Astragaloside IV Ameliorates Renal Fibrosis via the Inhibition of Mitogen-Activated Protein Kinases and Antiapoptosis In Vivo and In Vitro

Weijia Xu, Xinghua Shao, Lei Tian, Leyi Gu, Minfang Zhang, Qin Wang, Bei Wu, Ling Wang, Jufang Yao, Xiaoping Xu, Shan Mou, and Zhaohui Ni

Department of Nephrology, Molecular Cell Laboratory for Kidney Disease (W.X., X.S., L.T., L.G., M.Z., Q.W., B.W., L.W., S.M., Z.N.), Animal Centre (J.Y.), and Department of Biochemical Laboratory (X.X.), Ren Ji Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai, China

Received March 5, 2014; accepted June 19, 2014

ABSTRACT

Apoptosis of renal tubular cells plays a crucial role in renal fibrosis. Astragaloside IV (AS-IV), a compound extracted from Radix Astragali, has been shown to inhibit renal tubular cell apoptosis induced by high glucose, but its role in preventing chronic renal fibrosis as well as the underlying molecular mechanisms involved still remain obscure. In this study, human kidney tubular epithelial cells induced by transforming growth factor-β1 (TGF-β1) were used to investigate the protective role of AS-IV in antifibrosis. As an in vitro model, mice subjected to unilateral ureteral obstruction (UUO) were administered AS-IV (20 mg/kg) by intraperitoneal injection for 7 days. AS-IV significantly alleviated renal mass loss and reduced the expression of α-smooth muscle actin, fibronectin, and collagen IV both in vitro and in vivo, suggesting that this compound functions in the inhibition of renal tubulointerstitial fibrosis. Furthermore, transferase-mediated dUTP nick-end labeling assay results both in vitro and in vivo showed that AS-IV significantly attenuated both UUO and TGF-β1–induced cell apoptosis and prevented renal tubular epithelial cell injury in a dose-dependent manner. Western blotting results also revealed that the antiapoptotic effect of AS-IV was reflected in the inhibition of caspase-3 activation, which might be mediated primarily by the downregulation of mitogen-activated protein kinase effectors phospho-p38 and phospho-c-Jun N-terminal kinase. These data infer that AS-IV effectively attenuates the progression of renal fibrosis after UUO injury and may have a promising clinical role as a potential antifibrosis treatment in patients with chronic kidney disease.

Introduction

Chronic kidney disease (CKD) is recognized as a global public health burden that can progress into end-stage renal disease and consume a significant proportion of health resources. Predominate over glomerulosclerosis, tubulointerstitial fibrosis has been shown to be an important feature of CKD and is correlated with a loss of kidney function (Schainuck et al., 2002, Nlandu Khodo et al., 2012). The mitogen-activated protein kinase (MAPK) pathway operates as a key cell signaling pathway of apoptosis and inflammation. It has been shown that activation of the MAPK pathway may be involved in tubular atrophy, whereas inhibition of p38 MAPK attenuates the progression of renal fibrosis after UUO injury and may have a promising clinical role as a potential antifibrosis treatment in patients with chronic kidney disease (Wang et al., 2013).

The critical role of apoptosis in various renal injuries has been well established, and numerous studies have demonstrated that renal fibrosis is associated with enhanced tubular cell apoptosis (Gobé and Axelsen, 1987; Klahr and Morrissey, 2002, Nlandu Khodo et al., 2012). The mitogen-activated protein kinase (MAPK) pathway operates as a key cell signaling pathway of apoptosis and inflammation. It has been shown that activation of the MAPK pathway may be involved in tubular atrophy, whereas inhibition of p38 MAPK attenuates the progression of renal fibrosis after UUO injury and may have a promising clinical role as a potential antifibrosis treatment in patients with chronic kidney disease (Wang et al., 2013).

Astragaloside IV (AS-IV) is a saponin purified from Astragalus membranaceus Bge, a traditional Chinese herb that has been widely used in the treatment of kidney disease. It has been reported that AS-IV has antiapoptotic and anti-inflammatory effects in two rodent models: renal ischemic-reperfusion injury and contrast-induced nephropathy (Gui et al., 2013). However, the protective effects of AS-IV on CKD are poorly understood. Unilateral ureteral obstruction (UUO) is a classic model of renal tubular stress that induces kidney fibrosis (Nlandu Khodo et al., 2012, Smeets et al., 2013). Our previous work (Wang et al., 2013)
et al., 2014) demonstrated that AS-IV protected renal tubular cells from high-glucose–induced injury by reducing tubular cell apoptosis. To determine the effect of AS-IV on the progression of kidney fibrosis, mice were subjected to UUO as an in vivo model, and transforming growth factor-β1 (TGF-β1)–induced injury in renal tubular epithelial cells was used as an in vitro model. The aim of this study was to test our hypothesis that AS-IV prevents tubular epithelial apoptosis by inhibiting MAPK pathway activity, resulting in the prevention of renal fibrosis progression in obstructive nephropathy.

Materials and Methods

Drugs. Astragaloside IV (Fig. 1) was purchased from Shanghai PureOne Biotechnology (Shanghai, China), and its purity was greater than 98%. For the in vivo study, AS-IV was dissolved in 1% dimethylsulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO) and diluted in olive oil. For the in vitro study, AS-IV was stored as a stock solution at a concentration of 10 mg/ml and diluted with incubation medium as necessary; the final concentration did not exceed 0.1% (v/v). The compounds were prepared as a suspension in the medium prior to administration to the animals.

Murine Model of UUO. All animal procedures were approved by the Shanghai-Jiao Tong University School of Medicine. Male C57BL/6 mice (Shanghai Laboratory Animal Center, Shanghai, China), aged 6–8 weeks, were housed in an air-conditioned room at 23–1°C with 40% humidity on a 12-hour light/dark cycle. The animals were allowed free access to water and standard chow. The animals were randomly divided into three groups: sham-operated (Sham, n = 8), UUO model (UUO, n = 10), and AS-IV–treated UUO model (AS-IV, n = 10) groups. The UUO operation was performed on the UUO and AS-IV groups. Animals were anesthetized with 3.5% chloral hydrate (350 mg/kg i.p.) (Sangon Biotech, Shanghai, China). For the UUO operation, the right ureters were visualized through a flank incision and double-ligated with 6-0 silk. The sham group mice underwent the same operation as the UUO mice with the exception of the ligation. For the AS-IV group, AS-IV (20 mg/kg per day) was administered by intraperitoneal injection every day from day 9 (day of operation) to day 7. Both control and UUO mice were injected with an equal volume of vehicle (as described above).

Serum was obtained from the suborbital vein at either 7 or 14 days after the operation for renal function detection. Animals were sacrificed at either 7 or 14 days after surgery, and both kidneys were collected for histology, immunohistochemistry, Western blotting, and real-time polymerase chain reaction (PCR) analysis. One part of the unilateral kidney was fixed in 10% phosphate-buffered formalin for histologic studies. Another segment was fixed with 2.5% glutaraldehyde in 4°C phosphate-buffered saline overnight, postfixed in 1.0% OsO4, and embedded in LR White resin (London Resin Co., Basingstoke, Hampshire, UK) for transmission electron microscopy. The remaining tissue was snap-frozen in liquid nitrogen and stored at −80°C for protein and RNA extraction.

Cell Culture and Treatments. Human kidney tubular epithelial (HK2) cells were purchased from American Type Culture Collection (CRL-1571; Manassas, VA) and cultured in Gibco RPMI 1640 (Life Technologies, Grand Island, NY) with 10% Gibco fetal bovine serum (Life Technologies) in an atmosphere of 5% CO2 at 37°C. To determine the effects of AS-IV treatment on the epithelial-mesenchymal transition (EMT), HK2 cells were starved for 24 hours by incubation with RPMI 1640 containing 0.5% fetal bovine serum and exposed to various experimental conditions. The cells were divided into the following groups: 1) normal control group (Ct) incubated in RPMI 1640 containing 0.1% DMSO (i.e., vehicle); 2) TGF-β1 group stimulated with recombinant TGF-β1 (10 ng/ml; R&D Systems Europe, Abingdon, UK); and 3) AS-IV–treated group stimulated with recombinant TGF-β1 (10 ng/ml) and simultaneously treated with different concentrations of AS-IV (50, 100, and 200 μg/ml) for 24 or 48 hours. All experimental groups were cultured in quadruplicate.

Histologic Evaluation. Transverse kidney slices were sectioned at 2 μm and stained with hematoxylin and eosin and Masson trichrome to evaluate the severity of renal tubule injury in the corticomedullary junction. Ten nonoverlapping microscopic fields of each kidney section stained with Masson trichrome were randomly selected and observed under light microscopy at a magnification (400×) by an experienced expert who was blinded to the treatment each animal had received. For the Masson trichrome staining, poneoue and acid fuchsin (Shanghai SSS Reagent Co. Ltd, Shanghai, China) were used to stain the immune complex, with light green SF (China National Pharmaceutical Group Corporation, Shanghai, China) stained as green and phosophatugic acid (China National Pharmaceutical Group Corporation) stained as blue. Light green was used for staining collagen. Masson trichrome staining was performed as described previously (Long et al., 2012). The collagen levels in the tissue area were quantitatively measured using Image Pro-Plus Software (Media Cybernetics, Rockville, MD) by drawing a line around the perimeter of the positive staining area (green). The average ratio for each microscopic field (400×) was calculated and graphed.

RNA Preparation and Real-Time PCR. Total RNA was isolated from kidney cells using Trizol reagent (Invitrogen/Life Technologies). Total RNAs were isolated and reverse transcribed to cDNA using the RevertAid First Strand cDNA Synthesis Kit (ThermoScientific, Pittsburgh, PA). The PCR amplification was performed using the following primers: for GAPDH, sense, 5′-GGCGATGGTAAGATTCGGA-3′, antisense, 5′-GGGCCTCTCGTATTGTTGA-3′, for TGF-β1, sense, 5′-GGCGGTGCTCCTTTTGTAG-3′, antisense, 5′-GCCGAAATGCTGACGTATTGA-3′; for fibronectin, sense, 5′-AGGGCACAACAGACACACCAA-3′, antisense, 5′-CAGATTTCTCCACGATCGAATGGT-3′; and for β-actin, sense, 5′-GGCCCTCTGGTCATTGTTGA-3′, antisense, 5′-CACCATGCTTCTCTCTCTC-3′, for collagen IV, sense, 5′-AGGATGGATGGTCTCTGTA-3′, antisense, 5′-CAGATTTCTCAGGATCTCAGTA-3′, and for TGF-β1, sense, 5′-GGCCCTGTGTCCTTTTGTAG-3′, antisense, 5′-GCCGAAATGCTGACGTATTGA-3′. Real-time quantitative PCR was performed using SYBR Premix Ex Taq II (TaKaRa Bio Inc., Ohtsu, Japan), and the fluorescence was quantified with the PCR CFX-96 system (Bio-Rad, Mississauga, ON, Canada). The threshold cycle (Ct) was set within the exponential phase of the PCR. The relative quantities of the different mRNAs were calculated by comparing the cycle times for each target PCR. The target PCR Ct values were normalized by subtracting the Ct sample Ct sample from the Ct sample – ΔCt control method for each sample.

Immunoblot Analysis. SDS-PAGE and immunoblot analyses were carried out according to standard protocols and visualized using enhanced chemiluminescence on the immunoblot detection kits (Amersham Pharmacia Biotech UK, Little Chalfont, Buckinghamshire, UK). Polyvinylidene fluoride membranes were incubated at 4°C overnight with either rabbit polyclonal or monoclonal primary antibodies targeted to one of the following proteins (with their respective dilutions): α-SMA (1:1000; Abcam, Cambridge, UK), collagen-IV (1:500; Abcam), FGFR3 (1:1000; Abcam, Cambridge, UK), and PDGF-B (1:1000; Abcam, Cambridge, UK).
fibronectin (1:250; Abcam); and the following from Cell Signaling Technology (Danvers, MA): GAPDH (1:1000), cleave-caspase-3 (1:1000), caspase-3 (1:1000), phospho-p38 MAPK (1:1000), p38 MAPK (1:1000), c-Jun N-terminal kinase (JNK) MAPK (1:2500), phospho-JNK MAPK (1:2500), extracellular signal-regulated kinase 1/2 (ERK1/2) MAPK (1:1000), and phospho-ERK1/2 MAPK (1:1000).

Assessment of Cell Viability. Cell viability was determined using a cell counting kit-8 assay according to the manufacturer’s protocol (Beyotime Institute of Biotechnology, Jiangsu, China). The absorbance was measured using a microplate reader (ThermoScientific) at 450 and 630 nm. All of the recorded test group values were normalized to the control group and blank group values according to the protocol.

Apoptosis Assay. A transferase-mediated dUTP nick-end labeling (TUNEL) assay was performed to detect apoptotic nuclei in the kidney sections; the assay was conducted using an in situ cell death detection kit with fluorescein (Roche Diagnostics, Mannheim, Germany) according to the manufacturer’s instructions. Fluorescence microscopy was used for the qualitative detection of apoptosis at the single-cell level. Cells with nuclear positive staining were counted (original magnification, 400×) for at least three fields per section.

Statistical Analysis. All data are expressed as the mean ± S.D. The cell culture experiments were performed at least in triplicate and averaged. Statistics were analyzed using the SPSS 13.0 software (IBM, Armonk, NY). On the basis of the two-tailed Student’s t test, P < 0.05 was considered to be statistically significant.

Results

AS-IV Ameliorates the Kidney Mass Loss and Renal Tubulointerstitial Fibrosis Induced by UUO. The UUO rodent model was used to investigate the effect of AS-IV on renal tubulointerstitial fibrosis. The kidney morphology and histologic structures were vastly altered by UUO, which presented with a reduction of kidney mass and a markedly reduced volume of renal parenchyma (Fig. 2A). The kidney weight (obstructed side/body weight ratio from the UUO model was also significantly reduced compared with the control group (P < 0.01). AS-IV treatment prevented the morphologic and structural alterations of the kidney (P < 0.01) (Fig. 2B). AS-IV also significantly decreased the serum creatinine and blood urea nitrogen level after 7 days of ligation, suggesting that AS-IV played protective role after UUO injury (Fig. 2, C and D). Masson trichrome staining (Fig. 3A) and hematoxylin and eosin staining (Fig. 3B) showed severe structural damage and some tubulointerstitial fibrosis in the obstructed kidneys from the UUO group, which was characterized by extensively dilated tubules, a large amount of tubular epithelial cell apoptosis with shedding into the lumen, and inflammatory cell infiltration with adjacent accumulation of extracellular matrix. These presentations showed continued progression over the following 14 days after the UUO procedure. Kidney sections from the UUO mice treated with AS-IV showed markedly diminished histologic features consistent with renal tubular injuries.

It has been well established that α-SMA, fibronectin, and collagen IV are hallmarks of myofibroblasts and excessive extracellular matrix accumulation, both of which are increased in various models of chronic injury (Liu et al., 2011; Ma et al., 2011; Meng et al., 2011; Barrera-Chimal et al., 2013). Basal levels of fibronectin protein and mRNA were barely detected in

Fig. 2. Characterization of obstructed and nonobstructed kidneys. (A) Morphology of obstructed and nonobstructed kidneys. (B) Kidney weight/body weight (KW/BW) ratios after obstructive injury (mean ± S.E.M.). (C) Serum creatinine (Scr) of obstructed and nonobstructed kidneys (mean ± S.E.M.). (D) Blood urea nitrogen (BUN) of obstructed and nonobstructed kidneys (mean ± S.E.M.). **P < 0.01 versus sham-operated mice; *P < 0.05 versus sham-operated mice; #P < 0.01 versus UUO mice; P < 0.05 versus UUO mice.
the sham-operated mice; however, the levels on day 7 were significantly increased and showed additional elevation on day 14 (Fig. 4, A and F). Similar results were observed in the expression of α-SMA and collagen IV, for which the protein and mRNA levels increased over time after obstructive injury ($P < 0.05$). When compared with the UUO group, mice treated with AS-IV after the operation had lower expression levels of these proteins ($P < 0.05$) (Fig. 4). TGF-β1 has been identified as the most potent and universal growth factor closely associated with fibrosis in various types of models (Doi et al., 2011; Barrera-Chimal et al., 2013). We observed a significant time-dependent increase in the expression of TGF-β1 at the mRNA level ($P < 0.05$), which was ameliorated by administration of AS-IV after the obstructive injury ($P < 0.05$) (Fig. 4). These results were consistent with the histologic findings.

**AS-IV Inhibits TGF-β1-Induced Epithelial Mesenchymal Transition in HK2 Cells.** TGF-β1 is a well characterized inducer of EMT in renal tubular epithelial cells and was highly expressed in mice subjected to UUO in the current study. To investigate the protective effect of AS-IV on renal tubular epithelial cells, we cotreated HK2 cells with TGF-β1 and different concentrations of AS-IV. TGF-β1 alone (10 ng/ml) significantly upregulated the expression of α-SMA and collagen IV, both of which are EMT markers (Fig. 5). Upon cotreatment with AS-IV (50–200 μg/ml), this upregulation was inhibited in a dose-dependent manner ($P < 0.05$) (Fig. 5, A and B). As shown in Fig. 5B, TGF-β1 significantly increased fibronectin and α-SMA expression compared with normal cells after 12 hours, and this increase was more prominent after 24 hours. However, upon coadministration of AS-IV (200 μg/ml) with TGF-β1, the fibronectin and α-SMA expression levels were markedly decreased after 12 hours and were sustained after 24 hours (Fig. 5, C and D).

**AS-IV Protects against TGF-β1-Induced Cell Injury.** We tested the effect of AS-IV on cell proliferation with both a single dose and cotreatment with TGF-β1. In vitro, AS-IV showed a mild dose-dependent effect in stimulating tubular cell proliferation (Fig. 6A) at doses ranging from 50 to 200 μg/ml in normal tubular epithelial cells without cytotoxicity. The differences among these groups was not statistically significant ($P > 0.05$) (Fig. 6A). Cell viability was significantly inhibited by TGF-β1 stimulation after 24 hours ($P < 0.01$), whereas cotreatment with AS-IV notably shifted the population from apoptosis to active cell proliferation in a dose-dependent manner, with the highest dose of AS-IV (200 μg/ml) showing the greatest observed effect ($P < 0.05$) (Fig. 6B).

**AS-IV Has an Antiapoptotic Effect on Renal Tubular Cells In Vivo and In Vitro.** In vivo, TUNEL was performed to evaluate the effect of AS-IV on apoptosis after UUO. Figure 7, A and B, shows that the mice subjected to UUO had massive amounts of tubular cell apoptosis in obstructed kidneys on day 7 and additional evidence of cell death on day 14 ($P < 0.01$ compared with sham-operated mice), which was partly prevented by the administration of AS-IV ($P < 0.01$ compared with UUO mice on day 14) (Fig. 7, A and B). In vitro, HK2 cells subjected to various TGF-β1 and/or AS-IV treatments were photographed after TUNEL immunofluorescent staining. As shown in Fig. 7, C and D, treatment with TGF-β1 (10 ng/ml) alone significantly increased cell apoptosis after 12 hours with additional damage after 24 hours ($P < 0.05$ compared with the Ctl group). A single administration of the maximum dose of AS-IV (200 μg/ml) did not induce cell apoptosis and death ($P > 0.05$ compared with the Ctl group) (Fig. 7, C and D). However, AS-IV treatment inhibited the effects of TGF-β1–induced cell apoptosis in a dose-dependent manner ($P < 0.01$), with a maximal inhibitory effect at 200 μg/ml ($P < 0.05$) (Fig. 7, C and D).

We further investigated the effects of AS-IV on caspase-3 activity and cleavage (Fig. 8). The activity of caspase-3 was upregulated in obstructed kidneys compared with the kidneys in Sham group, and a reduced level of upregulation was observed in the AS-IV group ($P < 0.05$) (Fig. 8, A and B). Similarly to the in vivo results, the in vitro data showed that AS-IV reduced TGF-β1–induced caspase-3 activation in a dose-dependent manner ($P < 0.05$) (Fig. 8, C and D).

**AS-IV Partially Inhibits the Activity of the MAPK Pathway.** The MAPK pathway has been shown to be associated with apoptosis and inflammation in various studies (Pozdzik et al., 2008; Ma et al., 2011; Mulay et al., 2012; Qin et al., 2012). To address whether the MAPK pathway is involved in AS-IV–driven events, we evaluated the activity of JNK, ERK1/2, and p38 MAPK proteins both in vivo and in vitro. As shown in Fig. 9A, UUO injury markedly increased the phosphorylation of JNK, ERK1/2, and p38 in obstructed kidneys after 14 days. When AS-IV was administered after the obstruction, the phosphorylation of these three MAPKs was downregulated compared with the UUO group. Although UUO injury also increased the total protein levels of all three
kinases, treatment with AS-IV decreased the ratio of phosphorylated protein to total MAPKs (Fig. 9, A–D). These results suggest that AS-IV is able to suppress the activation of the MAPK pathway in the UUO injury model.

The in vitro study, however, yielded somewhat different results. As shown in Fig. 9, E–H, TGF-β1 significantly increased phospho-p38 and phospho-JNK expression levels compared with the control group. Phosphorylation of p38 was

Fig. 4. AS-IV inhibits the expression of fibronectin, collagen IV, α-SMA, and TGF-β1 induced by UUO. mRNA was extracted from sham-operated or obstructed kidneys of mice with/without AS-IV administration and subjected to quantitative real-time PCR. The mRNA expression of fibronectin (A), collagen IV (B), α-SMA (C), and TGF-β1 (D) are indicated as fold induction over the sham group. Kidney tissue lysates were subjected to immunoblot analysis with specific antibodies against fibronectin, collagen IV, α-SMA, or GAPDH (E). The expression levels of fibronectin (F), collagen IV (G), and α-SMA (H) were quantified by densitometry and normalized with GAPDH. The data are expressed as the means ± S.E.M. *P < 0.05; **P < 0.01 versus sham-operated mice; #P < 0.05 versus UUO mice on the same day. CNK, contralateral nonobstructed kidney.
markedly inhibited by AS-IV treatment in a dose-dependent manner (Fig. 9F), and AS-IV treatment inhibited the phospho-JNK/total JNK ratio at the highest dose (200 μg/ml) (Fig. 9G). However, we did not observe any significant variation of the phospho-ERK1/2 ratio after stimulation with TGF-β1. AS-IV mildly increased ERK phosphorylation at 200 μg/ml, which was thought to be associated with cytothesis (Fig. 9, E and H). This indicated that astragaloside IV plays an antiapoptotic role primarily by inhibiting the activation of p38 and JNK in the MAPK pathway (Fig. 9).

**Activation of the MAPK Pathway Plays a Prominent Role in the Antifibrotic Effect of AS-IV.** To further demonstrate the role of p38 MAPK in the antifibrosis process, we pretreated HK2 cells for 1 hour with 10 μM SB203580 (4-[4-(4-fluorophenyl)-2-[4-(methylsulfinyl)phenyl]-1H-imidazol-5-yl]-pyridine monohydrochloride; a specific inhibitor of phospho-p38

---

**Fig. 5.** AS-IV inhibits TGF-β1–induced α-SMA and fibronectin expression in renal tubular epithelial cells. (A and B) Immunoblot analysis shows the α-SMA, collagen IV, and fibronectin protein levels in HK2 cells after the indicated treatments. (C and D) Different time courses for the administration of TGF-β1 and AS-IV were observed to highlight the effect of the time of AS-IV administration. The data are expressed as the means ± S.E.M. *P < 0.05; **P < 0.01 versus sham-operated mice; †P < 0.05; ‡P < 0.01 versus UUO mice on the same day.
MAPK) and then stimulated the cells with TGF-β1 (10 ng/ml) for either 24 hours to detect apoptosis (Fig. 7, C and D) or 48 hours to measure the expression of fibronectin and α-SMA (Fig. 10). The results showed that apoptosis of renal tubular cells was markedly decreased (Fig. 7, C and D) and that the expression levels of fibronectin and α-SMA were clearly reduced, indicating that TGF-β1–induced EMT was reversed by inhibiting the phospho-p38 MAPK pathway. This suggests that activation of the p38 MAPK pathway appears to play a prominent role in the antifibrotic effect of AS-IV.

Discussion

*A. membranaceus* Bge, a traditional Chinese herb, has been used for over a thousand years to treat various chronic diseases. It has been reported that *A. membranaceus* Bge mitigates urine protein levels and exerts a protective effect on kidney function in patients with CKD (Chen, 2001; Li et al., 2011). Astragaloside IV, one of the major active components of *A. membranaceus*, has been detailed in various studies. Our previous work (Wang et al., 2014) demonstrated that AS-IV protected renal tubular cells from injury owing to high glucose by reducing tubular cell apoptosis. Gui et al. (2013) found that AS-IV prevented acute kidney injury in two rodent models by inhibiting oxidative stress and apoptosis pathways. Yet few studies have closely examined the effects of AS-IV on renal tubulointerstitial fibrosis. The present study found that AS-IV ameliorated renal tubulointerstitial fibrosis induced by UUO and reduced the deposition of extracellular matrix components as well as the...
were observed to have the most serious and persistent damage, and the subsequent tubulointerstitial fibrosis with regard to fibrotic correlated factors, overexpression, and accumulation. We observed that glomerulus injury recovered gradually, and injury in the corticomedullary junction and medullary areas persisted longer; fibrosis is more predisposed to develop in these two regions. After AS-IV treatment, tubulointerstitial fibrosis was obviously confined, especially in the corticomedullary junction. These results indicated that the focus should be directed on the long-term outcomes of obstructive nephropathy, and treatment with AS-IV might be a specific approach to protect renal tubular cells.

Cell apoptosis is involved in the injury and repair process of multiple kidney diseases. Injury leads to cell apoptosis, and excessive apoptosis can exacerbate tissue and cell injuries (Sanz et al., 2008). Truong et al. (1996) showed that apoptosis may play a significant role in tubular atrophy and renal weight loss in experimental chronic obstructive nephropathy. It has been reported that the level of renal tubular epithelial cell apoptosis is positively correlated with the degree of tubulointerstitial fibrosis. Moreover, tubulointerstitial fibrosis could be significantly decreased by AS-IV treatment. Additionally, studies are necessary to determine whether AS-IV has different effects on other cell lines compared with the protective effects observed in the kidney. Miyajima et al. (2000) showed that an antibody to TGF-β1 reduces tubular apoptosis in rats subjected to UUO as well as in vitro using tubular epithelial cells. Further, the antifibrotic effect of AS-IV may be associated with inhibiting TGF-β1-induced tubular apoptosis.

The MAPK pathway is a classic signaling pathway that regulates cell apoptosis and inflammation and is involved in serious tubular injury (Qin et al., 2012; Zheng et al., 2012; Wang et al., 2013). Qin et al. (2012) reported that A. membranaceus, from which AS-IV is purified, inhibited the phospho-p38 MAPK pathway in advanced glycation end-product–stimulated macrophages. Moreover, it has been reported that AS-IV could inhibit apoptosis partially through the p38 MAPK pathway in two rodent models of acute kidney injury, but that study did not evaluate other MAPKs such as JNK and ERK1/2 activity (Gui et al., 2013). Our data suggested that the phosphorylation levels of p38 and JNK MAPKs were prominently increased in the obstructed kidneys on the 14th day after UUO, and this observed increase was not attributable to upregulation of total p38 and total JNK. The effect of total MAPKs on UUO-induced injury is still unclear; however, the phosphorylation proteins were the active components. At 200 μg/ml, AS-IV significantly inhibited the phosphorylation of p38 and JNK pathways. Interestingly, the ERK1/2 pathway has different effects in vitro and in vivo. The in vitro results showed that the antifibrotic effect of AS-IV on renal tubular cells was associated with activation of renal JNK and p38 but not ERK. However, in UUO mice, the ratio of phospho/total ERK1/2 was increased, whereas in vitro ERK1/2 showed no significant

expression levels of TGF-β1, α-SMA, collagen IV, and fibronectin in kidneys. AS-IV also could significantly decrease the serum creatinine and blood urea nitrogen level after 7 days of ligation. However, the phenomenon was not very obvious after 14 days of ligation, which might be a result of the compensatory work of the unobstructed kidney.

Previous studies have commonly associated fibrosis with glomerular injury. Instead, tubular injury appears to be more closely associated with renal function deterioration and renal fibrosis than glomerulosclerosis (Venkatachalam et al., 2010; Chawla and Kimmel, 2012). Tubular atrophy is generally recognized to be associated with interstitial fibrosis. Tubular injury plays a crucial role in the progression of acute injury to fibrosis, and the areas that do not completely recover will probably develop into focal areas of fibrosis (Chawla and Kimmel, 2012). It is well known that obstructive nephropathy is pathologically a type of postrenal acute injury, which is a common clinical case, whereas UUO is an established model that has been implemented in many studies to induce renal fibrosis. Tubular injury is the primary feature of the UUO model. Thus, we focused on renal tubular cells, which were observed to have the most serious and persistent damage,
activation. This difference between the in vivo and in vitro results was considered to be associated with the cross-talk of a complex network of signals, and self-repair mechanisms might be different for the activation of the ERK1/2 pathway. These results suggest that increasing the activation of the JNK and p38 MAPK pathways might play a prominent role in renal tubular cell apoptosis (thus, subsequently accelerating the progression of renal tubulointerstitial fibrosis), and the inhibitory effect of AS-IV on cell apoptosis may be related to the inhibition of these two MAPK signaling pathways.

Fig. 9. AS-IV partially inhibits the activity of the MAPK pathway both in vivo and in vitro. Activation of p38, JNK, and ERK1/2 MAPK proteins were measured by Western blotting in UUO-operated kidneys on day 14 (A–D; **P < 0.01 versus sham-operated mice; ##P < 0.01 versus UUO mice on the same day) and in cell lysates stimulated by TGF-β1 and treated with AS-IV at various concentrations in vitro (E–H; **P < 0.01 versus control [untreated HK2 cells]; ##P < 0.01 versus TGF-β1 group).
Similar studies have been performed on the protective effect of AS-IV in renal injury and pathology (Meng et al., 2011; Zhang et al., 2011; Li et al., 2012; Qin et al., 2012; Gu et al., 2013; Qi et al., 2014). Meng et al. (2011) performed a study in UUO rats using AS-IV and ferulic acid as treatments. They concluded that AS-IV synergizes with ferulic acid to partially alleviate renal tubulointerstitial fibrosis by activating the JNK pathway. The dose of AS-IV used in Meng's study was lower than what was used in the current study and the animals and cell line used in Meng's study also differed. In addition, several reports have suggested that AS-IV might inhibit renal oxidative stress under various injury conditions (Meng et al., 2011; Gu et al., 2013; Qi et al., 2014). Further study is necessary to clarify whether AS-IV could regulate the oxidative stress response in addition to preventing renal tubulointerstitial fibrosis.

Moreover, other pathways are reported to be involved in the renal protective effect of AS-IV. Gu et al. (2012) performed in vitro and in vivo studies and found that AS-IV exerted an antiapoptotic effect on podocytes by regulating the balance between Bax and Bcl-2 under high-glucose conditions; however, the Bax protein levels published were not significantly different on the Western blot. Further studies are needed to determine whether the antiapoptotic effect of AS-IV is associated with either mitochondria-dependent (Bax/Bcl-2) or TGF-β/TGFβ-Smad apoptotic pathways.

In conclusion, the effects of AS-IV in this study indicated that AS-IV might be a novel drug with specificity for renal tubules to improve tubular cell survival and prevent cell apoptosis, thus delaying the progression of renal fibrosis in obstructive nephropathy.

References

Fig. 10. Comparison of the protective effects of AS-IV and SB203580. HK2 cells were pretreated for 1 hour with 10 μM SB203580, a specific inhibitor of phospho-p38 MAPK, and then stimulated with TGF-β1 (10 ng/ml) for 48 hours to measure the α-SMA expression and development of fibrosis. **P < 0.01 versus TGF-β1 group.

Authorship Contributions
Participated in research design: W. Xu, Shao, Mou, Ni.
Conducted experiments: W. Xu, Yao, X. Xu, Wu, Tian.
Performed data analysis: Shao, Gu, Zhang, Q. Wang, L. Wang.
Wrote or contributed to the writing of the manuscript: W. Xu, Shao, Mou.


Address correspondence to: Dr. Shan Mou, Ren Ji Hospital, School of Medicine, Shanghai Jiao Tong University, No.160 Pujian Road, Pudong New District, Shanghai, China. E-mail: shan_mou@126.com