Preventive Effects and Mechanisms of Rhein on Renal Interstitial Fibrosis in Obstructive Nephropathy

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Received January 3, 2011; accepted February 28, 2011; published online May 20, 2011

Renal interstitial fibrosis is a common outcome of a variety of chronic renal diseases. Here we evaluated the therapeutic efficacy of rhein on renal interstitial fibrosis induced by unilateral ureteral obstruction (UOO) and investigated the potential mechanisms. Mice underwent UUO, followed by orally administrated rhein (150 mg/kg/d) or control vehicle. Renal interstitial injury and the degree of fibrosis were evaluated by pathological staining and Western blot. The possible mechanisms were studied by Western blot, indirect immune-fluorescence and enzyme-linked immunosorbent assay. Our results showed that rhein therapy markedly ameliorated renal interstitial fibrotic lesions, reduced α-smooth muscle actin (α-SMA) expression, attenuated deposition of fibronectin (FN). Rhein also suppressed transforming growth factor-β1 (TGF-β1) and its type I receptor expression in obstructed kidneys. In vitro, rhein abolished the α-SMA and fibronectin expression of rat kidney interstitial fibroblasts cells (NRK-49F) induced by TGF-β1. These observations strongly suggest that rhein is a potent inhibitor of renal interstitial fibrosis, and its therapeutic mechanism is, at least in part, blocking interstitial fibroblasts cells activation.

Key words: rhein; renal interstitial fibrosis; interstitial fibroblast; transforming growth factor-β1

Chronic kidney disease (CKD) leading to end-stage kidney failure is associated with interstitial kidney fibrosis regardless of the underlying cause.¹ As of now, there are no specific treatments to target fibrosis in the clinic.¹,² Renal interstitial fibrosis is characterized by tubular atrophy/dilation, interstitial leukocyte infiltration, and increased interstitial matrix deposition.³ Many different cell types and cytokines are involved, α-smooth muscle actin (α-SMA)-positive myofibroblasts are the principal effect cells in fibrotic kidney and transforming growth factor-β1 (TGF-β1) plays the central role.⁴,⁵ For many years a common notion was that activated myofibroblasts arise primarily from resident fibroblasts, recent evidence has demonstrated that during fibrosis, activated fibroblasts can also arise from epithelial cells via epithelial-to-mesenchymal transition (EMT) and can be recruited from the bone marrow.⁶ Therefore, delineation of the originality and mechanism of activation of the matrix-producing myofibroblast cells may be indispensable for designing rational therapeutic strategies for effective treatment of renal interstitial fibrosis.

Earlier studies have identified hepatocyte growth factor (HGF) as a potent inhibitor of renal fibrosis. HGF can efficiently block myofibroblastic transition from interstitial fibroblast and tubular epithelial cells triggered by TGF-β1.⁷,⁸ Administration of HGF protein or its gene prevents renal interstitial fibrosis in numerous animal models of renal diseases.⁹–¹² But renal fibrosis is a long process, as for the short half life period and high price, it is impossible to treat renal fibrosis by HGF in clinic.

Rhubarb, one of popular traditional Chinese herbal medicine, has been widely used for the treatment of renal diseases in traditional Chinese medicine. Although the clinical efficacy of rhubarb has been established, the mechanisms against renal diseases were still unclear. Rhein is one of the most bioactive components from rhubarb. Earlier studies have identified rhein as a potent inhibitor of hepatic fibrosis induced by carbon tetrachloride, which is capable of reducing α-SMA expression, collagen synthesis and deposition in TGF-β1-stimulated hepatic stellate cells.¹³

In the present study we explored the therapeutic efficacy of rhein on renal interstitial fibrosis induced by unilateral ureteral obstruction (UUO) and investigated potential mechanisms. Our study showed that rhein could ameliorate renal interstitial fibrosis, reduce α-smooth muscle actin (α-SMA) expression, attenuate deposition of fibronectin and suppress transforming growth factor-β1 (TGF-β1) and its type I receptor expression in obstructive nephropathy. In vitro, rhein abolished the phenotypic conversion of rat kidney interstitial fibroblasts cells induced by TGF-β1. The results suggest that rhein may be a potent drug to reduce renal fibrosis during chronic kidney disease progress, and its therapeutic mechanism is, at least in part, blocking interstitial fibroblasts cells activation.

MATERIALS AND METHODS

Materials Rhein (purity 99%) was extracted and identified by the Chinese National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China), Recombinant human TGF-β1 was purchased from Peprotech, U.S.A. Antibodies against α-SMA, fibronectin and TGF-β1 type I receptor were from Sigma, U.S.A. Dulbecco’s modified Eagle’s medium/F12 (DMEM/F12), were from Invitrogen, U.S.A. and fetal calf serum (FCS) was from Sijiqing, China. Horseradish peroxidase-conjugated secondary antibodies and cyanine Cy2-conjugated secondary antibodies were from Sigma, U.S.A. TGF-β1 enzyme-linked immunosorbent assay (ELISA) kit was from R&D U.S.A. Bicinchoninic acid protein assay kit was from Sigma, U.S.A.

Animal Model Male CD-1 mice that weighed 18 to 22 g were obtained from animal center of Nanjing Medical Uni-

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versity. They were housed in the animal facilities of the Nanjing Medical University, with free access to food and water. Animals were treated humanely by use of the protocols that were approved by the Institutional Animal Use and Care Committee at the Nanjing Medical University. Mice were divided randomly into sham group, UUO with rhein therapy group and UUO with vehicle control group. All the unilateral ureteral obstruction (UUO) operations were performed using an established procedure in 3 h. Rhein was dissolved by 20% alcohol. Every day after UUO operation the mice were given rhein (150 mg/kg) or the same volume of vehicle until they were sacrificed. The first administration was done after mice recovered from anesthesia of operation immediately. The first day after operation, the mice had their second administration, and so on. Groups of mice (n=6) were sacrificed at days 3 and 7 after UUO with or without rhein therapy, respectively. One group of sham-operated mice (n=4) was killed at day 7 after surgery. One part of the kidneys was fixed in 10% phosphate-buffered formalin, followed by paraffin embedding for pathological studies. The remaining kidneys were snap-frozen in liquid nitrogen and stored at −80 °C for ELISA and protein studies.

**Histological and Pathological Staining** Kidney sections from paraffin-embedded tissues were prepared at 4-μm thickness using a routine procedure. Sections were stained with hematoxylin/eosin for general histology. Another set of sections was stained using the Masson’s trichrome staining method for identifying interstitial collagen by blue color. A morphological analysis was used for semi-quantitatively determining the extent of tubulointerstitial injury. Briefly, 5 vision (upper left, lower left, upper right, lower right and middle) were observed under low magnification per section, each of 8 parameters of tubulointerstitial injury (tubular epithelial cell degeneration, tubular dilatation, tubular atrophy, red cell cast, protein cast, interstitial edema, interstitial fibrosis and interstitial infiltration of inflammatory cells) was assigned a score from 0 to 3 according to severity (0=no abnormality, 1=mild, 2=moderate, 3=severe), and these scores added to yield an overall tubulointerstitial score (TIS) from 0 to 24 (arbitrary units). The pathologist was unaware of the group assignment of individual mice.

**Cell Culture and Treatment** Normal rat kidney interstitial fibroblast cells (NRK-49F) were obtained from American Type Culture Collection. The immortalized rat renal interstitial fibroblasts (NRK-49F) was cultured in DMEM/F12 containing 100 U/ml penicillin, 100 mg/ml streptomycin and 10% FCS at 37 °C in 5% CO2. The NRK-49F cells were seeded on six-well culture plates to 60 to 70% confluence in complete medium containing 10% fetal bovine serum for 20 min. Protein concentration was determined using a bichinchoninic acid protein assay kit, and whole tissue lysates were mixed with an equal amount 2×SDS loading buffer (125 mmol/l Tris–HCl, 4% SDS, 20% glycerol, 100 mmol/l dithiothreitol, and 0.2% bromphenol blue). Cells were lysed with SDS sample buffer. Samples were heated at 100 °C for 5 to 10 min before loading and were separated on 10% SDS-polyacrylamide gels. The proteins were electrotransferred to a nitrocellulose membrane in transfer buffer containing 49 mmol/l Tris–HCl, 39 mmol/l glycine, 0.037% SDS, and 20% methanol at 4 °C for 1 h. Nonspecific binding to the membrane was blocked for 1 h at room temperature with 5% non-fat milk in TBS buffer (20 mmol/l Tris–HCl, 150 mmol/l NaCl, and 0.1% Tween 20). The membranes were then incubated for 16 h at 4 °C with various primary antibodies in blocking buffer containing 5% milk at the dilutions specified by the manufacturer, followed by incubation with horseradish peroxidase-conjugated secondary anti-body for 1 h in 5% nonfat milk dissolved in Tris-buffered saline. Membranes were then washed with Tris-buffered saline buffer, and the signals were visualized using the enhanced chemiluminescence system.

**Indirect Immunofluorescence** Indirect immunofluorescence staining was performed using an established procedure. Briefly, cells that were cultured on coverslips were washed with cold phosphate buffered saline (PBS) twice and fixed with cold methanol:acetone (1:1) for 10 min at −20 °C. After extensive washing three times with PBS that contained 0.5% BSA, the cells were blocked with 20% normal donkey serum in PBS buffer and then incubated with the specific primary antibodies against α-SMA and fibrinectin. For visualizing primary antibodies, cells were stained with cyanine Cy2-conjugated secondary antibodies. Cells were double stained with DAPI (4',6-diamidino-2-phenylindole, HCl) to visualize the nuclei. Stained cells viewed under Nikon Eclipse E600 Epi-fluorescence microscope equipped with a digital camera.

**Enzyme-Linked Immunosorbent Assay** To measure renal TGF-β1 levels, kidneys from mice were homogenized in the extraction buffer containing 20 mm Tris–HCl, pH 7.5, 2 ml NaCl, 0.1% Tween 80, 1 mm ethylenediamine tetracacetate, and 1 mm phenylmethylsulfonyl fluoride, and the supernatant was recovered after centrifugation at 19000×g for 20 min at 4 °C. Renal tissue TGF-β1 level was determined by using the commercial Quantikine TGF-β1 ELISA kit in accordance with the protocol specified by the manufacturer. This kit measures the abundance of active TGF-β1 protein that binds to its soluble type II receptor precoated onto a microplate. Total protein levels were determined by using a bicinechonic acid protein assay kit as described above. The concentration of TGF-β1 in kidneys was expressed as picograms per milligram of total protein.

**Statistical Analysis** All data examined were expressed as mean±S.E. Statistical analysis was performed using SigmaStat software. Comparison between groups was made using one-way analysis of variance followed by Student-
Newman–Keuls test. A p value of less than 0.05 was considered to be statistically significant.

RESULTS

Rhein Reduces Renal Fibrosis in Obstructive Nephropathy To assess the potential effect of rhein on renal fibrosis, mice were given rhein (150 mg/kg/d) or control vehicle after common UUO. Figure 1 shows representative micrographs of the Hematoxylin–Eosin (HE) and Masson’s trichrome staining of renal tissue sections at 3 or 7 d after UUO. In mice treated with control vehicle, extensive periblial and interstitial collagen deposition was evident, as shown by positive Masson’s trichrome staining. However, delivery of rhein largely inhibited renal interstitial collagen accumulation after UUO. Collagen staining was much weaker in the enlarged periductal area.

To quantitatively evaluate the therapeutic efficacy of rhein on renal interstitial injury induced by UUO, we determined the tubulointerstitial score from 8 parameters as mentioned previously. As shown in Fig. 1M, the tubulointerstitial score was progressively increased at 3 and 7 d after UUO. However, rhein treatment substantially reduced the tubulointerstitial score in the renal tissue sections at different time points. The main difference between rhein treatment group and UUO group is mainly due to the interstitial fibrosis grading. The extent of tubular epithelial cell degeneration, tubular atrophy and interstitial infiltration of inflammatory cells are also reduced in the rhein treatment group. These results suggest that rhein is capable of protecting the kidney from the development of tubulointerstitial lesions after UUO.

Rhein Inhibits Myofibroblasts Activation and Extracellular Matrix Accumulation in Obstructive Nephropathy We further examined activation of myofibroblasts and the accumulation and deposition of the specific extracellular matrix components by Western blot. Because α-SMA-positive myofibroblasts are the effector cells that are primarily responsible for the overproduction of extracellular matrix at pathogenic conditions and there is almost no myofibroblasts in the normal renal tissues, we choose α-SMA as the mark of myofibroblasts activation. Extracellular matrix accumulation and deposition is the other character of interstitial fibrosis, fibronectin (FN) was selected as representative of extracellular matrix. As demonstrated in Fig. 2, renal tissues at 3 and 7 d after UUO displayed stronger protein expression of α-SMA and FN, suggesting activation of the renal myofibroblasts and extracellular matrix accumulation following UUO-induced injury. Consistent with the pathological staining, Western blot results showed that delivery of rhein largely blocked the renal α-SMA and FN expression, which means rhein treatment could suppress the activation of myofibroblasts and accumulation of the extracellular matrix.

Rhein Suppresses the Expression of TGF-β1 and Its Type I Receptor in the Obstructed Kidneys Because TGF-β1 is a fibrogenic cytokine that is believed to play a central role in regulating tissue fibrosis, we next investigated the effect of rhein on renal TGF-β1 and its type I receptor expression after UUO operation. As presented in Fig. 3A, UUO induced a dramatic increase in renal TGF-β1 protein expression, as determined by ELISA. The steady-state levels of TGF-β1 in the kidney at 7 d after UUO were increased about more than 10-fold, compared to the sham controls. However, rhein markedly suppressed renal TGF-β1 protein abundance. The expression of TGF-β1 type I receptor (Tβ1R-I) in the kidney was also examined at different time points after UUO by Western blot. As shown in Figs. 3B and C, rhein significantly altered renal Tβ1R-I expression at 3 and 7 d after UUO.

Rhein Abrogates TGF-β1-Induced Myofibroblastic Activation Because α-SMA expression is the hallmark for myofibroblasts, we investigated the myofibroblastic activation from renal interstitial fibroblast cells by examining the α-SMA protein expression after incubation with various agents. As shown in Fig. 4, TGF-β1 markedly induced de novo α-SMA expression in renal interstitial fibroblast NRK-49F cells. Simultaneous incubation with rhein dramatically repressed TGF-β1-initiated α-SMA expression of NRK-49F cells in a time and dose-dependent manner. This result was independently confirmed by an indirect immunofluorescence staining for α-SMA in NRK-49F cells. As shown in Fig. 4F, TGF-β1 induced de novo expression of α-SMA that was assembled into abundant α-SMA-positive microfilament fibers in the cytoplasm of NRK-49F cells. Simultaneous incubation with rhein primarily abolished the α-SMA staining induced by TGF-β1 (Fig. 4G). These results indicate that rhein elicits its anti-fibrotic actions by suppressing myofibroblastic activation of renal interstitial fibroblasts initiated by profibrotic cytokine TGF-β1.

Rhein Inhibits FN Expression in NRK-49F Cells Induced by TGF-β1 Western blot and indirect immuno-fluorescence staining were used to examine the effect of rhein on the expression of interstitial matrix components in NRK-49F cells activated by TGF-β1. As shown in Fig. 5, NRK-49F cells at basal conditions expressed trivial amount of FN. However, after myofibroblastic activation by TGF-β1, the NRK-49F cells became activated, principal matrix producing cells that expressed a striking amount of interstitial FN. Simultaneous incubation with rhein blocked the expression of FN induced by TGF-β1. Indirect immunofluorescence staining for FN in NRK-49F cells confirmed this result. Thus, on activation by TGF-β1, renal interstitial fibroblast cells are transformed into activated myofibroblasts that produce large amounts of interstitial matrix; and rhein completely blocks this process of myofibroblastic activation and its subsequent matrix accumulation and deposition.

Rhein’s Doesn’t Inhibit the Expression of TGF-β1 Type I Receptor in NRK-49F Cells Activated by TGF-β1 Western blot was used to examine the effect of rhein on the expression of TGF-β1 type I receptor (Tβ1R-I) in NRK-49F cells activated by TGF-β1. As shown in Fig. 6, after incubated with TGF-β1 (2 ng/ml) or/and rhein (1 ng/ml) for 24 or 48 h, the protein expression of Tβ1R-I in NRK-49F cells did have a little change, but the difference had no statistical significance.

DISCUSSION

Under most circumstances when patients are diagnosed with chronic kidney disease, their kidneys may already display different degrees of renal fibrosis. Hence, a key to an effective therapy for CKD is to develop a strategy that blocks the progression of an established renal fibrosis. The purpose
of this study was to test whether rhein, which has been shown to be preventive in retarding the onset of hepatic fibrosis induced by carbon tetrachloride, also has therapeutic effects to ameliorate renal fibrosis. Although the pathological mechanism underlying chronic obstructive nephropathy is not completely elucidated, the fibrogenic process clearly plays a critical role in ultimately leading to permanent loss of the normal structural and functional integrity of the kidney. Our results demonstrate that rhein markedly ameliorate renal tubulointerstitial fibrosis in UUO mice (see in Fig. 1). The character of renal tubulointerstitial fibrosis is the destruction of normal tissue and excess production and deposition of extracellular matrix components. And α-SMA-positive interstitial myofibroblast cells are responsible for relentless accumulation and deposition of extracellular matrix in the interstitial compartments of diseased kidneys. Suppress
renal myofibroblast activation can attenuates renal interstitial matrix deposition in the obstructed kidneys. Because myofibroblast cells are α-SMA-positive and not present in normal kidneys, we can evaluate the activation degree of myofibroblast by observing the α-SMA expression in the kidneys. As seen in Fig. 2, the protein expression levels of α-SMA in UUO mice renal tissues were significantly higher than the normal control group, rhein significantly reduced protein expression of α-smooth muscle actin in kidneys with unilateral ureteral obstruction. That means rhein can inhibit myofibroblast activation in obstructed kidneys. And consistent with reduction of α-SMA expression, the extracellular matrix protein expressions (FN) are also suppressed by rhein.

The origin of myofibroblast cells under pathological conditions is uncertain. They are often presumed to derive from local resident interstitial fibroblasts. Recently, emerging evidence suggests that these cells may also come from tubular epithelial cells via a process known as epithelial-to-mesenchymal transition.6) In response to tissue injury, renal interstitial fibroblasts undergo an activation process to become α-SMA-positive myofibroblast cells. Profibrotic TGF-β1 is the prime stimulator of this phenotypic activation, as shown in previous studies.18) We use rat kidney interstitial fibroblasts cells (NRK-49F) as an in vitro system, compare the expression of protein levels of α-smooth muscle actin and fibronectin between the normal control group, transforming growth factor-β1 activated group and rhein intervention group by Western blots and indirect immunofluorescence. Western blot detection showed that rhein significantly inhibited α-SMA protein expression of NRK-49F cells induced by TGF-β1 in a time and dose-dependent manner. As the same way, rhein reduced fibronectin expression of NRK-49F cells induced by TGF-β1. Indirect immunofluorescence results were consistent with Western blot. So rhein may reduce the extent of renal interstitial fibrosis of unilateral ureteral obstruction mice, at least in part, by inhibit renal interstitial fibroblasts activation.

Rhein blockade of myofibroblastic activation from interstitial fibroblast cells is likely mediated by antagonizing TGF-β1 signaling. TGF-β1, on binding to its type II and type I receptors that contains a cytoplasmic serine/threonine kinase domain, initiates a cascade of signaling transduction events involving intermediate mediator Smad proteins.23,24) There are three classes of Smads: the receptor-regulated Smads (R-Smads), the common Smad (Co-Smad), and the inhibitory Smads (I-Smads), each of which have distinct functions.21,22) Earlier studies reveal that TGF-β1 specifically initiates Smad-2 and -3 phosphorylation, which in turn bind to Co-Smad-4 and translocate into the nuclei, where they control the transcription of TGF-β1-responsive genes.21,24) Thus, disruption of any steps in this cascade of signal transduction processes may potentially lead to interception of TGF-β1 signaling that results in blockage of myofibroblastic activa-
In addition, induction of inhibitory Smad proteins could lead to suppression of R-Smad signaling by competitively binding to TGF-β1 receptors.25—27) We found that neither the phosphorylation of Smad-2/3 nor the expression of inhibitory Smad-6 and -7 were significantly altered by rhein (data not shown) and further research is needed.

We also examine the effect of rhein on the TGF-β1 and its type I receptor expression in vivo and in vitro. TGF-β1 plays a determinant role in the pathological accumulation of extracellular matrix in normal tissues after various injurious insults. Up-regulation of TGF-β1 expression is found in most, if not all, forms of chronic renal fibrotic diseases in animal models and in patients.28) Consistent with early study, the protein expression of TGF-β1 increased significantly after UUO operation (Fig. 3). TGF-β1 not only induces myofibroblastic activation from interstitial quiescent fibroblasts, but also initiates myofibroblastic transition from tubular epithelial cells at the advanced stage of chronic renal diseases.

Over-expression of TGF-β1 in transgenic mice induced increased expression of fibrotic matrix protein. Conversely, suppression of TGF-β1 signaling either by truncated, soluble receptor or by an antisense approach significantly retards the progression of renal interstitial fibrosis in animals.29,30) In present study, we observed that rhein therapy can reduce the TGF-β1 and Tβ1R-I levels of renal tissues in unilateral ureteral obstruction mice. But we did not observe the similar phenomenon in vitro. The protein expression of Tβ1R-I in NRK-49F cells was not significantly altered by rhein. So the effect of rhein on TGF-β1 axis expression is more complicated than what we have thought. The decreased TGF-β1 and its type I receptor expression in vivo may be due to an indirect effect of rhein or a secondary consequence resulting from the antifibrotic action of rhein. Many other cell types and cytokines must be involved, and more researches are needed. Together with the early study, rhein can indirectly reduce renal interstitial fibroblast activation and epithelial-to-

Fig. 4. Rhein Abrogates the Expression of α-SMA in Renal Interstitial Fibroblast NRK-49F Cells

Western blot and semi-quantitative analysis show rhein inhibit α-SMA expression of NRK-49F cells induced by TGF-β1 in a time- and dose-dependent manner. NRK-49F cells incubated with TGF-β1 (2 ng/ml) and rhein (1 ng/ml) for various periods of time as indicated (A, B), or increasing amounts of rhein for 48 h (C, D). Immunofluorescence staining shows the α-SMA expression in NRK-49F cells after various treatments for 48 h. C, H: control; F, I: 2 ng/ml of TGF-β1; G, J: 2 ng/ml of TGF-β1 and 1 ng/ml of rhein. *p, **p, #p and ##p < 0.05.
mesenchymal transition tubular by inhibit the expression TGF-\(\beta\)1 through an unknown mechanism. These observations highlight the effectiveness and efficacy of rhein as a potential therapeutic agent for inhibiting myofibroblast accumulation and interstitial fibrogenesis in the diseased kidneys.

In summary, this study suggests that rhein acts as a negative regulator of renal interstitial fibrosis that reduces renal fibrosis induced by UUO. And its therapeutic mechanism is, at least in part, blocking interstitial fibroblasts cells activation. In this regard, the present study not only underscores that the blockade of the activation of interstitial fibroblast cells is a novel strategy for prevention of fibrotic diseases but also sets a foundation for the rational utilization of rhein in combating renal fibrosis.

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