketamine, 100 mg/kg and xylazine, 10 mg/kg, i.p.). After surgeries, all animals received pentamético (0.1 mL/kg, intra-muscularly (i.m.)) and were returned to the cage with free access to standard lab chow and water ad libitum.

Kidney Function Study and Plasma Testosterone Level (PTL) Determination The animals in the SO, UUO+ORX, and UUO+ORX+TR groups were placed in metabolic cages for the collection of urine. Then, on day 21, blood was sampled as described above. Urinary and plasma creatinine levels were determined using a colorimetric method (Gold Analisa Diagnóstica Ltda.; Belo Horizonte, Minas Gerais, Brazil) to calculate the glomerular filtration rate (GFR) as previously reported. PTLs were determined by Tecsa Laboratory, Belo Horizonte-MG, using the electrochemiluminescence technique according to the specifications of commercial kits.

Histochemical and Immunohistochemical Techniques Kidney sections (4 µm) stained with hematoxylin–eosin (HE) were evaluated to determine general morphological changes. Toluidine blue staining was used to identify mast cells, and picrosirius red was used to evaluate total collagen. Mast cells were classified based on the levels of cell granule dispersion as previously reported; both total mast cell density (independently of its degranulating stage, expressed as mast cells per square millimeter) and the density of degranulating mast cells (cells per square millimeter) were determined. Analyses were performed using an optical microscope (40×objective). To determine mast cell density, the slides were scanned and the areas of tissue sections were determined. To evaluate total collagen, slides stained with picrosirius red were observed under a microscope equipped with a polarization filter; 20 random histological fields of kidney sections were analyzed (20×objective). All images were analyzed using ImageJ software, and the values obtained were expressed as percentages of marked areas.

Moreover, sections of renal tissue were incubated with monoclonal antibodies anti-α-smooth muscle actin (SMA) (1:1000, DAKO Corporation, Denmark) and anti-proliferating cell nuclear antigen (PCNA) (1:1000, Sigma Chemical, St. Louis, MO, U.S.A.). The sections were then incubated with anti-mouse immunoglobulin G (IgG) (1:200, monoclonal, Vector Laboratories, Burlingame, CA, U.S.A.) antibody. Immunohistochemical staining with 3,3-diaminobenzidine (Sigma, Israel) was detected with an avidin–biotin–peroxidase system (Vector Laboratories), and the sections were counterstained with methyl green. Nonspecific binding was blocked by incubation with 5% bovine serum albumin (Sigma Chemical) in phosphate buffered saline (PBS). Immunohistochrometry for α-SMA was assessed as the percentage of labeled glomerulus or renal cortex. A score from 0 to 4 was assigned: score 0 = 0–5% of the field scored; score 1 = 6–25%; score 2 = 26–50%; score 3 = 51–75%; and score 4 = 76–100%. The PCNA reaction was analyzed by counting positive cells. To quantify positive cells, 30-field photos of each slide were taken using an optical microscope coupled to a digital camera (40×objective). The cell counter tool in the Image J software program (ver. 1.4) was used to count marked cells. The mean counts were then calculated for each kidney. Collagen deposition and α-SMA expression were also analyzed in the pelvic tissue.

Fig. 1. Sections of Kidney Stained with HE Showing the Distension of the Calyx (+) and the Increase in the Renal Pelvis Wall (*) Induced by Unilateral Ureteral Obstruction (UUO) Compared to Sham Surgery (SO) (A) Mast cell density (regardless of the degranulation state) in the kidney parenchyma or pelvis in the UUO and SO groups (B). Section of the renal pelvis stained with toluidine blue of rats subjected to UUO (C); arrow: mast cell. Scale bars: 0.5 cm (A) and 50 µm (C). Values are mean ± S.E.M., n=5–6; *p<0.05 vs. SO pelvis.

Statistical Analysis Data are expressed as mean ± standard error of the mean (S.E.M.) and were compared using ANOVA followed by Tukey’s multiple-comparison test, Student’s t-test, or Student’s t-test with Welch’s correction, as appropriate. GraphPad Software was used for performing the statistical analyses and creating the artwork. A p value of less than 0.05 was considered to represent a statistically significant difference.

RESULTS

Although macroscopic inspections of kidneys from UUO groups immediately before their isolation suggested that the kidneys were swollen, presenting hydronephrosis, and that the ureters were distended, quantitative analyses of the total renal cross-sectional area (parenchyma+pelvis wall+pelvic space) and of the kidney parenchymal area evidenced no significant differences among groups (data not shown). However,
Macroscopic inspections clearly showed that both the renal medulla and cortex were compressed (Fig. 1A). Neither the GFR (mL/min) nor the urinary flux (µL/min) were altered by UUO (GFR: 1.44±0.15 vs. 1.06±0.22, urinary flux: 8.88±0.49 vs. 11.35±1.74; SO vs. UUO, p>0.05).

Mast cell populations observed in the pelvis wall of the SO and UUO groups were markedly more abundant than those observed in the kidney parenchyma (Figs. 1B, 1C); the mast cell population of the pelvis wall was approximately 60- and 400-fold greater than that of the cell population observed in the kidney parenchyma of the SO and UUO group, respectively (Fig. 1B). Moreover, mast cell density in the pelvic tissue was increased by obstruction of the urinary tract (Fig. 1B).

UUO also increased mast cell density in total renal tissue (i.e., parenchyma+pelvis) and CG administration and ORX partially or totally reversed this effect, respectively. However, CG did not enhance the effect of ORX. TR reversed the effect of ORX on mast cell density, and CG administration partially inhibited the effect of hormone replacement (Fig. 2A). Moreover, UUO also increased the density of degranulating mast cells, and both CG and ORX inhibited this effect. Again, CG administration did not enhance the effect of ORX. TR partially reversed the effect of ORX on mast cell degranulation, and CG treatment partially inhibited the effect of hormone replacement (Fig. 2B). Mast cell density in the renal parenchyma was extremely low, and none of the treatments significantly altered this parameter (Fig. 2C). UUO increased the deposition of collagen fibers in the renal parenchyma, and CG treatment or ORX abolished this effect. However, CG treatment did not enhance the effect of ORX. TR reversed the effect of ORX on the deposition of collagen fibers, and CG administration partially inhibited the effect of hormone replacement (Fig. 3).

Immunohistochemical staining for α-SMA expression showed that UUO increased the tubulointerstitial α-SMA expression. ORX significantly decreased the effect of UUO on tubulointerstitial α-SMA expression, and TR reversed the effect of ORX. Although CG did not enhance the effects of ORX, it partially reversed the effect of TR on tubulointerstitial α-SMA expression. Tubulointerstitial α-SMA expression in the CG-treated group differed neither from that in the UUO group nor from that in the ORX group (which differed from one another), suggesting a partially inhibitory effect of CG in this respect (Fig. 4). No significant difference in glomerular α-SMA expression was observed among groups (data not shown).

Renal expression of tubulointerstitial PCNA was increased by UUO; ORX and CG treatment partially inhibited this effect. TR reversed the effect of ORX on tubulointerstitial PCNA expression, and CG treatment partially inhibited the effect of TR (Fig. 5). No significant difference among groups was observed with respect to glomerular PCNA expression (data not shown).

Collagen deposition in the renal pelvic tissue was not altered by UUO, CG administration, or ORX (data not shown); however, α-SMA expression in the renal pelvic tissue was increased by UUO, but neither CG nor ORX altered this effect of UUO (data not shown).

ORX decreased PTls, and TR was effective in normalizing it (156.40±51.47, 4.92±0.59 [p<0.05 vs. SO], and 208.80±84.65 ng/dL in the SO, ORX, and TR group, respectively). Finally, neither ORX nor TR affected the body weight of the animals under our experimental conditions (310.70±15.83, 276.00±11.78, and 278.20±5.35 g in the SO, ORX, and TR group, respectively, p>0.05 vs. SO).

DISCUSSION

UUO determined kidney remodeling as characterized by cortical and medullary thinning and pelvic enlargement, alterations that have been associated with the increased pressure produced by urine accumulation in the pelvic space. The contralateral kidney appears to be under hyperfiltration, an adaption that may explain the maintenance of GFR and, subsequently, of the renal function observed after UUO.

The effects of CG treatment and ORX indicated the participation of testosterone and mast cells in inducing renal fibrosis after UUO, as previously reported. The observation that hormone replacement reversed the protective effect of ORX in
The effects of cromoglycate treatment (CG), orchietomy (ORX), or testosterone replacement (TR) as well as the effects of the association of CG with ORX or TR were evaluated in rats subjected to UUO. Sections of the renal parenchyma stained with picrosirius red, visualized in color (odd numbers) or viewed under polarized light (even numbers) demonstrating collagen deposition in the SO (B), UUO (C), UUO+CG (D), UUO+ORX (E), UUO+ORX+CG (F), UUO+ORX+TR (G), and UUO+ORX+TR+CG (H) groups. Scale bar: 50 µm. Values are mean±S.E.M., n=5–6. *p<0.05 vs. SO, †p<0.05 vs. UUO+CG, ‡p<0.05 vs. UUO+ORX, §p<0.05 vs. UUO+ORX+CG (p=0.0110 SO vs. UUO; p=0.0111 SO vs. UUO+TR; p=0.0056 UUO+CG vs. UUO; p=0.0021 UUO+ORX vs. UUO; p=0.0013 UUO+ORX+CG vs. UUO; p=0.0056 UUO+CG vs. UUO+ORX+TR; p=0.0021 UUO+ORX vs. UUO+ORX+TR; p=0.0013 UUO+ORX+CG vs. UUO+ORX+TR).

The effects of cromoglycate treatment (CG), orchietomy (ORX), or testosterone replacement (TR), as well as the effects of the association of CG with ORX or TR, were evaluated in rats subjected to UUO. Sections of the renal parenchyma demonstrating α-SMA immunoreactivity in the SO (B), UUO (C), UUO+CG (D), UUO+ORX (E), UUO+ORX+CG (F), UUO+ORX+TR (G), and UUO+ORX+TR+CG (H) groups. Scale bar: 50 µm. Values are mean±S.E.M., n=5–6. *p<0.05 vs. SO, †p<0.05 vs. UUO+ORX, ‡p<0.05 vs. UUO+ORX+CG (p<0.0001 SO vs. UUO; p=0.0189 SO vs. UUO+CG, p<0.0001 SO vs. UUO+ORX+TR; p=0.0149 SO vs. UUO+ORX+TR+CG; p=0.0162 UUO+ORX vs. UUO+ORX+TR; p=0.0421 UUO+ORX+CG vs. UUO+ORX+TR).

The effects of androgen replacement on the activation of these cell populations and their activity appear to involve resident precursor cells into mature mast cells and to induce cellular degranulation. The effects of testosterone on mast cell populations and their activity appear to involve resident cell activation, proliferation, and/or differentiation after UUO. In this respect, mast cell mediators have been proposed to stimulate the conversion of immature resident precursor cells into mature mast cells and to induce cellular degranulation. The effects of testosterone on mast cell populations and their activity appear to involve resident cell activation; CG treatment partially reversed the effect of testosterone on mast cell density and completely reversed the effect of androgen replacement on the activation of these cells.

In our study, mast cell density was increased in the pelvic tissue, but not in the kidney parenchyma, 14 d after UUO. Our data are in agreement with those reported by Summers et al., who observed increased mast cell activation in the kidney 6 h after UUO, followed by normalization 1 week later.
Those authors argued that “despite their early recruitment to the injured kidney, the pathological profibrotic properties of mast cells persist to (at least) 14 days.” Moreover, Pons et al.\(^\text{18}\) found that mast cells were not present in the kidney parenchyma some weeks after partial UUO in mice, but they were detected in kidney capsules; nonetheless, mast cells appear to be involved in the orchestration of collagen deposition in the kidney parenchyma, since mast cell-deficient mice did not present kidney fibrosis after UUO.\(^\text{19}\) Therefore, although we did not analyze mast cell populations early after UUO, our data and those reported by Pons et al.\(^\text{18}\) open another possibility: that mast cell mediators released in the pelvic tissue could reach the parenchyma and exert their profibrotic effects (i.e., long-distance modulation). Supporting this suggestion, it was shown that mast cells release mediators that can leave their sites of deposition and diffuse over long distances in tissues.\(^\text{14}\) Moreover, urine accumulating in the pelvic space due to ureteral ligation could also presumably serve as an alternative pathway for the diffusion of mast cell mediators from the pelvis to the kidney parenchyma. These hypotheses deserve further investigation.

The expression of renal \(\alpha\)-SMA increases after UUO, and the literature suggests that testosterone and mast cells are involved in this alteration.\(^\text{1,2,7}\) Our results not only show that testosterone modulates the increase in tubulointerstitial \(\alpha\)-SMA in the kidney after UUO but also suggest that this effect requires, at least in part, mast cell activation. ORX decreased \(\alpha\)-SMA in the kidneys of rats subjected to UUO, an effect that was abolished by TR, and CG treatment partially reversed this effect of hormone replacement. Although the elucidation of the molecular mechanisms involved in the interaction between testosterone and mast cells was beyond the scope of the present study, it is important to mention that mast cells were shown to express androgen receptors\(^\text{19}\) and they are able to stimulate \(\alpha\)-SMA expression in cultured fibroblasts.\(^\text{20}\) Those observations could explain the interaction between testosterone and mast cells in tubulointerstitial \(\alpha\)-SMA expression. Moreover, several renal cells can express \(\alpha\)-SMA, such as pericytes, vascular smooth muscle cells, and myofibroblasts.\(^\text{21}\) Myofibroblasts represent a fibroblast phenotype actively involved in the tubulointerstitial deposition of collagen under conditions that determine renal fibrosis,\(^\text{22}\) and interstitial \(\alpha\)-SMA accumulation has been used as an indicator of kidney fibrosis during renal obstruction.\(^\text{3}\) Therefore, the increase in \(\alpha\)-SMA observed in the present study might represent an important cellular mechanism by which testosterone, mast cells, and their interaction determine kidney fibrosis after UUO. The profibrotic effect of testosterone on the kidneys of animals subjected to UUO has been described in other studies.

The observation that the increase in PCNA-positive cells is partially reversed by ORX or CG treatment, and that TR abolished the effect of gonadal removal, suggest that both testosterone and mast cells stimulate, directly or indirectly, the cellular proliferation in the UUO model. This suggestion is based on the requirement of the PCNA protein for DNA replication, cell maintenance, and proliferation.\(^\text{22}\) Moreover, the effect of testosterone on PCNA-positive cells appears to be partially mediated by mast cells, since CG also partially reversed this effect of TR. Because the transition of fibroblasts into myofibroblasts after fibroblast proliferation is a mandatory mechanism involved in renal fibrosis, including after UUO,\(^\text{23}\) our results on kidney collagen deposition as well as PCNA and \(\alpha\)-SMA expression suggest that part of the PCNA-positive cells could be fibroblasts beginning proliferation before their differentiation into myofibroblasts.

The finding that in the renal pelvis \(\alpha\)-SMA expression was increased by UUO without any change in collagen deposition indicates that the kidney parenchyma and pelvis react to the increased pressure associated with UUO in different ways. While the kidney parenchyma adapts through collagen deposition, renal pelvic tissue seems to adapt through smooth muscle cell hyperplasia and/or hypertrophy.

In summary, the results of the present study suggest that both testosterone and mast cells have profibrotic effects on the kidney after UUO. Moreover, testosterone and mast cells
interact to increase tubulointerstitial fibrosis after ureteral ligation. This interaction appears to involve, at last partially, the differentiation of cells committed to extracellular matrix deposition as well as the permissive effect of testosterone on mast cell proliferation, differentiation, and/or activation in the pelvic tissue.

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Conflict of Interest  The authors declare no conflict of interest.

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