Oridonin protects against the inflammatory response in diabetic nephropathy by inhibiting the TLR4/p38-MAPK and TLR4/NF-κB signaling pathways

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ABSTRACT

Inflammation plays a pivotal role in the development and progression of diabetic nephropathy (DN). Oridonin (Ori), a component isolated from Rabdosia rubescens, possesses remarkable anti-inflammatory, immunoregulatory and antitumor properties. However, the renoprotective effects of Ori and the underlying molecular mechanisms have not been explored in DN. In this study, we aimed to investigate the protective effects and potential mechanisms responsible for the anti-inflammatory effects of Ori in diabetes-induced renal injury in vivo and in vitro. Our results showed that Ori significantly attenuated diabetes-induced renal injury and markedly decreased urinary protein excretion levels, serum creatinine concentrations and blood urea nitrogen concentrations in rats. Ori also significantly alleviated infiltration of inflammatory cells (cluster of differentiation (CD)68) in kidney tissues and reduced the levels of pro-inflammatory cytokines, including tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), IL-1β and monocyte chemotactic protein 1 (MCP-1), both in vivo and in vitro. TLR4 is a principal mediator of innate immune and inflammatory responses and participates in the development of DN. Our molecular studies indicated that Ori administration significantly down-regulated TLR4 overexpression in DN. Additional studies were conducted to investigate the effect of Ori on the p38-mitogen-activated protein kinase (p38-MAPK) and nuclear factor (NF)-κB pathways. The results showed that Ori inhibited IκBα, p65, and p38 phosphorylation, as well as NF-κB DNA-binding activity. In conclusion, these results demonstrated that Ori exerts protective effects in diabetes-induced renal injury in vivo and in vitro. These effects may be ascribed to its anti-inflammatory and modulatory effects on the TLR4/p38-MAPK and TLR4/NF-κB signaling pathways.

1. Introduction

Diabetic nephropathy (DN) is one of the most common and serious microvascular complications of diabetes mellitus. DN has become the single largest cause of end-stage renal disease (ESRD), and its prevalence has been increasing worldwide [1–4]. However, the pathogenesis of DN is not completely understood. Consequently, there is a lack of specific therapies capable of preventing DN development and progression. Therefore, studies aiming to elucidate the pathogenesis of DN and search for new therapies for DN are urgently needed. Accumulating evidence collected in recent years indicates that inflammatory processes facilitated by the innate immune response are of paramount importance with respect to the pathogenesis of DN [5,6]. Toll-like receptors (TLRs) are a conserved family of pattern recognition receptors that play a fundamental role in the innate immune system by triggering pro-inflammatory signaling pathways in response to microbial pathogens. In addition, TLRs are activated by endogenous agonists of nonmicrobial origin and participate in noninfectious inflammatory processes [7]. TLR4, a component of the primary innate immune receptor-mediated inflammatory signaling pathway, is mainly distributed in the glomerular mesangial cells and renal tubular epithelial cells of kidney tissues [8]. Accumulating evidence shows that TLR4 expression in glomerular mesangial cells and renal tubular epithelial cells can clearly increase in response to high glucose (HG) and angiotensin II, which can activate the downstream p38-mitogen-activated protein kinase (p38-MAPK) and nuclear factor (NF)-κB pathways and accelerate the secretion of pro-inflammatory cytokines, including tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), IL-1β and monocyte chemotactic protein 1 (MCP-1), thereby aggravating kidney injury [9–11]. The p38-MAPK and NF-κB pathways are two important inflammatory signaling pathways that are closely associated with renal tissue damage. Of these pathways, the p38-MAPK signaling pathway is the "classical" pathway...
through which anti-inflammatory drugs interfere with renal inflammatory tissue damage. Therefore, studying the natural immune inflammatory mechanisms underlying DN development and progressing and searching for drugs that modulate immune-mediated inflammation may yield promising strategies for curing DN.

Oridonin (Ori, Fig. 1), a famous diterpenoid isolated from the Chinese medicinal herb Rabdosia rubescens, possesses a variety of biological properties, including antitumor [12], anti-inflammatory [13–15], immunoregulatory [13,16], antioxidant [17] and antibacterial properties [18]. Ori has been administered for the treatment of inflammatory diseases for hundreds of years in China and has become one of the most popular herbs used clinically. Zhou et al. [19] reported that Ori efficiently increases survival, alleviates proteinuria, attenuates renal damage and ameliorates the serological and clinical manifestations of systemic lupus erythematosus (SLE) in MRL-lpr/lpr mice. However, few studies have investigated the effects of Ori on kidney injury in diabetic rats and glomerular mesangial cells. Therefore, we aimed to investigate whether Ori protects against kidney injury in diabetic rats and rat glomerular mesangial cells and further elucidate the anti-inflammatory mechanism that involves the TLR4/p38-MAPK and TLR4/NF-κB signaling pathways. Our findings may support the clinical application of Ori as a treatment for DN.

2. Materials and methods

2.1. Materials

Oridonin was purchased from Shanghai Yuanye Biological Technology Co., Ltd. (Shanghai, China). The purity of Ori, which was measured by high-performance liquid chromatography (HPLC), was > 98%. Streptozotocin (STZ) was purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). Dulbecco’s Modified Eagle’s Medium (DMEM/low glucose), DMEM/HG and fetal bovine serum (FBS) were purchased from HyClone (Logan, UT, USA). Commercial assay kits for Scr, BUN and 24-h urinary protein concentrations were determined using a creatinine assay kit, enzymatic assay kit and protein estimation kit, respectively, according to the protocols provided by the manufacturers.

2.2. Animals and treatment

A total of 32 specific pathogen-free (SPF) grade male Sprague Dawley (SD) rats (aged 6–8 weeks and weighing 200 ± 20 g) were purchased from the Center for Disease Control and Prevention in Hubei province (Hubei, China). All of the rats were maintained under a standard temperature (21 ± 2 °C), humidity (55 ± 2%) and 12-h light/dark cycle. The rats had free access to a standard rodent diet and drinking water. All the animal experiments were conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals published by the NIH.

After 1 week of adaptive feeding, all the rats were randomly assigned to the following four groups (8 rats per group): a normal control (NC) group, NC + Ori group, T2DM model (DM) group and DM + Ori group. The DM and DM + Ori groups were fed a high-fat diet (D12492) for 4 weeks to establish an insulin resistance model. All of the insulin resistance model rats were converted into DM rats by a single intraperitoneal injection of STZ (35 mg/kg dissolved in 0.1 mol/l citric acid buffer, pH 4.3). The NC and NC + Ori groups were fed normal chow (4% calories from fat) for 4 weeks and then intraperitoneally injected with the same dose of citric acid buffer (pH 4.3, 0.1 mol/l). At 72 h after STZ injection, we measured random blood glucose levels to confirm the successful establishment of the diabetes model. A random blood glucose level of > 16.7 mmol/l after STZ injection was indicative of the establishment of the model. All the rats in the model groups successfully developed T2DM. The rats in NC + Ori and DM + Ori groups were intraperitoneally injected with 10 mg/kg/day Ori, while the rats in the NC and DM groups received the equivalent dose of normal saline. The experiment lasted for 12 weeks. Body weights and random blood glucose levels were monitored weekly in all rats. All the rats were housed in metabolic cages for 24-h urine collection at the end of the 12th week and then sacrificed under chloral hydrate anesthesia. The data for the random blood glucose levels and body weights were compared among the groups. Blood samples were acquired by cardiac puncture at the time of sacrifice. Serum was then separated by centrifugation and stored at −20 °C for subsequent experiments. Kidney tissues were also excised, weighed, and stored in liquid nitrogen or fixed in 4% paraformaldehyde. The kidney weight-to-body weight ratio was calculated for each rat.

2.3. Assessment of renal biochemical markers

Scr, BUN and 24-h urinary protein concentrations were determined using a creatinine assay kit, enzymatic assay kit and protein estimation kit, respectively, according to the protocols provided by the manufacturers.

2.4. Histopathological examination of kidney tissues

Portions of the renal cortex that were fixed in 4% paraformaldehyde were embedded in paraffin and cut into 4-μm-thick sections. The tissue sections were stained with hematoxylin-eosin (H&E) and periodic acid-Schiff (PAS) for assessment under a light microscope (Olympus, Japan).

2.5. Immunohistochemistry for TLR4 and CD68 expression in kidney tissue

Following deparaffinization and hydration, 4-μm-thick renal cortical tissue sections were treated with 3% H2O2 for 5–10 min to inactivate endogenous enzymes and then treated with 5% BSA in phosphate-buffered saline (PBS) blocking solution for 30 min at room temperature. After an incubation with the appropriate primary antibodies (1:100) overnight at 4 °C, the sections were incubated with a horseradish peroxidase-labeled goat anti-rabbit polyclonal antibody for 30 min at 37 °C. The sections were then counterstained with hematoxylin, dehydrated, cleared, and mounted with Permount mounting solution. Ten fields in each section were randomly chosen for
examination at 400 × magnification and analyzed by a computer image analysis system (immunohistochemical (IHC) image analysis from Shanghai DaWeiKe Biotechnology). The positive integrated optical density (IOD) value of each field was calculated, and the mean values were compared among the experimental groups.

2.6. Cell culture and drug administration

A rat mesangial cell line (HBZY-1) was obtained from the China Center Type Culture Collection (Wuhan, China). Cells were cultured in low-glucose (5.6 mmol/l) DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C under a humidified atmosphere containing 5% CO₂. Cells between passage 5 and passage 12 were used for all experiments. When the cells exceeded 85% confluence, the medium was replaced with serum-free medium, and the cells were cultured for another 24 h. The cells were then divided into the following six groups: A, the normal glucose (NG) group, which received 5.5 mmol/l glucose; B, the NG + Ori group, which received 5.5 mmol/l glucose + 5 μmol/l Ori; C, the HG group, which received 30.0 mmol/l glucose; D, the HG + Ori group, which received 30.0 mmol/l glucose + 5 μmol/l Ori; E, the HG + TAK-242 group, which received 30.0 mmol/l glucose + 1 μmol/l TAK-242; and F, the HG + Ori + TAK-242 group, which received 30.0 mmol/l glucose + 5 μmol/l Ori + 1 μmol/l TAK-242.

2.7. Cell viability assay

Cell viability was assessed by CCK-8. Mesangial cells were seeded in 96-well plates at a density of 3 × 10⁴ cells/well. After the cells attached for 24 h, they were incubated with different concentrations of Ori (0, 2.5, 5, 10 and 20 μmol/l) under NG or HG conditions for 12, 24 or 48 h. Ten microliters of CCK-8 was subsequently added to each well, and the cells were incubated for 4 h at 37 °C. Absorbance was measured at 450 nm using a microplate reader (Bio-Tek, USA). Cell viability was calculated using the following equation: cell viability (%) = (drug group optical density (OD)-blank group OD)/(control group OD-blank group OD). The appropriate Ori concentration and treatment time were selected according to the results of the CCK-8 assay.

2.8. Macrophage migration assay

To confirm the effect of Ori on the migratory capacity of HG-mediated cell migration, we performed a cell migration assay using 6-well chemotaxis chambers (Corning 3422, USA). Polycarbonate filter inserts precoated with Matrigel were preincubated in DMEM supplemented with 1% FBS for 2 h before the cells were plated. Rat primary peritoneal macrophages were prepared and cultured according to previously described methods [20]. Cell suspensions containing 1 × 10⁶ macrophages per well were seeded in the upper chamber, and cell suspensions containing 4 × 10⁵ mesangial cells per well were seeded in the lower chamber. The cells were then divided into the NG group, NG + Ori group, HG group, HG + Ori group, HG + TAK-242 group, and HG + Ori + TAK-242 group. Next, the cells were incubated at 37 °C under a humidified atmosphere containing 5% CO₂. After 24 h incubation, the cells were washed with PBS three times, and non-migrating macrophages in the upper chamber of the filters were removed using cotton swabs. Macrophages that migrated and adhered to the other side of the filter were fixed in 4% formaldehyde for 20 min, stained with crystal violet and counted in five fields or quantitated by measuring absorbance at 570 nm after elution of crystal violet with 10% acetic acid. Data represent the mean of at least three independent experiments.

2.9. Cytokine assays

The levels of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6 and MCP-1) in renal tissues and mesangial cells were estimated by the appropriate ELISA kits, according to the manufacturer’s instructions.

2.10. Quantitative PCR analysis

Total RNA was extracted from renal tissues by TRizol (Invitrogen, USA), according to the manufacturer’s instructions. RNA concentrations were measured at an absorbance of 260/280, and cDNA was synthesized by reverse transcription. The specific primers for TNF-α, IL-1β, IL-6, MCP-1 and GAPDH were designed based on known sequences. The primers are shown in Table 1. The following amplification conditions were used for the experiment: 94 °C for 5 min, followed by 40 cycles of 95 °C for 10 s, 60 °C for 30 s, and 72 °C for 30 s. The polymerase chain reaction (PCR) system consisted of 10 μl of SYBR qPCR Mix, 1 μl of each primer, 1 μl of cDNA and 4 μl of nuclease-free water. The relative expression levels of the target genes were normalized to those of GAPDH using the 2-ΔΔCt comparative method. Every experiment was repeated 3 times.

2.11. Western blot analysis

Total protein was extracted from renal tissues and cells with the appropriate reagent, according to the manufacturer’s protocol (ASPEN, USA). Protein concentrations were quantitated using a bicinchoninic acid (BCA) protein assay kit (ASPEN, USA). Equal amounts of protein (50 μg) were separated by 10% sodium dodecyl sulfate (SDS) polyacrylamide gels and then transferred onto polyvinylidene fluoride (PVDF) membranes, which were blocked with 5% skim milk in Tris-buffered saline Tween-20 (TBST) for 1.5 h and then incubated with primary antibodies for the following proteins overnight at 4 °C: TLR4, t-α, IL-1β, p-kB, GAPDH and MCP-1. The membranes were then washed with TBST three times for 10 min each and incubated with the appropriate secondary antibody, which was conjugated to horseradish peroxidase, for 2 h at room temperature. Protein bands were detected using an ECL Plus Western Blotting Detection System (ImageQuant LAS 4000 mini, USA). GAPDH was used as a loading control.

2.12. EMSA

Nuclear proteins were prepared for an electrophoretic mobility shift assay (EMSA) using a Nuclear Extract Kit (KeyGEN BioTECH, China), according to the manufacturer’s protocol. Nuclear proteins (2.5 μg) were incubated with 10 × binding buffer (LightShift Chemiluminescent EMSA Kit, Thermo Scientific) in the presence of 1 μg/μl poly (dIdC), 1% Nonidet P-40, 100 mM MgCl₂ and 50% glycerol for 20 min. Next, the proteins were incubated with 1 pmol of biotin-labeled NF-κB (5’BIOTIN – TAGCATATGCTA…3’) consensus oligonucleotides (Thermo Scientific) for an additional 20 min at room temperature. The reaction mixture was subjected to 6.5% non-denaturing SDS-PAGE at 100 V for 60 min, transferred onto a nylon hybridization transfer membrane (GE

<table>
<thead>
<tr>
<th>Gene</th>
<th>Oligonucleotide primer sequences (5’-3’)</th>
<th>Product size(bp)</th>
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<tr>
<td>TNF-α</td>
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<tr>
<td>GAPDH</td>
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MCP-1) in renal tissues and mesangial cells were estimated by the appropriate ELISA kits, according to the manufacturer’s instructions.
HEALTHCARE), DNA cross-linked for 10 min, probed with horseradish peroxidase-conjugated streptavidin antibodies (1:300 dilution) for 15 min, visualized by enhanced chemiluminescence and captured by X-ray film. The OD values of the shifted bands were determined by AlphaEaseFC software.

2.13. Statistical analysis

SPSS 19.0 software was used to analyze the data, which are presented as the mean ± SD. Differences between the groups were assessed using one-way analysis of variance (ANOVA). P < 0.05 was considered statistically significant.

3. Results

3.1. Ori alleviated albuminuria and improved renal function without affecting blood glucose concentrations

There was no significant difference in body weight or blood glucose levels between the DM and DM + Ori groups (P > 0.05, Fig. 2A–B). Increases in the kidney weight-to-body weight ratio are a marker of renal swelling and injury [21]. As shown in Fig. 2C, the kidney-to-body weight ratio in the DM group was significantly higher than that in the normal and Ori control groups (P < 0.05); however, diabetic rats treated with Ori for 8 weeks had a much lower kidney weight-to-body weight ratio than did untreated diabetic rats (P < 0.05). Albuminuria and increases in the indicated renal functional parameters (Scr and BUN) are considered hallmarks of the progression of renal disease [22]. Urinary protein excretion and Scr and BUN concentrations in the DM group were higher than those in the normal and Ori control groups (Fig. 2D–F, P < 0.05). Remarkably, those parameters were significantly decreased by treatment with Ori in the DM + Ori group compared with those in the DM group, as shown in Fig. 2D–F (P < 0.05). These data showed that Ori protects the kidneys in a blood glucose-independent manner in diabetic rats.

3.2. Ori attenuated renal histopathological injury

The changes in renal histopathology in the different groups are shown in Fig. 3. H&E staining showed that the sections from the normal and Ori control groups displayed normally sized structures without any abnormalities. Conversely, the sections from the DM group displayed glomerular proliferation, diffuse mesangial matrix expansion, peripheral capillary wall thickening and capillary lumen diminution. However, Ori treatment significantly attenuated these diabetes-induced histopathological alterations. Similarly, PAS staining revealed that more materials displaying PAS-positivity, which is an indicator of glycogen accumulation, were present in diabetic glomeruli than in normal or Ori control glomeruli. However, markedly less PAS-positive materials were present in the DM + Ori group than in the DM group. These data suggested that Ori attenuated renal histopathological injury in diabetic rats.

3.3. Ori inhibited renal inflammatory cell infiltration

The IHC staining and quantification results showed that greater macrophage (CD68) infiltration was present in the DM group than in the normal and Ori control groups. However, treatment with Ori significantly reduced the numbers of infiltrating macrophages in the DM + Ori group compared with those in the DM group (Fig. 4A–B, P < 0.05).
3.4. Effects of Ori on cell viability

To determine a suitable Ori concentration and treatment time, we treated renal cells with various concentrations of Ori (0 μM, 2.5 μM, 5.0 μM, 10 μM, and 20 μM) for 12, 24 or 48 h and then used a CCK-8 assay to measure cell growth. As shown in Fig. 5A–B, cell viability was greatest in cells treated with Ori at a concentration of 2.5 μM for 24 h, suggesting that 2.5 μM and 24 h were the most suitable concentration for treatment.
and time period for treating renal cells with Ori, respectively. Thus, we choose 2.5 μM Ori as the concentration and 24 h as the treatment time.

3.5. Effects of Ori on macrophage migration

Cell migration was observed by the Transwell method. As the result indicated, the migratory capacity of macrophages in the HG group was much stronger than that of those in the NG group (Fig. 6, P < 0.05); however, treatment with Ori, as well as TAK-242, significantly inhibited the migratory capacity of macrophages (Fig. 6, P < 0.05).

3.6. Ori suppressed pro-inflammatory cytokine expression

In vivo results revealed that the mRNA and protein expression levels of several pro-inflammatory cytokines, including TNF-α, IL-1β, IL-6 and MCP-1, were significantly increased in the DM group compared with those in the normal and Ori control groups. However, these increases were significantly inhibited by Ori treatment (Fig. 7A–B, P < 0.05). Similarly, cell experiment results showed that HG induced increases in the levels of these pro-inflammatory cytokines, whereas treatment with Ori markedly decreased the levels of these pro-inflammatory cytokines, as shown in Fig. 7C (P < 0.05). To determine whether Ori inhibited the TLR4-mediated inflammatory response, we pretreated cells with or without the TLR4 inhibitor TAK-242 (1 μM) for 1 h before treatment with Ori for 1 h and then stimulated them with HG for 24 h. TAK-242 and Ori both partially suppressed HG-induced TNF-α, IL-1β, IL-6 and MCP-1 overexpression. Furthermore, pretreating the cells with a combination of TAK-242 and Ori synergistically reversed HG-induced increases in TNF-α, IL-1β, IL-6 and MCP-1 expression (Fig. 7C, P < 0.05). These results suggested that TLR4 is involved in Ori-mediated reductions in inflammation in diabetic rat renal tissues and HG-treated rat mesangial cells.

3.7. Ori down-regulated TLR4 expression

IHC analysis showed that the cytomembranes of both glomerular and renal tubular cells in the DM group displayed stronger positive

![Fig. 5. Effects of Ori on rat mesangial cell viability.](image)

(A and B) Cells were cultured in normal glucose, HG or HG with Ori (2.5, 5.0, 10.0 and 20.0) for 12, 24 or 48 h. Cell viability was detected by a CCK-8 assay. "p < 0.05 vs. NG, "p < 0.05 vs. HG, "p < 0.01 vs. HG.

![Fig. 6. Effects of Ori on macrophage migration.](image)

Cell migration was detected by a transwell method. Values are expressed as the mean ± SE of at least three independent experiments. "P < 0.05 vs. NG, "P < 0.05 vs. HG, "P < 0.01 vs. HG.
TLR4 signals than did those in the normal and Ori control groups (Fig. 8A–B, P < 0.05). However, treatment with Ori significantly reduced TLR4 expression in the DM + Ori group compared with that in the DM group (Fig. 8A–B, P < 0.05). Additionally, western blotting results showed that TLR4 expression was significantly increased in the DM and HG groups compared with that in the normal and Ori control groups and the NG group, respectively (Fig. 8C–E, P < 0.05). However, TLR4 expression was significantly decreased in the DM + Ori and HG + Ori groups compared with that in the DM and HG groups, respectively (Fig. 8C–E, P < 0.05). Moreover, in vitro studies showed that TAK-242 reduced TLR4 protein expression levels (Fig. 8D–E, P < 0.05).

3.8. Ori inhibited NF-κB activation

NF-κB is a key transcriptional factor that participates in the inflammatory process. To assess the inhibitory effects of Ori on the inflammatory response, we first examined the effects of Ori on NF-κB activity in renal tissues by western blotting. As shown in Fig. 8A, NF-κB protein expression levels in the DM group were significantly higher than those in the normal and Ori control groups (P < 0.05). The increase in NF-κB protein expression was attenuated by Ori treatment (Fig. 9A, P < 0.05). We also assessed the inhibitory effects of Ori in vitro. The EMSA results showed that Ori markedly inhibited HG-induced NF-κB DNA-binding activity in the HG + Ori group compared with that in the HG group (Fig. 9B, P < 0.05). In addition, the results showed that the phosphorylation levels of IκBα and p65 were significantly decreased by Ori in the HG + Ori group compared with those in the HG group (Fig. 9C, P < 0.05). Moreover, TAK-242 decreased the phosphorylation levels of IκBα and p65 and weakened NF-κB DNA-binding activity in HG-treated rat mesangial cells.

3.9. Ori inhibited the p38-MAPK pathway

To elucidate the mechanism underlying the effects of Ori on diabetes-induced kidney injury, we determined the effects of Ori on the p38-MAPK pathway. The western blotting results showed that the phosphorylation level of p38 in the DM group was significantly higher than that in the normal and Ori control groups; however, this increase was significantly inhibited by Ori treatment (Fig. 10A, P < 0.05). Similar results were observed in rat mesangial cell experiments. In these cells, the phosphorylation level of p38 was significantly higher in the HG group than in the control group but was clearly decreased by treatment with Ori and TAK-242 (Fig. 10B, P < 0.05).

4. Discussion

Despite improvements in glycemic and blood pressure control, as well as improvements in the efficacy of renin-angiotensin system blockades, which attenuate proteinuria, DN is the most common cause of ESRD worldwide [23]. Accumulating evidence collected in recent years indicates that immunity and inflammation play significant roles in initiating and aggravating renal injury in DN [8]; however, the mechanisms underlying these phenomena are not fully understood.
Therefore, further investigation into the mechanisms underlying intrarenal immunity and inflammation, as well as studies seeking to identify drugs that can suppress the immune inflammatory reaction, may lead to the identification of new targets for anti-inflammatory treatments for DN. Increasing numbers of studies have shown that increases in inflammatory marker levels are related to the anti-DN effects of some renoprotective molecules [24]. In addition, some research data have shown that glomerular injury in STZ-induced diabetic rats cannot be prevented by anti-inflammatory agents [25]. Natural anti-inflammatory products are attractive and safe alternatives to the conventional methods of modulating inflammatory disorders. Ori is an anti-inflammatory plant product that has been used in traditional medicine for centuries. Lin et al. demonstrated that Ori reduced proteinuria and attenuated renal damage in a spontaneous SLE mouse model by regulating the immune and inflammatory responses [19]. These results suggested that Ori attenuates proteinuria and protects the kidney from injury. Moreover, Ori also acts on a variety of cells, including immune cells, hepatocytes and vascular endothelial cells, to exert its protective effect. Zhao et al. [26] suggested that Ori could reduce inflammatory cytokines and restrict inflammatory responses stimulated by lipopolysaccharide (LPS) in RAW 264.7 cells. Bohanon et al. [27] reported that Ori inhibited hepatic stellate cell proliferation and fibrogenesis by suppressing endogenous and TGF-β1-induced ECM proteins. Dong et al. [12] suggested that Ori significantly suppressed human umbilical vascular endothelial cell (HUVEC) proliferation, migration, and capillary-like structure formation in vitro through blockade of VEGF-induced activation of Jagged2 and Notch. However, to date, whether Ori suppresses the inflammatory response and thus exerts beneficial effects on diabetes-induced renal injury has not been explored. To confirm these effects, we investigated the inhibitory effects of Ori on the inflammatory response in a diabetic rat model and rat mesangial cells.

For our in vivo studies, we used STZ-induced diabetic rats fed a high-fat diet to establish a T2DM animal model [28]. In our study, compared with rats in the control groups, rats in the DM group displayed increased plasma glucose levels, as well as an increased kidney weight-to-body weight ratio, which is indicative of renal injury. Significantly increased BUN, Scr, and 24-h urinary albumin concentrations were also noted in rats in the DM group. However, treatment with Ori effectively reversed these changes, as it lowered BUN and Scr concentrations, the kidney weight-to-body weight ratio and 24-h urinary albumin concentrations without affecting plasma glucose levels. The pathological changes typical of DN are mesangial cell proliferation, mesangial expansion accompanied by extracellular matrix accumulation and glomerular capillary wall thickening, and fully developed diabetic glomerulopathy accompanied by nodular sclerosis [29]. In this study, the histological results (as demonstrated by H&E and PAS staining) revealed that the DM group displayed glomerulosclerosis and glomerular expansion, capillary lumen diminution, diffuse mesangial matrix expansion, peripheral capillary wall thickening and significantly increased PAS-positive material (purple plaques) deposition, which is indicative of glycogen accumulation. However, Ori treatment significantly ameliorated these diabetes-induced histopathological alterations. These data indicated that Ori improves renal function, ameliorates diabetes-induced renal injury and delays progressive nephrotoxicity in rats with DM and that these improvements do not result from the body weight- and glucose-lowering effects of Ori.

Emerging evidence from experimental and clinical studies indicates that inflammatory processes facilitated by innate immune responses play a significant role in DN development and progression [30]. In the early stage of DN, macrophages and T cells migrate to and accumulate in the glomeruli and interstitium; these changes are facilitated by the...
local release of adhesion molecules and chemokines [31]. Increasing amounts of evidence suggest that the most prevalent infiltrating leucocytes found in diabetic kidneys are macrophages, which are associated with declining renal function in patients with DN [32]. According to analyses of animal models of type 1 and type 2 DN, CD68+ macrophages derived from either monocyte recruitment or local proliferation make up 90% of total kidney leucocyte infiltrates [33,34]. Similarly, our results demonstrated that compared with control rats, diabetic rats displayed significantly increased numbers of CD68+ macrophages in both interstitial and glomerular areas. However, treatment with Ori markedly attenuated these changes. Our results also showed that macrophages were involved in the pathogenesis of DN. Importantly, the infiltration of macrophages into diabetic kidney tissues was alleviated by Ori. Furthermore, Transwell assay results indicated that Ori significantly inhibited the migratory capacity of HG-treated macrophages. Macrophages are key inflammatory cells that mediate kidney inflammation in experimental and human diabetes. Current evidence shows that infiltrating macrophages are associated with chronic, low-grade inflammation. By interacting with resident renal cells, macrophages generate a pro-inflammatory microenvironment that enhances tissue injury and promotes scarring. As pleiotropic polypeptides, cytokines regulate inflammatory and immune responses though their actions on cells. Several studies have shown that resident renal cells, as well as macrophages, can synthesize pro-inflammatory cytokines [35]. Inflammatory cytokines, namely, IL-1β, IL-6, IL-18 and TNF-α, as well as MCP-1, are involved in DN development and progression. Specifically, MCP-1 regulates the migration and infiltration of macrophages. Therefore, decreasing the levels of these pro-inflammatory cytokines may be beneficial in DN. In the present study, we found that TNF-α, IL-1β, IL-6 and MCP-1 mRNA and protein expression levels were significantly increased in the DM group compared with those in the control groups. Similarly, rat mesangial cells stimulated by HG secreted more TNF-α, IL-1β, IL-6 and MCP-1 than did control cells. However, treatment with Ori effectively reduced the levels of these inflammatory cytokines in diabetic rats and rat mesangial cells. These results revealed that the protective effects of Ori on diabetes-induced renal injury may be due to its anti-inflammatory properties.

To explore the mechanism underlying the effects of Ori on inflammation, we assessed the effects of Ori on the NF-κB pathway. A growing number of studies have reported that NF-κB is a critical transcriptional factor and plays a significant role in the inflammatory response in the kidneys of patients with progressive DN [36]. A wide variety of HG-induced inflammatory genes, including genes encoding adhesion molecules, chemokines, and inflammatory cytokines, are mainly regulated by NF-κB activation, which results from the degradation of inhibitory IκB proteins, allowing NF-κB liberation and phosphorylation, followed by NF-κB nuclear translocation and DNA binding for target gene transcription [37]. Previous studies showed that IL-1β, IL-6 and TNF-α expression is partially NF-κB dependent [38]. Thus, HG-induced NF-κB activation apparently has far-reaching consequences in the pathogenesis of DN. More importantly, Ori inhibits NF-κB activation in LPS-stimulated cell lines [26] and several animal models [15]. In the present study, NF-κB activity was significantly increased in the kidney tissues of the DM group. However, treatment with

![Fig. 9. Ori inhibited NF-κB activation in vivo and in vitro.](image-url)
Ori markedly suppressed and thus normalized NF-κB activation. To elucidate the mechanism by which Ori inhibits TNF-α, IL-6 and IL-1β production, we determined the effects of Ori on NF-κB activation and NF-κB (p65) nuclear translocation in HG-treated rat mesangial cells. Li et al. found that HG increased NF-κB transcriptional activity by up-regulating IκB phosphorylation and nuclear p65 levels in HG-treated podocytes [39]. Consistent with these results, our results showed that HG stimulation dramatically increased NF-κB activation, IκBα and p65 phosphorylation and NF-κB DNA-binding activity, whereas treatment with Ori markedly decreased HG-induced NF-κB activation, as well as IκBα and p65 phosphorylation and NF-κB DNA-binding activity. Our results suggested that the inhibitory effects of Ori on inflammatory mediators and cytokine production are at least partially regulated by NF-κB signaling pathway suppression.

MAPK activation plays important roles in DN development [40]. p38-MAPKs are key MAPKs involved in inflammatory mediator production. As a member of the MAPK family, p38-MAPK is specifically activated by phosphorylation in response to inflammatory and stressful stimuli, including HG. Activated p38-MAPK modulates a variety of transcription factors via phosphorylation and induces the expression of genes encoding pro-inflammatory molecules, including TNF-α, IL-1β and IL-6 [41]. Hence, targeting the p38-MAPK signaling pathway is considered an attractive strategy for the development of anti-inflammatory drugs. p38-MAPK is activated by HG in human mesangial cells, mouse podocytes, and rat proximal tubular cells [31]. In the present study, p38-MAPK phosphorylation levels were significantly increased in both the DM group and HG-treated rat mesangial cells; however, treatment with Ori effectively reduced p38-MAPK phosphorylation and thus inhibited pro-inflammatory cytokine production in vivo and in vitro. These results suggested that Ori inhibits inflammation in DN in part by inhibiting the p38-MAPK pathway.

TLR4 is essential for adaptive immune responses [42], and TLR4 activation mediates inflammation-related signaling pathways, leading to pro-inflammatory cytokine and chemokine transcription, which may aggravate renal dysfunction in acute and chronic kidney diseases. Studies have shown that upon activation, TLR4 transmits a signal via an adaptor molecule, MyD88, leading to NF-κB and p38-MAPK translocation and subsequent pro-inflammatory cytokine and chemokine up-regulation, which initiates local inflammation and leukocyte accumulation. Pro-inflammatory cytokines and chemokines participate in the pathogenesis of DN [10]. Recent experimental studies reported that TLR4 deficiency attenuated the pro-inflammatory state in wild-type mice with STZ-induced diabetes [8,43]. Moreover, blockade of TLR4 signaling showed renoprotective effects in mice with T2DM [44]. Therefore, we surmised that TLR4 is involved in the pathogenesis of DN. Interestingly, Ori exerts its anti-inflammatory effects in LPS-treated RAW264.7 cells and acute lung injury by down-regulating TLR4 expression [26]. In the present study, immunohistochemistry results showed that TLR4 was highly expressed in the cytomembrane of both the glomerulus and renal tubules in the DM group. Furthermore, the western blotting results showed that TLR4 protein expression was markedly increased in both the DM group and HG-treated rat mesangial cells. However, Ori significantly decreased TLR4 expression in both kidney tissues and rat mesangial cells. To determine whether the target of Ori was TLR4, we used a TLR4 inhibitor, TAK-242, to interfere with TLR4 expression and analyzed the synergy between TAK-242 and Ori in vitro. The results showed that both TAK-242 and Ori partially suppressed HG-induced TLR4 overexpression; p65, IκBα, and p38-MAPK phosphorylation; and TNF-α, IL-1β and IL-6 production. Interestingly, TAK-242 and Ori exerted synergistic effects on TLR4 expression and
inflammatory cytokine production. These findings indicated that TLR4 down-regulation is the mechanism by which Ori exerts its anti-inflammatory effects in DN.

In conclusion, we assessed the therapeutic effects of Ori in DN using a T2DM rat model and HG-treated rat mesangial cells and observed that Ori reduced inflammatory cell infiltration, decreased inflammatory cytokine production, down-regulated TLR4 expression and inhibited NF-κB and p38-MAPK activation. These findings suggest that Ori is a novel therapeutic option for the treatment of DN or other inflammatory diseases.

Conflicts of interest

The authors declare no conflicts of interest.

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