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Astragaloside IV from *Astragalus membranaceus* ameliorates renal interstitial fibrosis by inhibiting inflammation via TLR4/NF-κB in vivo and in vitro

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ABSTRACT

Renal fibrosis is characterized by infiltration of inflammatory cells, activation and proliferation of fibroblasts, and accumulation of extracellular matrix (ECM). *Astragalus membranaceus* (AM) is traditional Chinese medicine and has a range of pharmacological effects. Astragaloside IV (As IV) is the main compound of AM and has anti-inflammation activities. Whether As IV ameliorates renal interstitial fibrosis by inhibiting inflammation remains unknown. Accordingly, this study investigated the ameliorating effect of As IV on renal fibrosis. Renal fibrosis was induced in vivo using the unilateral ureteral obstruction (UUO) model. UUO mice were administered intragastrically with As IV (20 and 40 mg/kg/day). After a week, ECM including fibronectin and collagen I was examined by Immunohistochemistry and Western blot, inflammatory cells (CD68 and CD3) were detected by Immunohistochemistry, the release of inflammatory cytokines (tumor necrosis factor-α and interleukin-1β) was inspected by polymerase chain reaction, and signaling pathway was determined by Western blot. In vitro, 100 ng/ml lipopolysaccharide (LPS) stimulated epithelial cells to construct the inflammatory model; these cells were treated by As IV (10 and 20 μM) with or without TAK-242 (1 μM) for 48 h. The released inflammatory cytokines were assayed by enzyme-linked immunosorbent assay, and signaling pathway was evaluated by Western blot. As IV decreased accumulation of ECM and infiltration of inflammatory cells in UUO-induced renal fibrosis. Furthermore, As IV markedly attenuated pro-inflammatory cytokines in UUO mouse and LPS-induced epithelial cells. As IV also inhibited the TLR4 and nuclear factor (NF)-κB signaling pathway in vivo and vitro. These results demonstrate that As IV protects against the progression of renal fibrosis by inhibiting inflammation via the TLR4/NF-κB signaling pathway.

1. Introduction

Renal fibrosis is a common pathogenic pathway of chronic kidney disease (CKD) [1]. However, the treatment for CKD is very limited. Therefore, clarifying the molecular cellular mechanisms underlying renal fibrosis is important to delay the development of CKD and find effective intervention strategies for this disease [2]. When the kidney incurs an injury, almost all kidney cells, including fibroblasts, renal tubular epithelial cells, pericytes, endothelial cells, and podocytes are mobilized [3]. These mobilized cells promote the repair and production of extracellular matrix (ECM). Meanwhile, lymphocytes, monocytes/macrophages, and other inflammatory cells are involved in the repair of the different pathways [4]. Renal fibrosis is abnormally repaired and is mainly characterized by infiltration of inflammatory cells, activation and proliferation of fibroblasts, accumulation of ECM, disappearance of intrinsic renal cells [5–7]. Inflammatory response occurs with renal fibrosis. Interleukin (IL)-1, IL-4, IL-6, IL-10, and tumor necrosis factor (TNF) participate in renal fibrosis [8]. Release of local inflammatory factors also aggravates the progression of fibrosis [9].

Astragalus membranaceus (AM) is a traditional Chinese herbal medicine and has been studied extensively in therapy of a various diseases. Astragaloside IV (As IV) is one of the main active ingredients in AM and exhibits various activities, including vasodilating effect, preventing endothelial dysfunction, improving cardiac cell energy metabolism ,anti-inflammatory effect, and antioxidant effects [10]. This study examined the anti-inflammatory effect of As IV on renal fibrosis and elucidated the underlying mechanism.

2. Materials and methods

2.1. Reagents

The purified natural product Astragaloside IV was obtained from Chengdu Conbon Bio-tech Co., LTD. (Chengdu, China). DMEM/F12

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culture medium and fetal bovine serum (FBS) were purchased from American Gibco Company. PrimeScript™ RT reagent kit was from TaKaRa, Japan. PCR primers were from Sangon Biotech, Shanghai, China. Collagen I and ELISA kit were from Boshide, Wuhan, China. CD68, CD3, and fibronectin were from Abcam, Cambridge, UK. TLR4, NF-κB, and IkBa were from Cell Signaling Technology Tech., Danvers, MA, USA. TAK-242 was from Millipore, USA.

2.2. Animals and experimental design

A total of 20 male C57BL6 mice (age, 8 weeks; weight, 18–24 g) were adaptively fed for 1 week. They were divided into the sham, UUO model, and As IV treatment groups (20 and 40 mg/kg/day). Food was forbidden 24 h before UUO operation. First, the mice were anesthetized by intraperitoneal injection of 10% chloral hydrate. Second, the surgical area was disinfected with 75% alcohol, the skin was cut layer-by-layer, and the left ureter was separated and exposed. Third, the left ureter was ligated with 4-0 silk sutures in the UUO model group. Mice from the sham group received the same incision and closure procedures without ligation. UUO mice were treated by intragastric administration of As IV (20 and 40 mg/kg/day) ($n = 5$ per group) for 1 week. Mice from the sham group were administered with equal volumes of sterile saline. The left kidney was taken out after 1 week, part of the kidney tissue was stored with 4% paraformaldehyde, and the remaining kidney tissue was stored in liquid nitrogen. The experiments were repeated five times.

2.3. Cell culture

HK-2 cells were obtained from Guge Bioengineering Co., Ltd. (Wuhan, China) and cultured in DMEM/F12 medium supplemented with 10% FBS at 37 °C in a humidified atmosphere containing 5% CO_2. When the cells were grown to 70%–80% confluence, 100 ng/ml LPS with or without As IV (10 and 20 μM) and TAK-242(1 μM) stimulated cells for 48 h.

2.4. Immunohistochemistry

The slides were incubated for 30 min at 60 °C to closely adhere the slicing. The sections were deparaffinized by xylene and alcohol before immersion in 0.01 M citrate buffer (pH 6.0). Subsequently, the sections were heated one to two times in a microwave oven for 5–10 min until boiling. After cooling naturally, the sections were washed two times with PBS, added with 30% H_2O_2 at room temperature for 5–10 min to inactivate the endogenous enzymes, washed three times with distilled water, and then added with 5% BSA blocking solution at room temperature for 20 min. The liquid was shaken off without washing. The sections were incubated overnight with primary antibodies at 4 °C. The sections were washed, further incubated with horseradish peroxidase-labeled goat anti-rabbit polyclonal antibody for 10 min at 37 °C, and developed with 3,3-diaminobenzidine for color reaction. The color reaction was stopped by washing two times with distilled water, and the sections were counterstained with hematoxylin.

2.5. PCR

A 50–100 mg sample of renal tissue was ground to a powder with liquid nitrogen and then mixed with 1 ml of Trizol. The supernatant was extracted by centrifugation at 12,000 × *g* for 5 min, added with 0.2 ml of chloroform, and then incubated on ice for 5 min. The upper phase was transferred to a new 1.5 ml tube after centrifugation at < 12.000 × *g* for 10 min, added with 0.5 ml of isopropanol, and then incubated on ice for 5 min. The supernatant was discarded via centrifugation at <12.000 × *g* for 5 min. A 1 ml aliquot of 75% ethanol was added, and the supernatant was discarded after centrifugation at 2000 × *g* for 5 min. DEPC water was added to dissolved RNA. RNA concentration was determined by 260/280 absorbance ratio. A cDNA copy was created with the PrimeScript™ RT reagent kit (TaKaRa, Japan). RT-PCR was performed on the resulting cDNA with the SYBR Green Mix and the AB7500 RT-PCR detection system. The primer sequences were as follows: forward 5′-TCCCCAAAGGGATGAGAAG-3′ and reverse 5′-CACTTGGTGGTTTGCTACGA′ for mouse TNF-α; forward 5′-GCAACTGTTCCTGAACTCAACT-3′ and reverse 5′-ATCTTTTGGGGTCCGTCCAACT-3′ for mouse IL-β; and forward 5′-AGTGGCAAAGTGGAGATT-3′ and reverse 5′-GTGGAGTCATACTGGAACA-3′ for mouse GAPDH. The threshold cycle (Ct) values of each sample were calculated with the $2^{-\Delta\Delta CT}$ data analysis method.

2.6. Western blot

Renal tissue and cells were lysed with PMSF and RIPA solutions. Protein concentration was determined using the BCA protein quantification kit. Each well was loaded with 25 μg of protein and initially electrophoresed at 60 V constant current to separate proteins. When the dye passed through the stacking gel, electrophoresis was continually increased to 100 V until the dye has run off at the bottom of the gel. The gel was soaked, and the polyvinylidene difluoride membrane was placed in the transfer buffer for 15 min. Electrophoresed transfer was conducted at 200 mA for 2 h. The membranes were blocked in 5% non-fat milk–TBST solution for 1 h while shaking. After removing the blocking solution, the membrane was incubated in 10 ml of 5% non-fat milk in TBST solution, added with primary antibodies, and then shaken overnight at 4 °C. The membrane was incubated in 10 ml of 5% non-fat milk in TBST solution and then added with secondary antibodies with shaking. The membranes were scanned under an infrared imaging system (Odyssey, LI-COR, USA).

2.7. ELISA

Cell culture medium was collected in sterile tubes via centrifugation at 2000–3000 r/min for 20. In accordance with the manufacturer's instructions, 100 μl samples and standards were added in each well, and the reaction took 90 min at 37 °C without washing. The wells were incubated with biotin-labeled antibodies at 37 °C for 60 min and then washed three times with 0.01 M TBS. The avidin–biotin–peroxidase complex (100 μl, ABC) was incubated at 37 °C for 30 min and then washed five times with 0.01 M TBS. TMB horseradish peroxidase color development solution was added in each well at 37 °C in a dark room for 30 min, and then the reaction was terminated. The absorbance at 450 nm was recorded.

2.8. Statistical analysis

Data were presented as mean ± SE. All experimental data were analyzed using SPSSl7.0. Statistical significance was determined using one-way ANOVA. $P < 0.05$ was considered statistically significant.

3. Results

3.1. As IV inhibited ECM accumulation in UUO mice

The results of Immunohistochemical staining and quantification showed that fibronectin and collagen I significantly increased in the UUO model group compared with the sham group. Fibronectin and collagen I significantly decreased in the treatment groups compared with the UUO group (Fig. 1A–C). The results of the Western blot were consistent with Immunohistochemical staining (Fig. 1D–F).

3.2. As IV inhibited inflammatory cell infiltration and inflammatory cytokine secretion in UUO mice

The results showed the infiltration of macrophages (CD68) and lymphocytes (CD3) in the UUO model group compared with the sham