ORIGINAL RESEARCH

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Rapamycin reduces renal hypoxia, interstitial inflammation and fibrosis in a rat model of unilateral ureteral obstruction

Abstract

Purpose: The purpose of this study was to explore effects of rapamycin on renal hypoxia, interstitial inflammation and fibrosis, and the expression of transforming growth factor β 1 (TGF- β 1), vascular endothelial growth factor (VEGF), Flk-1 and Flt-1 in a rat model of unilateral ureteral obstruction (UUO).

Methods: Male Sprague-Dawley rats (n=36) were randomly divided into three groups (n=12 per group): sham surgery, UUO and UUO plus rapamycin (0.2 mg/kg/d). Serum creatinine (Scr), blood urea nitrogen, uric acid, triglycerides, cholesterol and 24-h urine protein levels were measured. The extent of interstitial fibrosis was determined by Masson's trichrome staining. ED-1 positive macrophages, type III collagen, hypoxia, TGF-1, VEGF, Flk-1, and Flt-1 mRNA and protein expressions were detected using immunohistochemical staining, real-time PCR and Western blot.

Results: UUO induced an elevation in Scr, renal hypoxia, inflammation, interstitial fibrosis, TGF- β 1, VEGF, Flk-1, and Flt-1 mRNA and protein expression levels ($P < 0.05$). Rapamycin alleviated the UUO-induced renal hypoxia, infiltration of inflammatory cells and tubulointerstitial fibrosis (at days 3 and 7). Rapamycin also down-regulated the UUO-induced elevated expression levels of TGF- β 1 and Flt-1 mRNA and protein ($P < 0.05$). Rapamycin decreased VEGF mRNA and protein expression at day 3, and increased Flk-1 mRNA and protein expression at day 7, compared with the UUO group ($P < 0.05$).

Conclusion: Rapamycin shows beneficial effects by reducing UUO-induced renal hypoxia, inflammation and tubulointerstitial fibrosis.

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Currently chronic kidney disease (CKD) is a public health concern worldwide; however, the mechanism of kidney disease progression is still unclear. A hypothesis has been proposed that post-glomerular capillary injury or reduction in number of capillaries leads to chronic hypoxia of the tubular interstitium, which contributes to kidney disease progression and tissue fibrosis [1,2]. A number of growth factors can be affected by hypoxia, and some of those growth factors, including transforming growth factor $\beta 1$ (TGF- $\beta 1$), play a role in interstitial metabolism [1,3-5]. During periods of hypoxia, expression of vascular endothelial growth factor (VEGF) is markedly up-regulated through a hypoxia-inducible factor (HIF)-1 dependent mechanism [6]. VEGF has two transmembrane tyrosine kinase receptors: Flk-1 (VEGF-R2) and Flt-1 (VEGF-R1) [7]. It has been found that both VEGF and TGF- $\beta 1$ are involved in the pathogenesis of renal dysfunction and in the pathway mediating the progression to end-stage renal failure [8].

Rapamycin is an immunosuppressive drug and its target, mammalian target of rapamycin (mTOR), is a downstream effector of the phosphatidylinositol 3-kinase (PI3K) and Akt signaling pathways which regulate the protein translation process, interact with growth factors and their receptors and participate in cell cycle progression and membrane transport [9]. Rapamycin is an inhibitor of mTOR and it was found that it inhibited platelet-derived growth factor-induced proliferation and collagen synthesis in rat mesangial cells and alleviated tubulointerstitial inflammation and fibrosis in animal models [10-13]. It is known that mTOR is implicated in the hypoxia-induced HIF-1 signaling pathway and a recent study shows that rapamycin can reduce stability and transforming activity of HIF-1 α [14]. Therefore, the effects of rapamycin on renal hypoxia and chronic progressive nephropathy require further attention. The purpose of this study was to examine the effects of rapamycin on renal hypoxia, interstitial inflammation and fibrosis, and expressions of TGF- $\beta 1$, VEGF, Flk-1, and Flt-1 in a rat model of unilateral ureteral obstruction (UUO).

Methods

Animal preparation and experimental grouping

The ethics committee of Fudan University approved this study. Male Sprague-Dawley rats (n=36) weighing 170-206 g were purchased from the Experimental Animal Center of the School of Medicine, Fudan University. The animals were kept in a clean environment at $20 \pm 2^\circ\text{C}$ and provided with access to adequate food and water. The rats were randomly divided into three groups (n=12 in each group): sham operation, UUO and UUO treated with an intragastric gavage of rapamycin (0.2

mg/kg/d). The original concentration of rapamycin was 1 mg/ml, which was diluted 1:10 with sterile tap water (Wyeth-Ayers Pharmaceuticals, Philadelphia, PA, USA). Rapamycin was given every morning starting from one day prior to UUO with the last dose given on the day the rats were sacrificed. The procedures of UUO are as follows: after anesthetizing with 5% intraperitoneal sodium pentobarbitone (50 mg/kg), rats were fixed in a supine position. Routine abdominal hair removal and skin disinfection were performed. An incision was cut on the left side, 1 cm away from the midline, and the abdominal cavity was opened to expose the left ureter. The upper one third of the ureter was dissected and then ligated using a No. 4 silk suture at two places, 0.5 cm apart. The ureter was then cut between the ligations to prevent retrograde infection. The surgery was then complete and the abdominal cavity was closed. The sham group only received laparotomy and dissection of the upper one third of the left ureter. Half of the animals in each group were sacrificed at day 3 after surgery and the other half were sacrificed at day 7 after surgery.

Sample collection

The 24-hour urine samples of rats were collected one day prior to sacrifice using a metabolic cage to measure the 24-hour urine protein. Blood pressure was measured before the rats were anesthetized with intraperitoneal sodium pentobarbitone (50mg/kg). One and half hours prior to laparotomy, Hypoxyprobe-1 (Chemicon International, Dallas, TX, USA) 60 mg/kg was injected through the penile vein. Before nephrectomy was performed, a 3 mL venous blood sample was collected via the abdominal vein and centrifuged at 2000 rpm for 15 minutes. The serum was collected for measurement of serum creatinine (Scr), blood urea nitrogen (BUN), uric acid (UA), triglycerides and total cholesterol and measured with an ADVIA 1650 type automatic biochemical analyzer (Bayer, Tarrytown, NY, USA). Abdominal aortic cannulation was carried out and saline pre-chilled at 4°C was infused until the kidney turned a pale color. The renal pedicle was cut off and the kidney was removed. The capsule was rapidly removed while keeping the kidney on ice, and 2-3 mm thick transverse sections were made to prepare paraffin and frozen sections and to extract total RNA and tissue protein.

Hematoxylin and Eosin (HE) staining and Masson's trichrome staining

HE staining was used to determine the level of inflammation and Masson's trichrome staining was used to determine the extent of collagen deposition/fibrosis. Serial cross sections with

a thickness of 3 μm were prepared for staining. The visual fields (x200) were photographed and divided into 100 equal grids. The number of the fiber tissues with blue staining in each grid was calculated and scored 0 points for no interstitial fibrosis, 1 point for fibrosis extent $<1/3$, 2 points for fibrosis extent between $1/3$ and $2/3$, and 3 points for fibrosis extent $>2/3$. The score of interstitial fibrosis of each visual field was the total score of the 100 grids. For the cortex and the medulla of each specimen, five different visual fields were randomly-selected for examination and scoring.

Immunohistochemical staining

Formalin-fixed and paraffin-embedded tissue samples were used for ED-1, Hypoxyprobe-1, TGF- β 1, VEGF, Flk-1 (VEGF-R2), and Flt-1 (VEGF-R1) immunohistochemical staining and 4-micron sections were prepared. Immunohistochemical staining was achieved using primary antibodies including ED-1 (mouse monoclonal IgG, Serotec, Raleigh, NC, USA), Hypoxyprobe-1 (mouse anti-Hypoxyprobe-1, Chemicon), TGF- β 1 (rabbit polyclonal IgG, Santa Cruz, Dallas, TX, USA), VEGF (mouse monoclonal IgG, Santa Cruz), Flk-1 (VEGF-R2) (mouse monoclonal IgG, Santa Cruz), and Flt-1 (VEGF-R1) (rabbit polyclonal IgG, Santa Cruz) and secondary antibodies including Vectastain ABC kit, horse anti-mouse (purchased from Beijing Zhongshan Biological Technology, Beijing, China) and PowerVision two-step goat anti-rabbit IgG (Beijing Zhongshan). Because ED-1 is a marker of macrophages, the number of ED-1- positive cells reflects the degree of tissue inflammation. Five different non-overlapping high-power fields were selected from each of the cortex and medulla, and the number of ED-1 positive cells in those fields was counted. Pictures of Hypoxyprobe-1 immunohistochemical staining (x200) were taken and for each section, five visual fields were selected from each of the cortex and medulla. For each positive target, the optical density was measured and the percentage of positive area was calculated. The amount of target antigen was calculated as the optical density of the positive target multiplied by the percentage of positive area.

Real-time polymerase chain reaction (RT-PCR)

Total RNA was extracted using TRIzol (Shanghai Sangon, Shanghai, China). A sample of 1 μg total RNA was incubated at 42°C for 30 min. The samples were heated at 95°C for 5 min to inactivate the DNase. The first strand of cDNA was obtained by reversely transcribing RNA samples in a 20 μl reaction solution. The reaction system included 2 μl of template cDNA, 3 μl of MgCl_2 (25 mM), 5 μl of 10xR buffer, 1 μl of

dNTP (10mM), 1 μl of primers 1 and 2, 0.25 μl of TaqDNA synthase and nuclease-free water added to bring the final volume to 50 μl . PCR thermal cycler (PTC-100, MJ Research, Paris, France) was used for the PCR reaction. Primer sequences and reaction conditions are presented in Table 1. PCR products were separated by 1.2% agarose gel electrophoresis and stained with ethidium bromide staining. The band density of the PCR products on the agarose gel indicates the expression levels of target mRNA, which were calculated as a ratio with respect to the GAPDH band. Image acquisition under ultraviolet light and image analysis used a biological electrophoresis image analysis system (FR-200, Furi Technology, Shanghai, China).

Western blot

Tissue samples were homogenized on ice to prepare whole cell lysates and the protein concentration was determined using Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA). An equal quantity of protein from each sample was loaded on the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto a nitrocellulose membrane (Millipore, Bedford, MA, USA). The sample was blocked with 5% milk and incubated with one of the following primary antibodies: actin antibody (mouse monoclonal IgG, Santa Cruz), TGF- β 1 antibody (rabbit polyclonal IgG, Santa Cruz), VEGF antibody (mouse monoclonal IgG, Santa Cruz), Flk-1 antibody (VEGF-R2) (mouse monoclonal IgG, Santa Cruz), or Flt-1 antibody (VEGF-R1) (rabbit polyclonal IgG, Santa Cruz) at an optimized concentration (1:1000). The horseradish peroxidase-conjugated secondary antibodies (goat anti-mouse IgG (Shanghai Pufei Biotechnology, Shanghai, China) and goat anti-rabbit IgG (Shanghai Pufei Biotechnology) were then added into samples. The samples were washed three times between each addition of antibody. Enhanced chemiluminescent (ECL) substrates (Shanghai Pufei Biotechnology) were added and the images were developed on the X-ray Film. Image acquisition and analysis under white light used a biological electrophoresis image analysis system (FR-200, Furi Technology) and the actin band was used as a reference.

Statistical analysis

Data are presented as mean $\pm$ standard deviation (SD). Continuous variables were compared by one-way analysis of variance (ANOVA). When statistical significance among groups was found, post hoc multiple comparisons of means were performed. The Tukey's test method for post hoc pairwise comparisons was applied when the equal variance assumption

TABLE 1. Levels of serum biochemical indexes, 24-hour urine protein, and blood pressure by group

	Sham (n=6)	UO (n=6)	Rapamycin (n=6)	P-value
Day 3				
BUN (mmol/L)	4.33±0.57	5.90±1.48	7.15±1.47 [†]	0.032*
UA (μmol/L)	86.92±10.17	157.15±69.29	127.20±29.11	0.129
Chol (mmol/L)	2.04±0.23	2.09±0.24	2.60±0.35 [†]	0.036*
TG (mmol/L)	1.38±0.40	0.99±0.44	0.92±0.31	0.251
UP (mg/24 h)	6.33±2.28	6.33±2.28	5.08±2.06	0.664
BP (mmHg)	113.75±4.79	113.75±4.79	108.75±8.54	0.462
Day 7				
Scr (μmol/L)	40.65±2.38	56.68±1.98 [†]	55.82±4.01 [†]	< 0.001*
BUN (mmol/L)	4.33±0.57	7.50±0.42 [†]	6.93±1.89 [†]	0.009*
UA (μmol/L)	86.92±10.17	98.85±12.67	83.74±9.32	0.170
Chol (mmol/L)	2.04±0.23	2.60±0.20 [†]	2.33±0.25	0.021*
TG (mmol/L)	1.38±0.40	0.70±0.10 [†]	0.96±0.33	0.033*
UP (mg/24 h)	6.33±2.28	5.37±2.06	5.98±1.32	0.780
BP (mmHg)	113.75±4.79	113.75±4.79	113.75±4.79	1.000

Scr = serum creatinine, BUN = blood urea nitrogen, UA = uric acid, Chol = cholesterol, TG = triglyceride, UP = 24-hour urine protein, BP = blood pressure.

*Indicates a significant difference among the three groups, $P < 0.05$

[†]Indicates a significant difference between the treatment group and sham group, $P < 0.05$

Data were presented as mean ± standard deviation and tested by ANOVA followed by Tukey's test for post hoc pair-wise comparison.

seemed valid. Otherwise, Dunnett's T3 test was carried out. All statistical analyses were two-sided, and the significance level α was set at 0.05. Statistical analyses were performed using SPSS 15.0 statistics software (SPSS, Chicago, IL, USA).

Results

Serum biochemical indices, 24-hour urine protein, and blood pressure

The serum creatinine, blood urea nitrogen (BUN), uric acid, cholesterol and triglyceride levels, 24-hour urine protein, and blood pressure of different groups are compared in Table 1. Compared with the sham group, the serum creatinine levels were significantly increased in the UO group at day 3 and day 7 and the BUN levels were significantly increased in the UO group at day 7 ($P < 0.001$). Administration of rapamycin at 0.2 mg/kg/d significantly increased the serum creatinine

levels and blood urea nitrogen at day 3 and day 7 compared with the sham group ($P < 0.05$).

Hypoxia of the kidney cortex and medulla

Fig. 1A and 1B shows the quantitative results of Hypoxyprobe-1 immunohistochemical staining. When compared with the sham operation, UO caused an increase in hypoxia in the kidney cortex and medulla at day 3 and day 7 ($P < 0.05$). Administration of rapamycin significantly alleviated the hypoxia in both the cortex and medulla following UO at day 3 and day 7 ($P < 0.05$; however, the level of hypoxia in the rapamycin group did not return to the level seen in the sham group ($P < 0.05$).

FIGURE 1. Hypoxia of the kidney cortex and medulla by group as indicated by positive Hypoxypoint-1 immunohistochemical staining at (A) day 3 and (B) day 7 (n=6 per group). One-way ANOVA followed by Tukey's test for post hoc tests were performed. *Indicates a significant difference between the treatment and UUO groups, $P < 0.05$. †Indicates a significant difference between the treatment group and sham group, $P < 0.05$. (C) Hypoxypoint-1 immunohistochemical staining pictures at day 3 and day 7 ($\times 40$).

FIGURE 2. Number of infiltrated ED-1-positive cells among the three groups at (A) day 3 and (B) day 7 ($n=6$ per group). One-way analysis of variance followed by either Tukey's test or Dunnett's T3 test for post-hoc test was performed. *Indicates a significant difference between the treatment and UUO groups, $P < 0.05$. †Indicates a significant difference between the treatment and sham groups, $P < 0.05$. (C) Immunohistochemical staining pictures ($\times 400$). Yellow arrows indicate the ED-1-positive cell.

Kidney cortex and medulla inflammation and interstitial fibrosis

The sham group had normal HE staining results at both day 3 and day 7 (data not shown). Fig. 1C shows the Hypoxyprobe-1 immunohistochemical staining results of UUO group and rapamycin treated group. In the UUO group, the glomeruli were normal, but diffuse dilation of the tubular lumens was observed at day 3. The tubular epithelial cells had incomplete brush borders and some of them ($< 30\%$) were necrotic. Scattered inflammatory cell infiltration was observed in the interstitium. The glomeruli in the UUO group remained normal at day 7,

but the tubular lumens were diffusely dilated, and the brush border of the tubular epithelial cells was incomplete. More than half of the tubular epithelial cells were necrotic. Vacuolar degeneration was observed, with aggravation of interstitial inflammatory cell infiltration including more severe inflammatory cell infiltration in the medulla.

In the rapamycin group, the glomeruli remained normal and the tubular lumen was diffusely dilated at day 3. The brush border of the tubular epithelial cells remained more intact and the tubular epithelial cell necrosis was reduced com-

FIGURE 3. The mRNA expression of type III collagen by group at (A) day 3 and (B) day 7 ($n=6$ per group). One-way ANOVA followed by Tukey's test for *post hoc* tests were performed. †Indicates a significant difference between the treatment and sham groups, $P < 0.05$.

pared with the UUO group. Scattered inflammatory cell infiltration was still observed in the interstitium in the rapamycin group. The HE staining results of the rapamycin group at day 7 were not apparently different from that at day 3.

The number of infiltrated ED-1 positive cells in the cortex and medulla was used to assess the degree of kidney cortex and medulla inflammation (Fig. 2). As indicated by the increased number of infiltrated ED-1-positive cells in the kidney cortex and medulla, UUO caused an increase in the inflammatory reaction in the cortex and medulla at day 3 and day 7 compared with the sham group (Fig. 2A and 2B, $P < 0.05$). Administration of rapamycin reduced the inflammatory reaction in the kidney cortex and medulla at day 3 and day 7 as indicated by the reduced number of infiltrated ED-1 positive cells (Fig. 2A and 2B, $P < 0.05$). Furthermore, administration of rapamycin reduced the inflammatory reaction in the kidney cortex and medulla to a level that was not significantly different from that in the sham group at day 7 (Fig. 2B). The mRNA expression levels of type III collagen were used to reflect the degree of interstitial fibrosis. UUO increased the type III collagen mRNA levels in the kidney tissues at day 3 and day 7 compared with the sham group (Fig. 3, $P < 0.05$). Administration of rapamycin failed to significantly reduce the elevated type III collagen mRNA levels induced by UUO at day 3 or day 7 (Fig. 3). The interstitial fibrosis scores were compared among groups (Fig. 4). The interstitial fibrosis scores of the kidney cortex and medulla were elevated in the UUO group compared with the

sham group (Fig. 4A and 4B, $P < 0.05$). The interstitial fibrosis scores were significantly reduced by rapamycin at day 3 and day 7 (Fig. 4A and 4B, $P < 0.05$), but the interstitial fibrosis scores of the rapamycin group were still higher than those of the sham group at day 3 and day 7 (Fig. 4A and 4B, $P < 0.05$).

Expression of TGF- β 1 mRNA and protein

Figure 5 shows the expression of TGF- β 1 mRNA and protein in the three groups. The expression levels of TGF- β 1 mRNA and protein were elevated in the UUO group compared with the sham group at day 3 and day 7 (Fig. 5A and 5B, $P < 0.05$). At day 3 (Fig. 5A), administration of rapamycin lowered the expression of TGF- β 1 mRNA and protein compared with the UUO group ($P < 0.05$). The level of TGF- β 1 mRNA expression in the rapamycin group was reduced to a level that was even lower than that in the sham group ($P < 0.05$), and the level of TGF- β 1 protein expression in the rapamycin group was reduced to a level that was not significantly different from that in the sham group. At day 7 (Fig. 5B), administration of rapamycin lowered the expression of TGF- β 1 mRNA and protein compared with the UUO group ($P < 0.05$), but the lowered levels of TGF- β 1 mRNA and protein expression were still higher than those in the sham group ($P < 0.05$).

Expression of VEGF mRNA and protein

Figure 6 shows that expression of VEGF mRNA and protein were increased in the UUO group compared with the sham group at day 3 and day 7 (Fig. 6A and 6B, $P < 0.05$). At day 3, administration of rapamycin lowered the levels of VEGF mRNA and protein compared with the UUO group ($P < 0.05$), with no significant difference found between the rapamycin and the sham groups (Fig. 6A). At day 7, administration of rapamycin did not change the expression of VEGF mRNA

or protein significantly compared with the UUO group (Fig. 6B).

Expression of Flk-1 and Flt-1 mRNA and protein

The expression levels of Flk-1 and Flt-1 mRNA and protein are shown in Figure 7. Expression of Flk-1 mRNA and protein were elevated in the UUO group compared with the sham group at day 3 (Fig. 7A, $P < 0.05$). At day 7 (Fig. 7B), the Flk-1 mRNA levels in the UUO group returned to a level that was

FIGURE 4. The scores of interstitial fibrosis in the kidney cortex and medulla by group at (A) day 3 and (B) day 7 ($n=6$ per group). One-way analysis of variance followed by Dunnett's T3 test for post-hoc test was performed. *Indicates a significant difference between the treatment and UUO groups, $P < 0.05$. †Indicates a significant difference between the treatment and sham groups, $P < 0.05$. (C) Masson staining pictures ($\times 200$).

FIGURE 5. Expression of TGF- β 1 mRNA and protein by group at (A) day 3 and (B) day 7 ($n=6$ per group). Data were shown as mean $\pm$ standard deviation. One-way ANOVA followed by Tukey's test for *post hoc* tests were performed. *Indicates a significant difference between the treatment and UUO groups, $P < 0.05$. †Indicates a significant difference between the treatment and sham groups, $P < 0.05$. (C) Western blot analyses of TGF- β 1 proteins (1= UUO group; 2= UUO+ rapamycin group). Actin was used as a control.

FIGURE 6. Expression of VEGF mRNA and protein by group at (A) day 3 and (B) day 7 ($n=6$ per group). Data were shown as mean $\pm$ standard deviation. One-way analysis of variance followed by either Tukey's or Dunnett's T3 *posthoc* tests was performed. *Indicates a significant difference between the treatment and UUO groups, $P < 0.05$. †Indicates a significant difference between the treatment and sham groups, $P < 0.05$. (C) Western blot analyses of VEGF proteins (1= UUO group; 2= UUO+ rapamycin group). Actin was used as a control.

FIGURE 7. Expressions of Flk-1 and Flt-1 mRNA and protein by group at (A) day 3 and (B) day 7 ($n=6$ per group). Data were shown as mean $\pm$ standard deviation. One-way ANOVA followed by Tukey's test for *post hoc* test were performed. *Indicates a significant difference between the treatment and UUO groups, $P < 0.05$. †Indicates a significant difference between the treatment and sham groups, $P < 0.05$. (C) Western blot analyses of Flk-1 and Flt-1 proteins (1= UUO group; 2= UUO+ rapamycin group). Actin was used as a control.

not significantly different from that in the sham group, but the Flk-1 protein levels in the UUO group were still higher than that in the sham group ($P < 0.05$). At day 3 and day 7, administration of rapamycin significantly increased the levels of Flk-1 mRNA and protein compared with the UUO and sham group, ($P < 0.05$). The only exception to this was that the increase in

Flk-1 mRNA levels of the rapamycin group compared with the UUO group at day 3 was not significant (Fig. 7A).

Compared with the sham group, UUO increased the expression levels of Flt-1 mRNA and protein at day 3 and day 7 ($P < 0.05$). At day 3 and day 7, administration of rapamycin reduced the levels of Flt-1 mRNA and protein compared with the UUO group ($P < 0.05$). The expression levels of Flk-1

mRNA and protein in the rapamycin group were still significantly higher than those in the sham group (Fig. 7A and 7B).

Discussion

In this study, UUO induced infiltration of inflammatory cells in the interstitium as well as interstitial fibrosis, as indicated by the HE staining, the increased number of infiltrated ED-1 positive cells and the upregulated type III collagen mRNA levels. Hypoxyprobe-1 immunohistochemical staining results indicate that UUO caused an increased degree of hypoxia in the kidney cortex and medulla at day 3 and day 7, accompanied by elevated TGF- β 1, VEGF, Flk-1, and Flt-1 mRNA and protein expressions. Administration of rapamycin alleviated the UUO-induced renal hypoxia, infiltration of inflammatory cells and tubulointerstitial fibrosis, and it down-regulated the UUO-induced elevated expression levels of TGF- β 1 and Flt-1 mRNA and protein ($P < 0.05$). Compared with the UUO group, the rapamycin group showed decreased VEGF mRNA and protein expression at day 3, and increased Flk-1 mRNA and protein expression at day 7 ($P < 0.05$). Rapamycin can inhibit lymphocyte proliferation and differentiation, tumor angiogenesis, replication of cytomegalovirus, growth factor-mediated proliferation of vascular smooth muscle and endothelial cells and fibroblast proliferation [15]. It may also inhibit proliferation and collagen synthesis induced by platelet-derived growth factor in rat mesangial cells [10,11]. Our study found that rapamycin can alleviate UUO-induced renal hypoxia and fibrosis, which may be related to its anti-inflammatory and anti-proliferative effects. In addition, the PI3K signaling pathway is involved in the HIF-1 signaling pathway, and the downstream effector of the PI3K is mTOR [9], which can be inhibited by rapamycin. This may be another mechanism by which rapamycin alleviates renal hypoxia. In a recent study conducted by Tamura et al., mTOR/ (phosphorylation of ribosomal protein S6 kinase 1) S6K/HIF-1 α signaling was found to be activated by hypoxia in ovarian clear cell adenocarcinoma cells cultured *in vitro* [16]. As an inhibitor of mTOR, rapamycin decreased the level of HIF-1 α and blocked the transcriptional regulator of mTOR, S6K [16]. In a mouse model of B-cell lymphoma xenograft, mTOR inhibitors mediated tumor cell apoptosis and it was found that the apoptotic effects were caused by the reduced VEGF protein expression and the downregulation of angiogenesis [17]. In a rat kidney model of chronic allograft dysfunction, another mTOR inhibitor, rapamycin attenuated tubulointerstitial damage, glomerulosclerosis, leucocyte accumulation and vasculopathy, which was associated with diminished expression of VEGF and VEGF receptor mRNA and protein [18]. In podocytes, endothelial and mesangial cells cultured *in*

vitro, rapamycin not only reduced VEGF production in podocytes but also inhibited VEGF-induced proliferation of podocytes, endothelial and mesangial cells [18]. The specific mechanism by which rapamycin alleviates renal hypoxia still needs to be further explored.

Compared with the sham group, rapamycin significantly downregulated the VEGF mRNA and protein expression levels at day 3 ($p < 0.05$), but not at day 7. This early-stage decline might be the result of mTOR inhibition and improved renal hypoxia resulting in decreased HIF-1 accumulation. The relative increase at a later stage might be explained by the overall increase in the VEGF production caused by alleviation of renal tubular epithelial cell injury. As for the VEGF receptors, rapamycin down-regulated the elevated expression levels of Flt-1 mRNA and protein induced by UUO at day 3 and day 7, but it increased Flk-1 mRNA at day 7 and protein expression at day 3 and day 7. The role of these changes in the VEGF and its receptors in UUO-induced renal injury is still unknown, and further study is warranted.

In early-stage diabetic nephropathy, the levels of VEGF and its receptors are elevated, which promotes the hypertrophy of glomeruli and tubules and the early progression of nephropathy [16,17]. The VEGF suppression at that time may improve glomerular hyperfiltration, proteinuria and glomerular hypertrophy [19,20]. Since rapamycin can inhibit the expression of VEGF, its potential effects delaying the progression of diabetic nephropathy requires further attention. The main adverse effects of rapamycin are thrombocytopenia and hyperlipidemia in a dose-dependent manner [21].

Our RT-PCR data demonstrates that rapamycin (0.2 mg/kg/day) significantly lowered the expression of TGF- β 1 mRNA. In a previous study by Wu et al., rapamycin treatment, at a dose of 1 mg/kg/day, did not reduce TGF-beta mRNA expression at day 7 after UUO operation as assessed by Northern blot analysis [12]. One explanation for this discrepancy in results may be the sensitivity and accuracy of the experimental methods used. As RT-PCR is more sensitive than Northern blot for detection of mRNA levels [22], smaller differences in expression are more likely to be detected by RT-PCR. Furthermore, over-saturation of signal will lead to an overestimation of the expression level in a Northern blot analysis.

This study had some limitations. First, whether rapamycin plays a role in the regulation of HIF-1 α and mTOR in this model was not addressed. Since HIF-1 α is a key regulator of hypoxia and VEGF expression, future studies should address this topic. Platelet changes were not measured, but the cholesterol elevation caused by rapamycin at day 3 returned to a level not significantly different from that in the

sham group at day 7. At the same time, the triglyceride levels in the rapamycin group did not change significantly compared to the sham group. The rapamycin dose (0.2 mg/kg/d) in our study was low, and that might be the reason that the blood lipid changes were not salient.

In summary, rapamycin shows beneficial effects in improving UUO-induced renal hypoxia, inflammation, and tubulointerstitial fibrosis in rats. It alleviated the UUO-induced renal hypoxia, infiltration of inflammatory cells and tubulointerstitial fibrosis at day 3 and day 7. It also altered the elevated mRNA and protein expressions of a series of growth factors and receptors after UUO including TGF- β 1, VEGF, Flt-1, and Flk-1. These findings suggest that rapamycin might have the potential as a protective or therapeutic agent for progressive renal fibrosis in human.

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