Full Paper

Total Glucosides of Paeony Attenuate Renal Tubulointerstitial Injury in STZ-Induced Diabetic Rats: Role of Toll-Like Receptor 2

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Abstract. Accumulating evidence suggested that macrophages induce tubulointerstitial injury. Total glucosides of paeony (TGP), extracted from *Paeonia lactiflora*, has presented anti-inflammatory activities in diabetic kidney disease. This research will investigate the protective effect of TGP on renal tubulointerstitium and its mechanism in streptozotocin-induced diabetic rats. TGP was administered orally at a dose of 50, 100, and 200 mg·kg⁻¹·d⁻¹ for 8 weeks. Tubulointerstitial injury was quantified, followed by immunohistochemistry analysis of renal α -smooth muscle actin (α -SMA), E-cadherin (E-cad) expression, nuclear factor kappa B (NF- κ B)-p-p-65⁺, Toll-like receptor (TLR)2⁺, and ED-1⁺ cell infiltration in renal tubulointerstitium. Renal TLR2⁺ macrophages were detected by double immunohistochemical staining. Western blotting was used to detect the TLR2 expression. Histologically, there was marked accumulation of TLR2⁺, NF- κ B-p-p-65⁺, ED-1⁺ cells, and ED-1⁺TLR2⁺ cells (macrophages) in the diabetic kidney and TGP treatment could alleviate it. Accompanying with that, the tubulointerstitial injury was ameliorated, α -SMA expression was lower, and E-cad expression was higher compared with the diabetic rats. Western blot analysis showed that the expression of TLR2 protein was significantly increased in the kidney of the diabetic rats, whereas TGP treatment reduced it. Our study showed that TGP could prevent renal tubulointerstitium injury in diabetic rats through a mechanism that may be at least partly correlated with suppression of increased macrophage infiltration and the expression of TLR2.

Keywords: diabetic nephropathy, total glucoside of paeony, macrophage, Toll-like receptor, tubulointerstitium

Introduction

Diabetic nephropathy (DN) is one of the leading causes of end-stage renal disease in developing countries. Current investigations on DN have focused on the pathological changes within the glomerulus, and in particular the mesangium. Indeed, as with other primary glomerular diseases, the extent of tubulointerstitial injury in the diabetic kidney is a more important predictor of renal impairment. Therefore, it is important to investigate what induces the kidney interstitial lesions and how to interfere with it. In DN, the tubulointerstitium lesions are expanded in the early stage. Without effective control, it will gradually progress to renal tubule atrophy

and tubulointerstitium fibrosis (1), and some evidence showed that tubular epithelial myofibroblast trans-differentiation (TEM) might promote interstitium fibrosis in DN. Accumulating evidence have suggested that macrophages induced tubulointerstitial injury. Indeed, macrophages can produce a variety of substances which can promote renal injury such as adhesion molecules, nitric oxide, reactive oxygen species, and so on (2, 3). Examination of human biopsies and animal models has shown the presence of macrophages in diabetic kidney (4, 5).

Toll-like receptors (TLRs) are an innate family of receptors that can induce tissue damage and a cascade of inflammation following obstruction. Recent reports have shown reduced fibrosis in TLR2 and TLR4-deficient mice in the kidney (6, 7). TLRs modulate the immune system through the production of different cytokines, and influence the differentiation of immune cells. TLRs

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modulate macrophage and CD4⁺ T-cell activity, thereby inducing renal fibrosis (8). In vitro, the TLR4 stimulates profibrotic changes in renal tubular epithelial cells (9). Devaraj et al. (10, 11) provided evidence of the pro-inflammatory role of TLR2 and TLR4 in DN. Lin et al. (12) recently showed that a TLR4 antagonist attenuates renal tubulointerstitial inflammation and that slowed the progression of advanced DN. However, the role of TLRs in renal tubulointerstitial injury remains to be further studied.

Paeonia lactiflora pall root is a Chinese traditional herbal medicine that has a long history of use for gynecological problems, pain, and giddiness. Total glucosides of paeony (TGP) is extracted from *Paeonia lactiflora* pall by alcohol refluxing extraction and then was separated and purified by macroporous absorption resin and column chromatography. It has been shown that TGP has anti-inflammatory, antioxidative, hepatoprotective, and immuno-regulatory activities (13–15). It is a disease-modifying drug for rheumatoid arthritis (RA) that has been approved by the State Food and Drug Administration (SFDA) in 1998. Based on the therapeutic role of TGP in amelioration of inflammation and oxidative stress, we have previously validated its role in experimental DN. Our data suggested that TGP inhibited DN progression via anti-inflammation, antioxidation, and amelioration of macrophage activation (16–18). Recently, Nakagawa and his coworkers demonstrated that keishibukuryogan, which contains TGP, protected the function of kidney in diabetic WBN/Kob rats (19). However, the effects of TGP on renal interstitium injury in DN remains to be defined. In the present study, we further elucidated the mechanism by which TGP prevents renal tubulointerstitial injury in diabetic rats.

Materials and Methods

Drugs and reagents

TGP was provided by the Chemistry Lab of the Institute of Clinical Pharmacology of Anhui Medical University (Hefei, Anhui, China). It was extracted and isolated from *Paeonia lactiflora* pall by the methods of ethanol reflux, *n*-butanol extraction, and macroporous absorption resin chromatography. Effective parts and chemical constituents of TGP have been extracted and identified. It was determined to contain paeoniflorin at 90.2% by high performance liquid chromatography (HPLC) analysis (Waters e2695 Alliance HPLC System; Waters Corp., Milford, MA, USA) was used. The separation was performed on a Kromasil C18 column (250 mm × 4.6 mm, 5.0 μm) eluted with 0.1% phosphoric acid and acetonitrile (86:14) at a flow-rate of 1.0 mL/min. The detection wavelength was set at 230 nm and the

Fig. 1. The chemical structures of major components in total glucosides of paeony.

column temperature was set at 30°C. TGP also contained other components such as hydroxyl-paeoniflorin, paeonin, albiflorin, benzoylpaeoniflorin, and so on (17) (Fig. 1). Streptozotocin (STZ) was from Sigma Chemical Co. (St. Louis, MO, USA). The microalbumin assay kit was from Exocell, Inc. (Philadelphia, PA, USA). Anti-TLR2, nuclear factor kappa B (NF-κB)-p-p-65, E-cadherin (E-cad), and α-smooth muscle actin (α-SMA) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA); mouse anti-macrophage monoclonal (ED-1) antibodies were from Abcam Biotechnology (Abcam, Cambridge, MA, USA). Nitrocellulose membrane and chemiluminescence kit were purchased from Amersham Life Science (Little Chalfont, UK). The immunohistochemistry kit (PV-9000) and double immunohistochemistry kit (DS-0001) were purchased from Beijing Zhongshan Biotechnology Inc. (Zhongshan, China).

Animals

Male Munich-Wistar rats (weight; 180–200 g) were obtained from the Experimental Animal Center of Anhui Medical University. A research protocol in accordance with the principles was approved by the animal ethics committee of Anhui Medical University. Animals were housed in a wire-bottomed cage under a 12 h light/dark cycle. Room temperature (about 24°C ± 1°C) and humidity (about 60%) were controlled automatically. The rats were allowed free access to standard laboratory chow and tap water.

Experimental design

After several days of adaptation, the rats were intraperitoneally injected with STZ diluted with citrate buffer (0.1 M, pH 4.0) at a dose of 65 mg/kg following over-

night fasting. Two days later, the diabetic state was confirmed by measurement of tail blood glucose levels using a reflectance meter (One Touch II; Lifescan LTD, Jinan, China). Blood glucose (BG) levels were measured twice a week. Diabetic rats (BG > 16.7 mmol/L) were then randomly divided into 4 groups (n = 10 per group), avoiding any inter-group differences in blood glucose levels. A group of normal rats was also included (n = 10). These rats were administered 0.5% sodium carboxymethylcellulose (CMC-Na), while the rats used in the experiments were given orally TGP (suspended in 0.5% CMC-Na) at a dose of 50, 100, or 200 mg·kg⁻¹·d⁻¹ using a stomach tube for 8 weeks. In the preliminary experiments, the effects of TGP 50, 100, and 200 mg·kg⁻¹·d⁻¹ concentrations on albuminuria in diabetic rats proved to be effective.

Blood sample and tissue collection

After the rats were treated for 8 weeks, their body weights were measured at the end of the experiment. Rats were fasted for 12 h and subsequently anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg) and then placed on a temperature-regulated table. The right jugular artery was catheterized for blood sampling. Blood glucose levels were determined with a glucose analyzer. The kidney was perfused in vivo via the abdominal aorta with 100 ml of normal saline at 4°C, while the renal vein was punctured to permit the perfusate to drain. After that, the kidneys were removed immediately and sectioned vertically into 2 pieces. One portion was fixed in 10% formalin and processed in paraffin for subsequent histologic assessment and immunohistochemical studies, while the other was stored at -70°C and used for other assays (see below).

Detection of urinary albumin excretion

Prior to sacrifice, rats were placed in metabolic cages for collection of urine over a 24-h period of time and used for measurement of albumin levels. Briefly, urine samples from each rat were combined and measured. After centrifugation, aliquots of the supernatant were frozen at -70°C for subsequent analysis of albumin levels by using an enzyme-linked immunoabsorbent assay with an anti-rat albumin antibody. The 24 h albumin excretion was calculated by multiplying the urinary albumin excretion by the 24-h urine volume.

Renal pathology

Formalin-fixed kidney sections (2 μm) were stained with periodic acids Schiff (PAS) reagent to identify kidney structure and hematoxylin to distinguish cell nuclei. Digital images of interstitial areas without glomeruli were obtained from microscopy (magnifica-

tion, ×400). We choose 25 consecutive interstitial fields randomly, and tubulointerstitial area in the cortex was evaluated and graded as follows: 0, normal; 1, the area of interstitial inflammation and fibrosis, tubular atrophy and dilation with cast formation involving < 25% of the field; 2, lesion area between 25% and 50% of the field; and 3, lesions involving > 50% of the field. The tubulointerstitial injury index (TII) were calculated by averaging the grades assigned to all tubule fields. All measurements and scoring were performed on blinded slides.

Immunohistochemistry

Immunoperoxidase staining was performed on 10% formalin-fixed paraffin sections (2-μm-thick). Briefly, the deparaffinized and rehydrated tissue sections were incubated with 3% hydrogen peroxide to block endogenous peroxidase and then subjected to antigen retrieval through microwave oven heating in 0.1 M sodium citrate (pH 6.0) for 10 min. After that, tissue sections were incubated with 10% normal goat serum for 10 min followed by an overnight incubation with anti-TLR2 (1:100), anti-E-cad (1:100), anti-α-SMA (1:100), anti-phosphorylated NF-κB-p-65 (anti-NF-κB-p-p-65) (1:100), and anti-ED-1 antibodies (1:50) in 10% normal goat serum at 4°C. The sections were washed, further incubated with horseradish peroxidase (HRP)-labeled goat anti-rabbit/mouse polyclonal antibody for 20 min at 37°C, and developed with 3,3-diaminobenzidine (DAB, Sigma) for color reaction. The sections were then counterstained with hematoxylin. After that, the immunostained sections were reviewed under a light microscope. To score the sections, we randomly selected 20 tubulointerstitial regions (0.22 × 0.18 mm²) under a high magnification. Image-Pro-Plus 6.0 image analysis software was used to calculate the number of positive cells and percentage of positive area of the tubular-interstitial area, and the mean was taken. All measurements and scorings were performed blindly.

Double immunohistochemical staining for simultaneous detection of TLR2⁺ macrophages was also performed on formalin-fixed paraffin sections (2-μm-thick). Immunoperoxidase staining of ED-1 was performed as described above. After development with DAB, the sections were placed in 500 ml of 0.1 M sodium citrate buffer (pH 6.0) and microwave-treated for 10 min and then incubated sequentially with 10% normal goat serum for 10 min followed by the anti-TLR2 antibody (1:100) overnight at 4°C. The sections were subsequently incubated with alkaline phosphatase-labeled goat anti-rabbit IgG antibody and developed with AP-Red to produce a red color. The method for calculating positive double-stained cells is described above.

Western blot analysis

Kidney samples were homogenized and lysed in SDS-PAGE sample buffer, boiled, centrifuged, and the supernatant recovered. The protein content was estimated by the dye binding assay of Bradford, with bovine serum albumin used as a standard. Samples were separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE), electroblotted onto nitrocellulose membranes, incubated with blocking buffer for 1 h, and incubated with primary antibody overnight at 4°C. After the blots were washed, they were incubated with a HRP-labeled goat anti-rabbit IgG. The bound secondary antibody was visualized with an enhanced chemiluminescence (ECL) detection kit. Housekeeping protein β -actin was used as a loading control. Positive immunoreactive bands were quantified densitometrically (Leica Q500IW image analysis system) and expressed as ratio of TLR2 to β -actin in optical density units.

Statistical analyses

Data were expressed as the mean $\pm$ S.E.M. and median unless otherwise specified. One-way analysis of variance with pairwise comparisons according to the Tukey method was performed. Since urinary albumin excretion rate followed a non-normal distribution, log transformation analysis was performed prior to statistical analysis of this parameter. Differences were considered significant if the *P*-value was less than 0.05.

Results

Characteristics of the rats in clinical and metabolic parameters

The diabetic control rats exhibited reduced body weight and increased blood glucose levels. However, there were no effects on body weight and blood glucose observed in TGP-treated rats. The ratio of kidney weight to body weight (relative kidney weight) was significantly higher in the diabetic control than that in normal rats. TGP treatment for 8 weeks did not have any effect on the increase in the ratio of kidney weight to body

weight of diabetic control rats. In diabetic control rats, albuminuria was significantly increased when compared to normal rats; treatment with TGP dose-dependently attenuated the increase in albuminuria from the diabetic rats, but this level was still higher than that observed in normal rats (Table 1).

Renal histology

Compared with the normal rats, the tubulointerstitial injury was detected in diabetic rats. Histological observations of kidney sections stained with PAS showed that in diabetic rats, renal tubular epithelial cells exhibited hypertrophy, tubular basement membranes were thickened, and the extracellular matrix (ECM) was proliferating. This confirmed that rats in the diabetic control group had an increase in TII when compared to the normal group (Fig. 2 and Table 2). Rats in the diabetic control group had an increase in TII when compared to that in the normal group; the increased TII was only ameliorated by TGP at 100 and 200 mg·kg⁻¹·d⁻¹.

Renal E-cad and α -SMA expression

Compared with the normal group, the E-cad was decreased in the kidney of the diabetic control group, and it was partly recovered with the TGP (50, 100, 200 mg·kg⁻¹·d⁻¹) administration (Fig. 3: A, C). In the normal group, α -SMA was only expressed in vascular smooth muscle cells. Immunostaining for α -SMA was increased significantly in the diabetic control group in the tubulointerstitium. α -SMA immunostaining was mostly observed not only in renal tubular epithelial cells but also in tubulointerstitium. The increasing expression of α -SMA in DN was also seldomly found in glomeruli and periglomeruli. In comparison with the diabetic control, TGP administration at the dose of 50, 100, or 200 mg·kg⁻¹·d⁻¹ can suppress the α -SMA expression in the kidney of diabetic rats. (Fig. 3: B, C)

Immunohistochemical analysis of NF- κ B-p-p-65 in the kidney

The results of immunohistochemistry staining showed

Table 1. Characteristics of clinical and metabolic parameters of the rats

Group	Dose (mg/kg·d)	Blood glucose (mg/dl)	Body weight (g)	Kidney weight / body weight (g/100 g BW)	Albumin excretion rate ^a (mg/24 h)
Normal		123.53 $\pm$ 29.19	458 $\pm$ 27.47	0.30 $\pm$ 0.04	0.38 $\times$ / $\div$ 1.3
Diabetic control		469.07 $\pm$ 74.58**	271.75 $\pm$ 16.86**	0.56 $\pm$ 0.05*	1.87 $\times$ / $\div$ 1.1**
Diabetic + TGP	50	445.04 $\pm$ 77.43	281.75 $\pm$ 25.01	0.52 $\pm$ 0.02	1.32 $\times$ / $\div$ 1.1 [#]
	100	470.49 $\pm$ 75.47	266.4 $\pm$ 27.87	0.50 $\pm$ 0.06	1.15 $\times$ / $\div$ 1.1 [#]
	200	484.16 $\pm$ 75.65	318.0 $\pm$ 17.8	0.50 $\pm$ 0.04	0.65 $\times$ / $\div$ 1.1 ^{##}

^aShown as geometric mean $\times$ / $\div$ tolerance factor. Number of rats in each group was 10. **P* < 0.05, ***P* < 0.01, compared with the normal group; [#]*P* < 0.05, ^{##}*P* < 0.01, compared with the diabetic control group.

that compared with normal rats, NF- κ B-p-p65 on the epithelial cells of renal tubules of the diabetic control group was significantly increased. The levels of NF- κ B-

p-p65 in TGP groups were significantly lower than that of the diabetic control group (Fig. 4).

Renal TLR2 expression

To detect molecular changes in these experimental rats, we performed western blot analysis of renal TLR2 expression. The data showed an increase in amount of

Fig. 2. Pathomorphology of renal tissue in rats to evaluate tubulointerstitial injury (PAS $\times$ 400). A: normal group, B: diabetic control group, C: diabetic + TGP ($50 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$), D: diabetic + TGP ($100 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$), E: diabetic + TGP ($200 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$).

Table 2. Tubulointerstitial injury index

Group	Dose (mg/kg·d)	Tubulointerstitial injury index ^a (score)
Normal		0 (0 – 1)
Diabetic control		3 (2 – 3)**
Diabetic + TGP	50	2 (1 – 3)
	100	1.5 (0 – 3) [#]
	200	0.5 (0 – 1) ^{##}

^aData are shown as the median. Number of rats in each group was 10.

** $P < 0.01$, compared with the normal group; [#] $P < 0.05$, ^{##} $P < 0.01$, compared with the diabetic control group.

Fig. 3. Representative micrographs of immunostaining and percentage of positive area analysis in the kidney. Immunostaining for E-cad (A), α -SMA (B) in renal tissue from 5 groups of rats (Original magnification $\times$ 400), and percentage of positive area analysis (C, tubule-interstitium %). A: normal rats, B: control diabetic, C: diabetic + TGP ($50 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$), D: diabetic + TGP ($100 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$), E: diabetic + TGP ($200 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$). ** $P < 0.01$, compared with the normal group; ^{##} $P < 0.01$, compared with the diabetic control group.

Fig. 4. Representative micrographs of immunostaining for positive cells in the kidney. Immunostaining for NF- κ B-p-p-65 in the kidney (A, Original magnification $\times 400$), positive cells analysis (B, cells/ $0.22 \times 0.18 \text{ mm}^2$). A: normal group, B: diabetic control group, C: diabetic + TGP ($50 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$), D: diabetic + TGP ($100 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$), E: diabetic + TGP ($200 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$). ** $P < 0.01$, compared with the normal group; ## $P < 0.01$, compared with the diabetic control group.

Fig. 5. Western blot detected the expression of TLR2 in the kidney. Renal TLR2 expression (A), densitometric analysis (B). A: normal rats, B: control diabetic, C: diabetic + TGP ($50 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$), D: diabetic + TGP ($100 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$), E: diabetic + TGP ($200 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$). ** $P < 0.01$, compared with the normal group; ## $P < 0.01$, compared with the diabetic control group.

immunoreactive peptide in the kidney of diabetic control rats compared to that of normal rats. Specifically, densitometric analysis of the western blot showed an increase in the amount of TLR2 in diabetic control rats compared to normal rats. In contrast, TGP treatment at 50, 100, or 200 $\text{mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ reduced the levels of TLR2 protein (Fig. 5).

Detection of renal TLR2⁺, ED-1⁺, and ED-1⁺TLR2⁺ macrophages

TLR2⁺, ED-1⁺, and ED-1⁺TLR2⁺ macrophages in renal tubulointerstitium were detected by immunohistochemistry. The average percentages of the TLR2-positive staining area was increased in the diabetic control group when compared with the normal group ($6.64\% \pm 0.80\%$ vs. $1.29\% \pm 0.16\%$, $P < 0.01$) and treatment with TGP ($50, 100, 200 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$) significantly attenuated it when compared with diabetic control group ($3.61\% \pm 0.66\%$, $2.01\% \pm 0.41\%$, $1.86\% \pm 0.37\%$ vs. $6.64\% \pm 0.80\%$, $P < 0.01$) (Fig. 6A). We detected ED-1⁺ macrophages and the TLR2 in these macrophages in the rats renal tubulointerstitium. There were rare ED-1-positive macrophages accumulated in normal rat kidneys; however, there was a significant increase of macrophage infiltration in the diabetic control rats. TGP treatment significantly decreased the number of macrophages in the tubulointerstitial areas (Fig. 6: B, D). We also found that in the tubulointerstitium ED-1⁺TLR2⁺ cells were significantly increased in the diabetic rats compared to those of normal rats. In contrast, TGP treatment significantly attenuated the elevated ED-1⁺TLR2⁺ cells

Fig. 6. Representative micrographs of immunostaining for positive cells in the kidney. Immunostaining for TLR2⁺ (A), ED-1⁺ (B), and ED-1⁺TLR2⁺ (C) cells in the kidney (Original magnification $\times 400$) and positive cells (D, cells/0.22 $\times$ 0.18 mm²). A: normal rats, B: control diabetic, C: diabetic + TGP (50 mg·kg⁻¹·d⁻¹), D: diabetic + TGP (100 mg·kg⁻¹·d⁻¹), E: diabetic + TGP (200 mg·kg⁻¹·d⁻¹). * $P < 0.05$, ** $P < 0.01$, compared with the normal group; # $P < 0.05$, ## $P < 0.01$, compared with the diabetic control group.

in tubulointerstitium compared to those of diabetic rats (Fig. 6: C, D).

Discussion

In DN, hyperglycaemia is the initial factor of tubulointerstitial injury, and in addition, there are many other etiological factors such as albuminuria, growth factors, transdifferentiation of tubulointerstitium cells, infiltration of lymphocyte, macrophage, and so on (20). Proteolytic disruption of E-cadherin/ β -catenin mediated cell-to-cell adhesion has been recognized as an important mechanism responsible for the induction of Epithelial Myofibroblast Transdifferentiation (EMT). In our research, we found by immunohistochemical staining that the disruption of E-cad in DN was ameliorated by TGP. α -SMA is also the most common marker used to localize the myofibroblast. Our observation showed that

in DN, α -SMA immunostaining was detected in some glomeruli, tubules, and interstitium, but in normal rats, it was only expressed in vascular wall. The expression in DN was obviously depressed by the administration of TGP. These observations suggested that TGP could restrain the trans-differentiation of renal tubular epithelial-mesenchymal cells in DN. Therefore trans-differentiation might play a considerable role in the interstitial fibrosis of DN (21).

Previous reports suggested that renal macrophage accumulation in the tubulointerstitium may play a central role in the tubulointerstitial fibrosis; this was also found in DN (22). The interstitial macrophage is involved in both the initiation and continuation of this tubulointerstitial injury. Direct damage to resident cells is caused through the generation by macrophages of radical oxygen species (ROS), nitric oxide (NO), varied inflammatory mediators, profibrogenic cytokines, and proinflammatory

cytokines such as interleukin (IL)-1, tumor necrosis factor (TNF)- α , transforming growth factor (TGF)- β , and so on. As consequence, it improved the inflammation and induced fibroblast proliferation, ECM enhancement, and the TEMT. These led to kidney injury advancement (3, 20). An in vitro study provided evidence that matrix metalloproteinases (MMPs), specifically MMP-9, secreted by effector macrophages can induce TEMT and thereby contribute to renal fibrosis (23). Research suggested that macrophage infiltration could directly or indirectly contribute to the pathogenesis of renal fibrosis by inducing TEMT (23, 24). Our results showed that TEMT and tubulointerstitial injury in DN occurred in parallel with the macrophage infiltration. This hinted that macrophages may contribute to renal fibrosis via TEMT in DN. Recently, Lange-Sperandio et al. (25) showed that blockade of macrophages recruitment reduced tubular cell EMT and renal fibrosis. The in vitro study by Tan et al. (26) showed that conditioned medium derived from activated macrophages can induce TEMT in C1.1 cells and primary tubular epithelial cells (TECs), providing direct evidence for a role for macrophages in TEMT induction.

In this study we found that renal tubular epithelial cells overexpressed TLR2 in STZ-induced diabetic kidney, which was correlated with ED-1-positive macrophage infiltration and activation, and this promoted tubulointerstitial inflammation and TEMT. Moreover, TGP administration reduced the macrophage infiltration in diabetic rats accompanied with the expression of TLR2. Investigations by others also demonstrated that TLR2 will induce macrophages activity, promote tubulointerstitial inflammation, and aggravate TEMT and renal fibrosis (12, 27). Just as TGP decreased the macrophages, Fujisawa et al. (28) suggested that spironolactone ameliorates renal fibrosis, presumably via the inhibition of macrophage infiltration, and TGF- β 1 expression in streptozotocin-induced early diabetic injury.

TLRs are expressed in macrophages, neutrophils, dendritic cells, intestinal epithelial cells, and endothelial cells. They trigger the myeloid differentiation factor 88 (MyD88)-related downstream signaling cascade, and in turn, results in the activation of mitogen-activated protein kinases (MAPKs) and NF- κ B, further leading to the regulation of the proliferation and immune responses (29). In DN, the TLR/NF- κ B signaling pathway induced the kidney interstitial macrophage infiltration (30). In agreement with this, our current study indicated that the NF- κ B activation was accompanied with TLR2 expression in diabetic tubulointerstitium, and as a result, the macrophage infiltration increased. TGP treatment could depress the expression of TLR2 and NF- κ B in DN tubulointerstitium and also it could reduce the

ED-1⁺ and ED-1⁺TLR2⁺ cells.

We had found that TGP could obviously reduce the expression of IL-1 and TNF- α in kidney of DN rats (16). IL-1 and TNF- α are proinflammatory cytokines, mainly produced by leukomonocytes and activatory macrophages. As a consequence, activatory macrophages regenerate IL-1 and TNF- α , and the inflammatory reaction is amplified. Further, the amplified inflammatory reaction, which was caused by the IL-1 and TNF- α generated by activatory macrophages, was also blocked by TGP. Our in vivo data demonstrate that TGP possesses an anti-inflammatory effect and inhibits TLR2 activation and consequently reduces TNF- α and IL-1 expression.

In conclusion, our research confirmed that TGP had a protective effect against tubulointerstitium injury in DN, the mechanism of which may be at least partly correlated with the amelioration of TEMT through the pathway of TLR2-related macrophage infiltration.

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