Ulinastatin attenuates renal interstitial inflammation and inhibits fibrosis progression in rats under unilateral ureteral obstruction

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Abstract. The aim of the present study was to examine the protective effects of the urinary trypsin inhibitor ulinastatin (UTI) on renal interstitial inflammation and fibrosis in rats subjected to unilateral ureteral obstruction (UUO). A total of 24 male Wistar rats were randomly divided into the three groups; the sham operation (SOR) group (n=8), the UUO group (n=8) and the UUO+UTI group (post-UUO UTI treatment, n=8). UUO was performed with complete ligation of the left ureter. As a medical intervention, saline (4 ml kg^{-1} d^{-1}) and UTI (40000 units kg^{-1} d^{-1}) were injected, respectively, into the animals of the corresponding groups on day one following surgery. The rats in all three groups were euthanized on day seven post surgery. Blood samples were harvested for blood urea nitrogen (BUN) and serum creatinine (Scr) content measurements. The degree of interstitial pathological changes in the tissues from the obstructed kidneys were observed through hematoxylin and eosin (H&E) and Masson staining. The CD68+ macrophage amount, tumor necrosis factor-α (TNF-α), interleukin 1β (IL-1β), nuclear factor-κB (NF-κB), transforming growth factor-β1 (TGF-β1) and type I collagen (Col-I) levels were examined immunohistochemically. The protein expression levels of NF-κB were examined using western blot analysis. Total superoxide dismutase (SOD) activity and malondialdehyde (MDA) content of homogenates were measured spectrophotometrically. The results revealed that ulinastatin had no statistically significant effect on the BUN and Scr levels (P>0.05). However, in comparison with the SOR group, the UUO group exhibited significantly more severe renal interstitial pathological injury in terms of tubular dilation, epithelial atrophy, renal interstitial inflammatory cell infiltration and proliferation of fibrous tissues, as well as significantly elevated levels of interstitial CD68+ macrophages, IL-1β, TNF-α, NF-κB, TGF-β1 and Col-I (P<0.01). UTI treatment significantly reduced UUO-induced renal interstitial damage with reduced levels of interstitial CD68+ macrophages, IL-1β, TNF-α, NF-κB, TGF-β1 and Col-I and MDA (P<0.05), and increased SOD levels (P<0.05). In conclusion, the present study indicated that UTI is able to effectively inhibit UUO-side renal interstitial inflammatory reaction and fibrosis in UUO-inflicted rats.

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Introduction

Inflammation has a crucial role in the progression of numerous acute and chronic renal injuries. The persistence of an inflammatory response may lead to renal fibrosis, and inhibiting such inflammatory reactions is effective in delaying the progression of fibrosis (1,2). The unilateral ureteral obstruction (UUO) model is a classic animal model that is used to study obstructed renal interstitial inflammation and fibrosis (3). The renal damaging effects caused by UUO mainly include interstitial inflammatory response, apoptosis and progressive interstitial fibrosis (4). Among these, inflammatory responses are characterized by an excessive generation of cytokines, including interstitial pro-inflammatory cytokines and growth factors, as well as reactions involving inflammatory cell packages, actuate renal tubular atrophy and interstitial fibrosis. Therefore, inhibiting the inflammatory response may facilitate attenuating renal tubular epithelial cell apoptosis and interstitial fibrosis (5).

Ulinastatin (UTI) is a glycoprotein separated and purified from human urine. As a typical Kunitz-type wide spectral and highly effective protease inhibitor (6), it is able to eradicate oxygen free radicals and inhibit the release of inflammatory mediators and package inflammatory cells (7,8). Its clinical applications include treatment of acute pancreatitis, shock and extracorporeal circulation injury. Several recent studies have indicated that UTI has protective effects on numerous types of acute renal injury (9,10). In one study by Katoh *et al* (11), it was confirmed that ulinastatin effectively reduced the TGF-β1 content in the lung tissues of rats with radioactive lung injury, and prevented radioactive lung injury and fibrosis. However, to the best of our knowledge, there have been no investigations on the effectiveness of UTI in reducing renal interstitial inflammatory responses and fibrosis in UUO-inflicted rats. Therefore, the present

study aimed to examine the effects of UTI on renal injury of UUO-inflicted rats, to further investigate its potential clinical utility as a renal protective strategy.

Materials and methods

Reagents and antibodies. The chemicals and reagents of analytical grade were purchased from ZSGB-BIO (Beijing, China) unless otherwise stated. UTI was purchased from Techpool Bio-Pharma Co., Ltd. (Guangdong, China) and the Sircol Collagen Assay kit was purchased from Biocolor Ltd. (Carrickfergus, Antrim, UK). The following primary antibodies were used for immunohistochemistry: Rabbit polyclonal antibody against transforming growth factor-β1 (TGF-β1) was obtained from Abcam (Cambridge, UK); mouse monoclonal antibody to CD68 and rabbit polyclonal antibody against nuclear factor (NF)-κBp65 were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA); rabbit polyclonal antibody against type I collagen, rabbit monoclonal antibodies against tumor necrosis factor-α (TNF-α) and interleukin 1β (IL-1β) were purchased from Boster Biological Technology Co., Ltd. (Wuhan, China); malondialdehyde (MDA), superoxide dismutase (SOD) and the Coomassie Brilliant Blue Assay kit were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

Experimentation animals. A total of 24 male Wistar rats, 8-12 weeks old and weighing 180-220 g, were purchased from Vital River Laboratories Co., Ltd. (Beijing, China). The present study was conducted in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal experimental procedure was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the Second Affiliated Hospital Harbin Medical University (Harbin, Heilongjiang, China). All of the rats were bred and maintained in accordance with the Care and Use of Laboratory Animals guidelines published by the China National Institute of Health.

Grouping. The 24 Wistar rats were randomly divided into three groups: The sham operation group (SOR group, n=8), the UUO control group (UUO group, n=8) and the UTI treatment group (UTI group, n=8). The UUO surgery followed the established procedure (12). Under intraperitoneal anesthesia, the left abdominal cavity of the rats was accessed through left lateral incision. The left ureter was separated and exposed, and thereafter ligated at two locations using size 5-0 sutures. The ureter was cut in between the two ligation points and layered suture was performed. For the SOR group, the left ureter was separated following abdominal incision, but no ligation was performed. The UTI group was injected with 40 kU kg^{-1} d^{-1} UTI into the abdomen following surgery, while the SOR and UUO groups were injected the same amount of 1 ml kg^{-1} d^{-1} normal saline in the abdomen for comparison. All of the rats were sacrificed in each group on the 7th day following surgery. The samples were harvested and frozen at -80˚C. Blood was obtained from abdominal cardinal veins. The serum from the centrifugation was refrigerated at -20˚C.

Biochemical indicator measurement. BUN and Scr levels between the different groups of rats were measured using a Roche Automatic Biochemical Analyzer.

Histopathological examination. Paraffin-embedded sections of nephridial tissues were stained with hematoxylin and eosin (H&E) and Masson trichrome for the morphological studies. A total of 20 consecutive high power fields of each renal cortex section were examined under light microscopy. H&E staining provided semi-quantitative scoring on the degree of interstitial injury that assigned points (0 to 3) for the extent of interstitial fibrosis, tubular atrophy (defined as luminal dilation and flattened tubular epithelial cells) and interstitial inflammatory cell infiltration, while Masson staining calculated the renal interstitial fibrosis area (the percentage of blue staining in the whole area). The analysis was conducted by two independent observers and the average of all values was obtained.

Sircol collagen assay. Analysis of total soluble collagen concentration within each renal cortical tissue sample was performed using the Sircol Collagen Assay according to the manufacturer's instructions (Biocolor Ltd.) The tissue samples were dissolved in 0.5 M acetic acid (wet weight, 20 ml/g) and heated for 120 min at 60˚C. The tissue suspensions were centrifuged, supernatants collected and the total collagen concentration measured at 540 nm. Soluble collagen content was expressed as the tissue wet weight (mg/g).

Immunohistochemical analysis. Streptavidin horseradish peroxidase biotin staining (streptavidin-peroxidase method) was employed to measure CD68+ macrophage count and TNF-α, IL-1β, NF-κB, TGF-β_1 and Col-I expression. In the negative control group, the primary antibody was replaced with phosphate-buffered saline (PBS; pH 7.4). Paraffin-embedded tissue sections were sliced at 4-μm intervals following formalin fixation, and thereafter deparaffinized in xylene and rehydrated through a graded ethanol series. Antigen was retrieved with boiling under high pressure and endogenous peroxidase activity was suppressed by placing the slide-mounted tissues in H_2O_2. Following natural cooling, the sections were rinsed in PBS and then incubated with polyclonal antibodies overnight at 4˚C: CD68 (diluted 1:100), TNF-α (diluted 1:100), IL-1β (diluted 1:100), NF-κB (diluted 1:150), TGF-β_1 (diluted 1:100) and Col I (diluted 1:200). Following rinsing, the section slides were incubated at room temperature with biotinylated goat anti-rabbit immunoglobulin G secondary antibody for 20 min. The immune complexes were detected using a diaminobenzidine (DAB) substrate. The sections were subjected to a final 40 sec counterstaining of H&E and PBS rinsing, before they were mounted onto transparent neutral balsam. Red-stained areas were positive. The percentage of positive stained areas in the tubule-interstitum on the section slides was measured with an image analyzer (Lumina Vision, Version 2.0)

Assessment of oxidative stress [lipid peroxidation (MDA) and antioxidant enzyme activities (SOD)] estimation. The rat tissues were homogenized in 1.15% potassium chloride (KCl) buffer (1:9, w/v) using a manual glass homogenizer (Tempest

Virtishear, model 278069; VirTis, Gardiner, NY, USA) for ~5 min. The supernatant was used for analysis of the MDA and the content of homogenates was determined spectrophotometrically by measuring the presence of thiobarbituric acid-reactive substances (13). A total of 3 ml of 1% phosphoric acid and 1 ml of 0.6% thiobarbituric acid solution were added to 0.5 ml of plasma and pipetted into a tube. The mixture was heated in boiling water for 45 min. Following cooling, the color was extracted into 4 ml of *n*-butanol. Absorbance was measured in spectrophotometer (Ultraspec Plus; Biochrom, Ltd., Cambridge, UK) at a 532 nm wavelength. The quantities of lipid peroxides were calculated as thiobarbituric acid-reactive substances of lipid peroxidation and were provided in units of nM/g tissue.

Total (Cu-Zn and Mn) SOD (EC 1.15.1.1) activity was determined according to the method described by Shin *et al* (14). The principle of the method is based on the inhibition of nitroblue tetrazolium (NBT) reduction by the xanthine-xanthine oxidase system as a superoxide generator. One unit of SOD is defined as the enzyme amount causing 50% inhibition in the NBT reduction rate. SOD activity was expressed as the U/mg protein.

The activity of SOD was measured using a SOD assay kit (Trevigen, Gaithersburg, MD, USA). This method is based on the reduction of nitro blue tetrazolium by SOD. Superoxide ions convert nitro blue tetrazolium into blue formazan, which absorbs light at a wavelength of 550 nm. SOD reduces the superoxide ion concentration and thereby lowers blue formazan formation. The extent of reduction in terms of appearance of blue formazan reflects the amount of SOD activity in a sample.

Western blot analysis. The protein levels were assessed by western blot analysis. Rat nephridial tissue total protein was extracted to measure its protein content using the Bicinchoninic Acid (BCA) method. A total of 50 μg of the protein was sampled, separated on 12% SDS-PAGE and transferred onto a nitrocellulose membrane with a 100 mA current. The membrane was then blocked for 1 h in 5% skimmed milk powder in Tris-buffered saline with Tween-20 (TBS-T) at room temperature and incubated overnight at 4˚C with primary antibodies (rabbit anti-rat NF-κB/p65 antibody diluted at 1:1,000). Following washing the membrane three times in 0.05% Tween-20/PBS for 60 min, it was incubated in HRP-anti rabbit IgG (diluted 1:10,000) secondary antibody for 1 h at room temperature and then washed in 0.05% Tween-20/PBS again. Enhanced chemiluminescence reagent was added and incubated with the membrane for one minute before it was exposed to an X-ray film, developed and fixed. Using GAPDH as the internal control, the relative ratios in all groups were measured using National Institutes of Health Image software (NIH, Bethesda, MD, USA; version 1.6).

Statistical analysis. SPSS 17.0 statistical software (SPSS, Inc., Chicago, IL, USA) was used for analysis. Statistical data are presented as the mean ± standard deviation. The results were assessed by analysis of variance between the groups for comparison among groups. A 95% confidence level was used for statistical significance. P<0.05 was considered to indicate a statistically significant difference.

Table I. Biochemical test results among SOR, UUO and UTI groups (mean ± standard deviation).

Group	Sample size (n)	BUN (mmol l^{-1})	Scr (μmol l^{-1})
SOR	8	5.12±0.65	40.30±5.54
UUO	8	6.18±1.15	42.35±5.19
UUO+UTI	8	6.05±1.74	42.10±5.37

[a]P>0.05, [b]P>0.05, compared with the SOR group; [c]P>0.05, [d]P>0.05, compared with the UUO group. SOR, sham operation; UUO, unilateral ureteral obstruction; UTI, ulinastatin; BUN, blood urea nitrogen; Scr, serum creatinine.

Results

UUO and UTI do not affect rat renal function. There was no statistically significant difference (P>0.05) between the levels of blood urea nitrogen (BUN) and serum creatinine (Scr) among the rats in the different groups (Table I).

Ulinastatin improves renal interstitial pathological injury in UUO. No apparent interstitial damage was observed in the SOR group at either observation point. Interstitial inflammatory cell infiltration, partial renal tubular expansion, loose arrangement, swelling and vacuolar degeneration were observed in the epithelial cells seven days following UUO; at 14 days after the surgery, the above changes became significant. In addition, partial epithelial cell exfoliation and partial renal tubular atrophy were observed at this time-point. As compared with the UUO group, the UTI group exhibited an attenuated inflammatory cellular infiltration and markedly reduced tubular expansion and atrophy at the two observation points (Fig. 1A). The renal interstitial injury score was significantly higher in each UUO group as compared with that in the SOR groups (P<0.01). The score was significantly lower in the UTI group than that in the UUO group at the respective observation point (P<0.05), although it remained higher than that of the SOR group (P<0.01; Fig. 1B).

There was no evidence of interstitial fibrosis in the rats' kidneys following SOR as determined by Masson's trichrome staining for the degree of fibrosis. The interstitial fibrosis area was significantly increased in each UUO group, particularly on day 14. The administration of UTI markedly decreased the area of interstitial fibrosis in the UUO group (Fig. 1A). The percentage of renal interstitial fibrosis area was significantly higher in every UUO group, as compared with the SOR groups (P<0.01). It was significantly lower in the UTI group than that in the UUO group at the respective observation point (P<0.05), although it remained higher than that in the SOR group (P<0.01; Fig. 1C). The renal total soluble collagen (I-IV), which was measured with the Sircol collagen assay kit, in the UTI group was significantly lower than that in the UUO group (P<0.05; Fig. 1D).

Ulinastatin reduces the expression of CD68+ macrophages, TNF-α, IL-1β and NF-κB in the obstructed rat kidney. The present study then examined whether UTI treatment inhibited

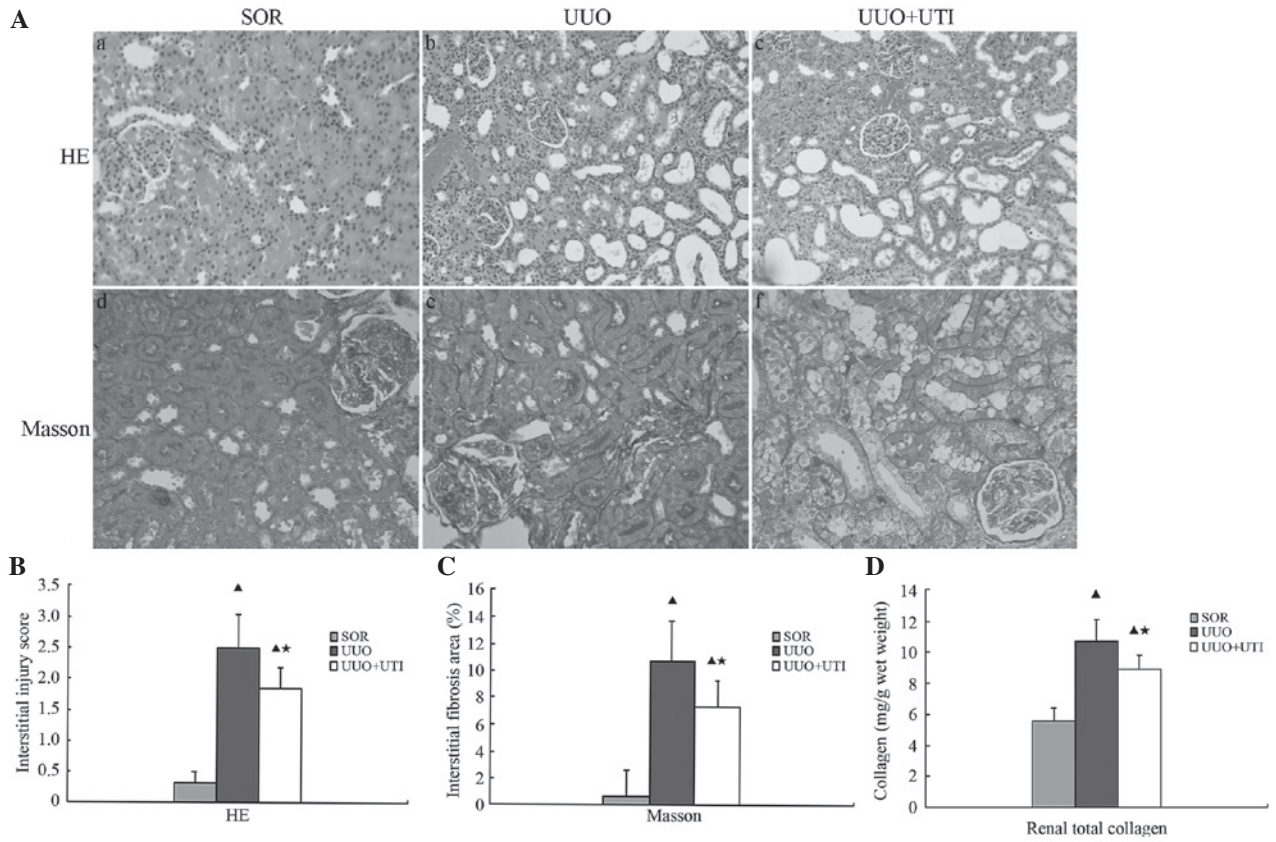

Figure 1. UTI attenuates tubulointerstitial injury and fibrosis in the obstructed kidneys following UUO. (A) H&E and Masson staining in the SOR, UUO and UUO+UTI groups (magnification, x200). (B) Histogram of the semiquantitative analysis result of tubulointerstitial injury in the kidneys. (C) Histogram of the semiquantitative analysis result of tubulointerstitial fibrosis area in the kidneys. (D) Histogram of the analysis results of renal cortical total soluble collagen using the Sircol Collagen Assay. All data are presented as the mean ± standard deviation. The number of rats in each group was 8. ▲P<0.01, UUO and UTI vs. SOR; ★P<0.05, UTI vs. UUO. UTI, ulinastatin; UUO, unilateral ureteral obstruction; H&E, hematoxylin and eosin; SOR, sham operation.

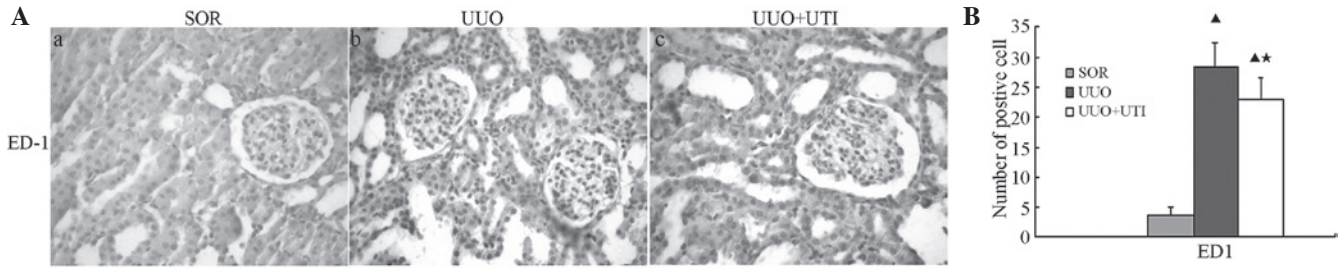

Figure 2. UTI decreases the number of ED-1+ cells in the obstructed kidneys following UUO (IHC magnification, x400). (A) IHC for localization of ED-1+ cells in SOR, UUO and UUO+UTI groups. The histogram (B) is the semiquantitative analysis result of ED-1+ cells in the kidneys. All data are presented as mean ± standard error. The number of rats in each group was 8. ▲P<0.01, UUO and UTI vs. SOR; ★P<0.05, UTI vs. UUO. UTI, ulinastatin; UUO, unilateral ureteral obstruction; IHC, immunohistochemistry; SOR, sham operation.

renal interstitial inflammation. For this, the expression of indicative pro-inflammatory and cytotoxic mediators, namely CD68+ macrophages, IL-1β and TNF-α, were assessed in the whole kidney by immunohistochemistry. CD68+ macrophages were detected on day seven following UUO in the cortical and medullar areas of the kidney. Treatment with UTI led to a clear reduction in the interstitial monocyte/macrophage levels (Fig. 2). In the UUO group, the tissue expression levels of IL-1β and TNF-α were significantly higher than those in the SOR group. Of note, the levels of these mediators in the UUO rats treated with UTI were significantly lower than those in the obstructed kidneys (Fig. 3). Semi-quantitative analyses using immunohistochemistry demonstrated significantly enhanced IL-1β and TNF-α expression in the UUO group compared with the SOR group (P<0.05). In comparison with the UUO group, the UTI group exhibited significantly reduced IL-1β and TNF-α expression (P<0.05; Fig. 3B).

NF-κβ is a ubiquitous transcription factor involved in the upregulation of numerous pro-inflammatory genes (15). NF-κβ binding sites in the promoter region have been demonstrated in a variety of inducible cell adhesion molecules and cytokines, including TNF (15). At least five genes belong to the NF-κβ family, but the most common dimers are composed of the Rel A (p65) and NF-κβ1 (p50) or NF-κβ2 (p52) subunits (15).

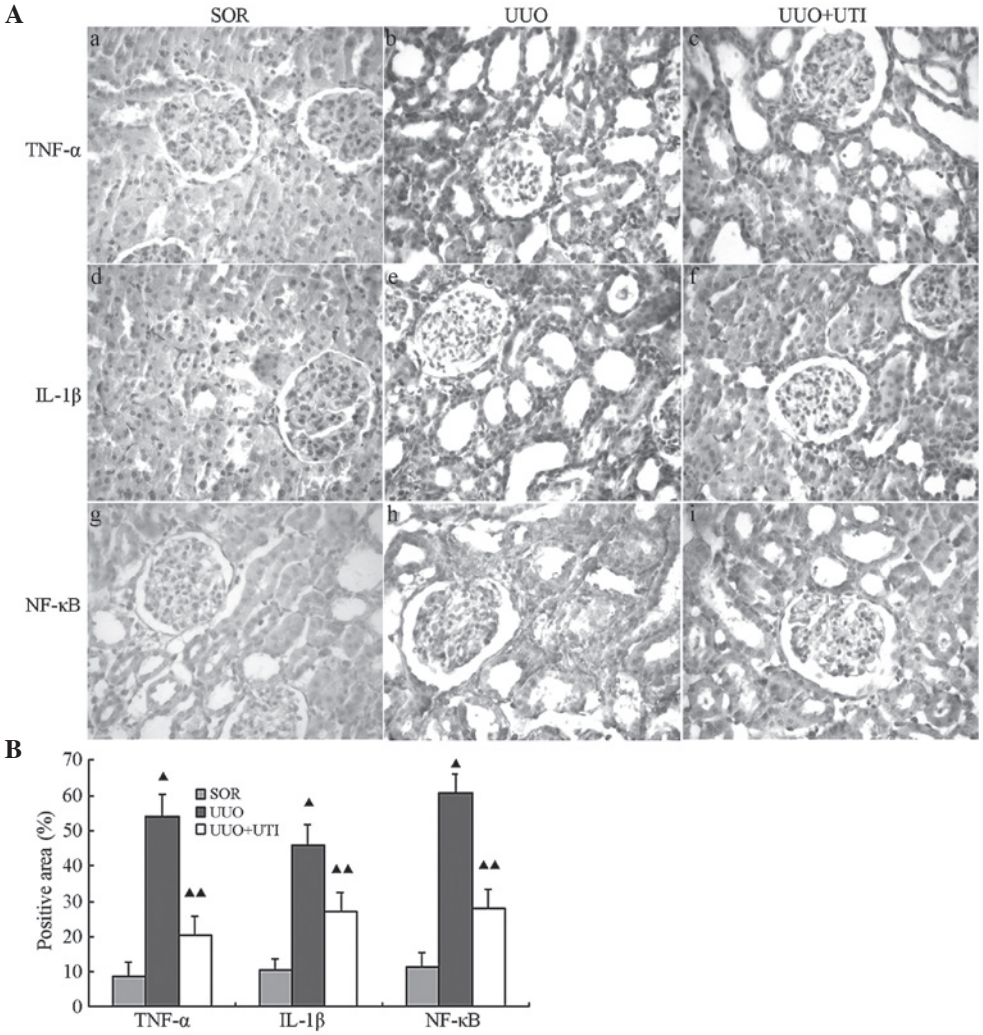

Figure 3. UTI suppresses TNF-α, IL-1β and NF-κB expression in the UUO model at day seven. (A) Immunohistochemical detection of TNF-α, IL-1β and NF-κB in kidney tissue following SOR, UUO, UUO+UTI (magnification, x400). (B) Histogram of the semiquantitative analysis results of TNF-α, IL-1β, NF-κB deposition in the kidneys. All data are represented as the mean ± standard deviation. The number of rats in each group was 8. ▲P<0.01, UUO and UTI vs. SOR and for UTI vs UUO. UTI, ulinastatin; TNF-α, tumor necrosis factor-α; IL-1β interleukin 1β; NF-κB, nuclear factor-κB; UUO, unilateral ureteral obstruction; IHC, immunohistochemistry; SOR, sham operation.

In the majority of cell types, NF-κβ dimers are sequestered in an inactive cytoplasmic complex by binding to its inhibitory subunit, inhibitor-κB (I-κB). Upon stimulation, I-κB undergoes phosphorylation, followed by ubiquitination and rapid degradation through a proteasome-dependent pathway (16). This pathway allows translocation of free active NF-κβ complexes (including p65 and p50 subunits) into the nucleus, where they bind to specific DNA motifs in the promoter/enhancer regions of target genes and activate transcription (16).

NF-κB expression is located in the cell nucleus. The SOR group showed low amounts of NF-κB protein expression in renal glomerular endothelial and tubular epithelial cell nuclei. However, the UUO group exhibited a highly positive expression for NF-κB, while the expression following UTI treatment was notably lower (Fig. 3Ag-l). Semi-quantitative analyses using immunohistochemistry demonstrated significantly enhanced NF-κB expression in the UUO group, as compared with the SOR group (P<0.05), whereas in comparison with the UUO group, the UTI group showed significantly reduced NF-κB expression (P<0.05; Fig. 3B).

Ulinastatin reduces expression of TGF-β1 and Col-I in obstructed kidneys. TGF-β1 is an important profibrotic growth factor in the progression of interstitial fibrosis. The activation of TGF-β1 has an important role in epithelial mesenchymal transition (EMT) and renal fibrosis. The protein deposition of active TGF-β1 was measured by immunohistochemistry. As revealed in Fig. 4A, the SOR group showed low amounts of TGF-β1 protein expression in the tubular epithelial cell cytoplasm of rat nephridial tissues and almost no expression in the renal corpuscles. The post-operative UUO group exhibited TGF-β1 expression distributed throughout the renal tubule with cortical and medullar damages, and the expression levels were significantly augmented with increments in the tubular damage range and deterioration of interstitial fibrosis. Following UTI treatment, the expression of TGF-β1 and the degree of interstitial injury were markedly reduced. At each observation point, the UUO group showed notably higher TGF-β1 expression levels, as compared with the SOR group (P<0.01). By contrast, the UTI treatment group exhibited a notably lower TGF-β1 expression than the UUO group (P<0.01; Fig. 4B).

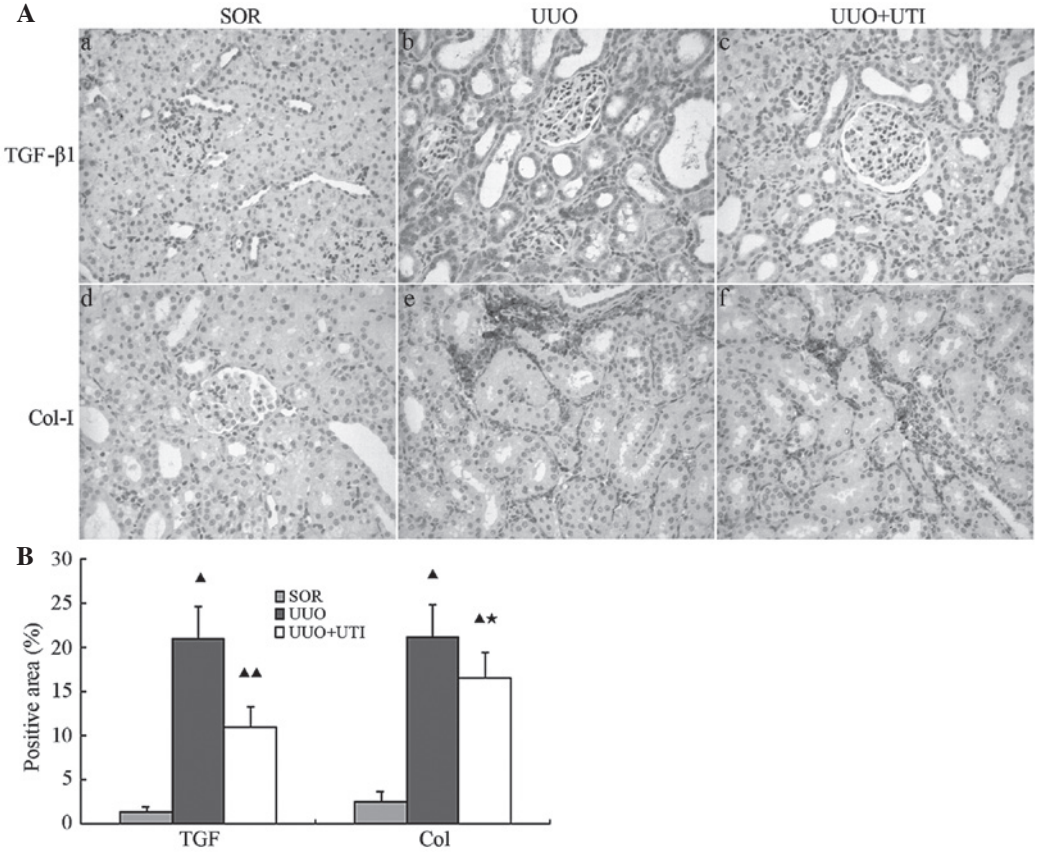

Figure 4. UTI suppresses TGF-β1 and Col-I expression in the UUO model. (A) Immunohistochemical assessment of TGF-β1 and Col-I in kidneys following SOR, UUO and UUO+UTI (magnification, x200). (B) Histogram of the semiquantitative analysis results of TGF-β1 and Col-I deposition in the kidneys. All data were presented as the mean ± standard deviation. The number of rats in each group is 8. ▲P<0.01, UUO & UTI vs. SOR; ★P<0.05, UTI vs. UUO; ▲P<0.01, UTI vs. UUO. UTI, ulinastatin; TGF-β1, transforming growth factor-β1; Col-I, type I collagen; UUO, unilateral ureteral obstruction; IHC, immunohistochemistry; SOR, sham operation.

Col-I is one of the extracellular matrix (ECM) proteins and is most commonly expressed in the renal arterioles. However, it is rarely present in the renal interstitium. There was almost no Col-I protein expression in the renal interstitium following SOR, whereas large quantities of interstitial Col-I deposition were observed in the UUO group, the expression of which increased with time. The Col-I levels were higher in the UUO group as compared with those in the SOR group at each observation point. Following UTI treatment, Col-I expression was reduced (Fig. 4Ad-f). Semiquantitative analyses of the immunohistochemistry of Col-I in the obstructed renal tissues revealed significantly higher Col-I expression levels in the UUO group than those in the SOR group (P<0.01). Although the UTI treatment group exhibited notably lower levels of Col-I expression (P<0.01) in comparison with those in the UUO group, they remained higher than those in the SOR group (P<0.01; Fig. 4B).

UTI suppresses oxidative stress in UUO. To confirm whether UTI reduced renal tissue oxidant enzyme levels and lipid peroxidation, MDA levels were measured in the kidney tissue. In the UUO group, the average MDA levels were significantly higher and the SOD levels were lower than those in the SOR group (Table II). Furthermore, the average levels of MDA in the UTI-treated obstructed kidneys were significantly lower than those in the obstructed kidneys, whereas SOD levels were significantly higher. This indicated that UTI treatment was partially but significantly capable of preventing oxidative stress.

Ulinastatin reduces expression of NF-κB by western blot analysis. To elucidate whether UTI is able to suppress NF-κB expression in the obstructed kidney, NF-κB expression was examined by western blot analysis. At each observation point, the SOR group of the rats exhibited lower NF-κB protein levels. The UUO group of rats exhibited significantly elevated NF-κB expression, while the expression levels of the above proteins following UTI treatment were notably lower (Fig. 5A).

Semiquantitative analyses of the western blots of protein from the obstructed nephridial tissues of rats in the three groups revealed that NF-κB expression levels were significantly higher in the kidneys of rats in the UUO group than those in the SOR group at each observation point (P<0.05). In comparison with the UUO group, the UTI group showed notably lower NF-κB protein expression levels (P<0.05; day seven; Fig. 5B).

Discussion

Among all causes of kidney damage, the lack of blood or oxygen in renal tissue and subsequent activation of kidney intrinsic cells releases a great amount of chemokines. Inflammatory cell infiltration in the circulation is localized to the damaged tissue, inducing a renal inflammatory response,

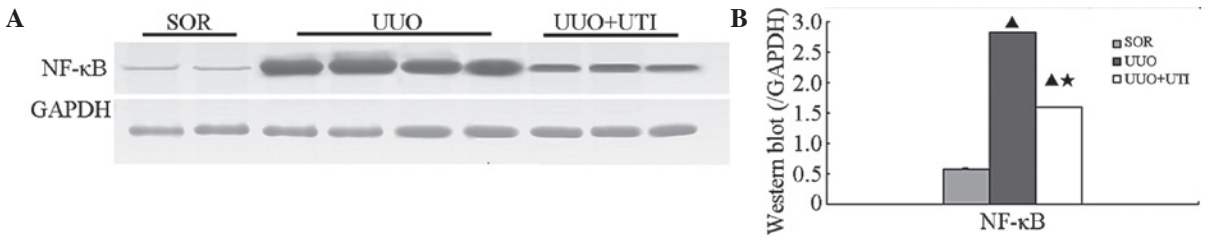

Figure 5. Western blot analysis demonstrating the effects of UTI on renal NF-κB protein expression in an UUO model. (A) Expression levels of NF-κB were quantified by densitometry and normalized with GAPDH. (B) Histogram of the analysis results of NF-κB protein expression in the kidneys. All data are presented as the mean ± standard deviation. The number of rats in each group is 8. ▲P<0.01, UUO & UTI vs. SOR; *P<0.05, UTI vs. UUO. UTI, ulinastatin; NF-κB, nuclear factor-κB; UUO, unilateral ureteral obstruction; SOR, sham operation.

Table II. Results of MDA and SOD among SOR, UUO and UTI groups (mean ± standard deviation).

Group	Sample size (n)	MDA (mmol/mg)	SOD (U/mg)
SOR	8	2.89±0.33	278.00±25.31
UUO	8	5.59±1.25[a]	161.70±21.77[a]
UUO+UTI	8	3.83±0.97[a,b]	207.20±44.16[a,b]

[a]P<0.01, UUO & UTI vs. SOR; [b]P<0.05, UTI vs. UUO. MDA, malondialdehyde; SOD, superoxide dismutase; SOR, sham operation; UUO, unilateral ureteral obstruction; UTI, ulinastatin.

which in turn leads to the generation and secretion of inflammatory mediators and profibrotic cytokines. Inflammatory mediators mediate the cascade amplification and sustenance of inflammatory responses, and cause apoptosis of renal tubular cells and kidney fibrosis. As such, the renal inflammatory response is one of the actuating links in the pathogenesis of kidney fibrosis (1,2). Numerous studies have demonstrated that inhibition of UUO renal interstitial inflammatory cell infiltration (12), as well as a reduction in the expression of certain cytokines, including TNF-α, IL-1, NF-κB and TGF-β1 (5), alleviate apoptosis of renal tubular epithelial cells and the subsequent development of interstitial fibrosis.

UTI has inhibitory effects on a number of enzymes, including serine protease-like trypsin, α-chymotrypsin, neutrophil elastase, hyaluronidase, sulfhydral enzymes and plasmin (6). Its functions include eradication of oxygen free radicals, inhibition of inflammatory mediator production, neutrophil activation and microvascular permeability (7). It has numerous clinical applications ranging from the treatment of acute pancreatitis, shock, ischemia reperfusion injury, multiple organ dysfunction (MODS) and acute respiratory distress syndrome (ARDS) to administration post organ transplantation and extra-corporal circulation operation, to inhibit inflammation and apoptosis, protect cells, and improve circulation and blood coagulation disturbance. In addition, UTI has immunomodulatory effects. It has also been identified that by attenuating excessive inflammatory reactions, ulinastatin protects lipopolysaccharide (LPS) and severe burn-induced lung injury (7,17). In another study, it was also able inhibit NF-κB activity and alleviate live ischemia reperfusion injury (18). Furthermore, it was demonstrated to decrease the expression of TNF-α, IL-6, caspase-3 and thus reduced lipopolysaccharide/D-gal-induced acute liver damage (13) and myocardial ischemia reperfusion injury (14). A previous study by our group demonstrated that UTI improves renal fibrosis (19); however, the mechanism underlying this effect remained elusive.

Animal studies have indicated that UTI significantly increases superoxide dismutase (SOD) levels in the kidney tissue of rats with kidney damage and lowers its serum creatinine (Scr) and BUN (9) levels. It may also reduce renal tubular epithelial cell necrosis, inflammatory cell filtration and protect kidney function in rats with zymosan-induced MODS (10). Biochemical examination in the present study revealed no significant impact of UUO or UTI on the kidney functions of the experimental rats. HE and Masson staining results indicated that following UTI treatment, UUO-inflicted rats had a significantly lower renal interstitial injury index with significantly reduced amounts of renal interstitial inflammation infiltration and area of fibrosis. This indicated that UTI had protective effects on injuries in the rat obstructed kidney tissues.

As one of the actuating links of obstructed kidney injury, the inflammatory response involves the infiltration of inflammatory corpuscles (primarily monokaryons/macrophages) (20) and the release of inflammatory mediators. Active radical oxygen species are important inflammatory mediators. MDA is a product of the ROS lipid peroxidation reaction, whose amount directly reflects that of ROS. The main function of SOD is anti-oxidative activity. It indirectly reflects the organism's ability to eradicate ROS. The present study revealed that UTI reduces the amount of renal interstitial infiltration of CD68+ macrophages in rats with UUO, while simultaneously lowering the expression of the primary inflammatory factors IL-1β and TNF-α (P<0.05). With UTI intervention, the MDA content was reduced and the SOD content increased again (P<0.05) in the renal tissues, as compared with that in the UUO group. These data demonstrated that UTI inhibited the generation of ROS and hence it is concluded that ulinastatin is able to significantly inhibit inflammatory responses in obstructed rat kidneys.

NF-κB, as a general nuclear transcription factor, is a homo- or hetro-dimer consisting of two glutenin subunits, p65 and p50, that resides in the center of inflammatory response. NF-κB controls the gene expression of numerous inflammation-associated substances, including downstream inflammatory cytokines (IL-1α, IL-1β, IL-2, IL-6, IL-8, TNF-α, nitric oxide), inflammatory chemokines (monocyte chemotactic protein-1 and regulated on activation, normal T cell expressed and secreted),

cell adhesion molecules (intercellular adhesion molecule 1, vascular cell adhesion molecule 1, E-selectin), a number of inducible enzymes (cyclooxygenase-2, nitric oxide synthase), growth factors and certain acute reactive proteins (21,22). At the same time, it is regulated by the positive feedback of certain cytokines (IL-1β, TNF-α), ROS as well as other molecules, such as angiotensin II (16). As such, NF-κB and its regulatory molecules together form the NF-κB signaling pathway.

NF-κB is activated at an early stage of UUO and participates in the pathological formation and progression of kidney obstruction (22). By inhibiting NF-κB activity, mononuclear cell infiltration and inflammatory gene over-expression are reduced, and concurrently, apoptosis and interstitial fibrosis are attenuated (23). In the present study, immunohistochemistry and western blot analysis confirmed significantly elevated levels of NF-κB deposition and protein expression in the obstructed kidney tissues post UUO operation, as well as reduced NF-κB expression with UTI intervention. It is therefore concluded that UTI reduces the UUO-induced kidney interstitial inflammatory response by inhibiting the NF-κB signaling pathway. This also proves the existence of a positive feedback regulation of IL-1β, TNF-α, ROS and NF-κB.

The ECM includes numerous components, including collagen I, III, fibronectin and others. Under normal physiological conditions, the generation and degradation of the ECM is balanced. Excessive deposition of the ECM is one of the main pathological changes in renal interstitial fibrosis. Currently, TGF-β1 is recognized as one of the key factors in the formation and progression of interstitial fibrosis. It may induce renal tubular epithelial cell apoptosis, transdifferentiation of tubular epithelial cells into myofibroblasts (MFB), as well as excessive concentration of the ECM proteins, and eventually lead to interstitial fibrosis (24-26). The present study observed a significant increase in TGF-β1, Col-I and α-SMA expression levels in nephridial tissue, as well as interstitial fibrosis in the UUO group of rats. In comparison, the UTI treatment group showed a significantly reduced TGF-β1 and ECM concentration and ameliorated renal interstitial fibrosis.

In conclusion, UTI is able to inhibit the inflammatory response and fibrosis in rats with UUO and appears to have certain protective effects on the injury of the obstruction-side kidney. As an endogenous protease inhibitor, UTI has complex biological actions and thus, further studies are required to elucidate the detailed mechanism underlying its renal protective effects.

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