Arctigenin suppresses renal interstitial fibrosis in a rat model of obstructive nephropathy

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\textbf{A B S T R A C T}

\textbf{Background:} Renal tubulointerstitial fibrosis (TIF) is commonly the final result of a variety of progressive injuries and leads to end-stage renal disease. There are few therapeutic agents currently available for retarding the development of renal TIF.

\textbf{Purpose:} The aim of the present study is to evaluate the role of arctigenin (ATG), a lignan component derived from dried burdock (Arctium lappa L.) fruits, in protecting the kidney against injury by unilateral ureteral obstruction (UUO) in rats.

\textbf{Methods:} Rats were subjected to UUO and then administered with vehicle, ATG (1 and 3 mg/kg/d), or losartan (20 mg/kg/d) for 11 consecutive days. The renoprotective effects of ATG were evaluated by histological examination and multiple biochemical assays.

\textbf{Results:} Our results suggest that ATG significantly protected the kidney from injury by reducing tubular dilatation, epithelial atrophy, collagen deposition, and tubulointerstitial compartment expansion. ATG administration dramatically decreased macrophage (CD68-positive cell) infiltration. Meanwhile, ATG downregulated the mRNA levels of pro-inflammatory chemokine monocyte chemoattractant protein-1 (MCP-1) and cytokines, including tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)), interleukin-1\(\beta\) (IL-1\(\beta\)), and interferon-\(\gamma\) (IFN-\(\gamma\)), in the obstructed kidneys. This was associated with decreased activation of nuclear factor \(\kappa\)B (NF-\(\kappa\)B). ATG attenuated UUO-induced oxidative stress by increasing the activity of renal manganese superoxide dismutase (SOD2), leading to reduced levels of lipid peroxidation. Furthermore, ATG inhibited the epithelial-mesenchymal transition (EMT) of renal tubules by reducing the abundance of transforming growth factor-\(\beta\)1 (TGF-\(\beta\)1) and its type I receptor, suppressing Smad2/3 phosphorylation and nuclear translocation, and up-regulating Smad7 expression. Notably, the efficacy of ATG in renal protection was comparable or even superior to losartan.

\textbf{Conclusion:} ATG could protect the kidney from UUO-induced injury and fibrogenesis by suppressing inflammation, oxidative stress, and tubular EMT, thus supporting the potential role of ATG in renal fibrosis treatment.

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\textbf{Introduction}

Chronic kidney disease (CKD) has emerged as a worldwide public health problem with a rapid growth in prevalence (Couser et al., 2011). Progressive tubulointerstitial fibrosis (TIF) is the common pathological presentation of nearly all kinds of CKD leading to end-stage renal failure (Liu, 2006). Although tremendous efforts have been made to prevent or retard the progression of TIF,

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specific drug therapies to delay the progression of TIF toward end-stage renal failure are limited.

CKD can initially manifest as inflammatory responses characterized by infiltration of immune cells, mainly monocytes/macrophages and T lymphocytes, into the glomeruli and tubulointerstitium (Lopez-Novoa and Nieto, 2009). On one hand, these inflammatory cells secrete various pro-inflammatory cytokines and chemokines such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interferon-γ (IFN-γ), and monocyte chemotactant protein-1 (MCP-1), which further contribute to recruitment of circulating inflammatory cells. This creates a malignant positive feedback loop of inflammation (Diamond et al., 1994). On the other hand, activated macrophages produce profibrotic cytokines such as transforming growth factor-β1 (TGF-β1), which have been shown to induce activation of matrix-producing myofibroblasts (Leask and Abraham, 2004). The stimulated myofibroblasts cause accumulation of extracellular matrix (ECM) proteins and lead to complete destruction of renal parenchyma and irreversible renal failure. Renal inflammation—and in particular the accumulation of macrophages in renal interstitium—are a critical element in the mechanism responsible for the initiation and development of renal fibrogenesis. Therefore, inhibiting inflammatory responses may significantly attenuate renal TIF.

Recent studies show that a significant portion of synthetically active myofibroblasts arise from renal tubular epithelial cells (TECs) via the epithelial-mesenchymal transition (EMT) in fibrotic kidney diseases (Liu, 2010). The pro-fibrogenic effect of inflammation depends, at least partially, on triggering EMT (Wynn, 2008). Several studies have shown that sustained stimulation of pro-inflammatory cytokines TNF-α or IL-1 can induce EMT in the epithelial cell lines (Takahashi et al., 2010). During EMT, differentiated TECs lose their epithelial characteristics and undergo multiple biochemical changes, which enable them to assume a mesenchymal phenotype. This phenotypic conversion involves the de novo synthesis of mesenchymal cytoskeletal biomarkers such as α-smooth muscle actin (α-SMA), fibronectin and vimentin, a down-regulation of epithelial biomarkers such as E-cadherin and zonula occuludens-1, and the acquisition of a fibroblastic morphology with a concomitant invasive phenotype. TGF-β1 has been identified as the main inducer of EMT and consequent interstitial ECM production in kidney and other organ systems (Acloque et al., 2009; Pan et al., 2015). The intracellular Smad pathway is important for TGF-β1 to initiate tubular EMT and fibrotic responses (Lan and Chang, 2011). To prevent progression of TIF, the inhibition of tubular EMT mediated by TGF-β1/Smad signaling pathway may be helpful.

Unilateral ureteral obstruction (UUO) is a well-established experimental model used to elucidate pathogenic mechanisms of chronic obstructive nephropathy (Chevalier et al., 2009). Oxidative stress plays a central role in the progression of renal damage in obstructed nephropathy (Kawada et al., 1999). Oxidative stress occurs when the production of reactive oxygen species (ROS) exceeds the capacity of intrinsic antioxidant defense. In UUO kidneys, the superoxide anion (O₂⁻⁻) and its derivative hydrogen peroxide (H₂O₂) are increased, while the antioxidant enzyme catalase (CAT) and copper-zinc superoxide dismutase (SOD1) mRNA correspondingly decreased (Ricardo et al., 1997). Increased ROS concentrations can cause tubulointerstitial injury by increasing lipid peroxidation, hydrogen peroxides, leukocyte activation, DNA breakdown, protein oxidation, and apoptosis. Furthermore, ROS generated in TECs induce nuclear factor kappa B (NF-κB) activation (Gloire et al., 2006) leading to transcription of some pro-inflammatory mediators such as MCP-1, TNF-α, INF-γ, and IL-1β, infiltration of monocytes/macrophages, proliferation of fibroblasts, and ECM accumulation in the renal interstitium. Initial oxidative stress and inflammation contribute to the progression of renal fibrosis. Thus, antioxidant and/or anti-inflammatory agent have been used to protect the kidney from injury induced by UUO.

Burdock (Arctium lappa L.) has been used in traditional and folk medicine in oriental countries for centuries. Various experimental models have shown that arctigenin (ATG) is a main bioactive ingredient derived from dried fruit of A. lappa. ATG exhibits anti-inflammatory (Hyam et al., 2013), anti-oxidant (Zhang et al., 2015), and antineoplastic (Awale et al., 2006) properties in various disorders. Our preliminary study demonstrated that oral administration of 95% (v/v) hydroalcoholic extract from A. lappa containing a higher amount of ATG and its glycoside Arctin versus aqueous and petroleum ether extracts, exhibited the strongest inhibitory effect on ECM component synthesis in the obstructive kidneys of rats with UUO (Supplementary Fig. S1). Our previous data also showed that ATG could reverse TGF-β1-triggered renal tubular ECM-like phenotypic changes in vitro using human proximal tubular epithelial cells (HK-2 cells)—mainly due to the inhibition of TGF-β1-induced up-regulation of MCP-1 (Li et al., 2015). Because the regulatory role of ATG in renal fibrosis in vivo has not yet been studied, we studied here a 14-day UUO rat model to assess the anti-fibrotic efficacy of ATG and further delineated the potential molecular mechanisms by which ATG elicits its effects on experimental renal fibrosis.

Materials and methods

Chemicals

Arctigenin (MW: 372.41) was provided by Nanjing Zelang Medical Technology Co. Ltd. (Nanjing, China). The purity of ATG was determined to be > 8% using high performance liquid chromatography (HPLC; Supplementary Fig. S2). Losartan was purchased from Merck Sharp & Dohme Ltd. (Hangzhou, China). Antibodies against the following proteins were used: NF-κB p65 (Signalway Antibody, Pearlard, TX, USA); fibronectin (BD Biosciences, San Jose, CA, USA); Smad2/3, phosphorylated Smad2/3 (p-Smad2/3) and vimentin (Cell Signaling Technology, Beverly, MA, USA); E-cadherin, lamin B1, k-Bcr, TGF-β1, and TGF-β type I (TβR-I) and type II (TβR-II) receptors (Santa Cruz Biotechnology, Santa Cruz, CA, USA); α-SMA, SOD1, manganese superoxide dismutase (SOD2) and Smad7 (Epitomics, Burlingame, CA, USA); β-actin (Bioworld Technology, Minneapolis, MN, USA); collagen type I and CD68 (Boster biological technology, Wuhan, China). Serum creatinine, blood urea nitrogen (BUN), urinary protein, hydroxyproline (HYP), malondialdehyde (MDA), and SOD assay kits were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Glutathione peroxidase (GPx), glutathione reductase (GR), CAT, and biconcinchoninic acid (BCA) protein assay kits were obtained from Beyotime Institute of Biotechnology (Jiangsu, China). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise specified.

Animals

Eight-week-old male Sprague-Dawley rats (180–200 g; certification No: SCXK-Yu-2012-0005) were supplied by the Experimental Animal Center of Third Military Medical University (Chongqing, China). All studies were performed in accordance with the National Institutes of Health guidelines for the Care and Use of Laboratory Animals (8th Edition, 2011). The experimental protocols were approved by the Institutional Ethics Committee of Chongqing University of Technology. All rats were housed in an air-conditioned room at 21 ± 2°C and 50 ± 5% relatively humidity under a 12-h light/dark cycle and had access to standard rodent chow and water ad libitum throughout the study period.
UO model and treatment protocols

A UUO kidney disease model was induced in rats by ligation of the left ureter as described previously (Kinter et al., 1999). All rats were randomly divided into 6 groups (6 rats in each group): (1) sham group where the rats underwent sham operations and were administered with vehicle; (2) sham + ATG group where the rats underwent sham operations and were treated with ATG (3 mg/kg/d); (3) UUO model group where the rats were subjected to UUO but were treated with vehicle; (4) UUO + ATG low-dose group where the rats underwent UUO and were treated with ATG (1 mg/kg/d); (5) UUO + ATG high-dose group where the rats underwent UUO and were treated with ATG (3 mg/kg/d); (6) UUO + losartan group where the rats underwent UUO and were administered with losartan (20 mg/kg/d, an angiotensin II receptor antagonist). The dosage of ATG and losartan was selected based on previous report (Awale et al., 2006) and our pilot study. The ATG and losartan were first dissolved in dimethyl sulfoxide (DMSO) at 4 or 12 mg/ml and kept at −20°C. The stock solution of ATG was diluted in saline to a final concentration of 0.1 or 0.3 mg/ml until immediately before administration. The final volume of DMSO in saline is 2.5%, and this served as the vehicle. On day 4 after obstructive surgery, rats were administered vehicle, ATG, or losartan by gastric gavage once daily for 11 consecutive days. All rats were sacrificed under pentobarbital sodium anesthesia on day 14 postoperatively.

Urine, blood and tissue preparation

The day before euthanasia, the urine samples were collected from rats that were housed individually for 24 h in metabolic cages to measure urinary protein excretion. Whole-blood samples were collected from the inferior vena cava, and creatinine and BUN concentrations were analyzed from those serum samples. The left kidney was harvested, de-capsulated, and separated into two parts—one part of the renal tissues were fixed in 10% (v/v) buffered formaldehyde and embedded in paraffin for histopathologic and immunohistochemical examinations. The remaining tissues were snap-frozen using liquid nitrogen and stored at −80°C for either protein or RNA analysis.

Renal function evaluation

The 24-h urinary protein excretion was measured using the Bradford method (Nanjing Jincheng, China). Serum concentrations of creatinine and BUN were measured with commercially available colorimetric kits (Nanjing Jincheng, China). These were expressed as μmol/L and mmol/L, respectively.

Histopathological examination

Paraffin-embedded sections of renal tissues (4μm thickness) were deparaffinized and stained by hematoxylin and eosin (H&E) using standard methods. Renal tubulointerstitial lesions characterized by tubular dilation and epithelial desquamation with interstitial expansions were graded on a scale from 0 to 4 according to the extent of cortical involvement: 0, normal; 1, changes affecting <25% of the cortex; 2, changes affecting 25–50% of the cortex; 3, changes affecting 50–75% of the cortex; 4, changes affecting >75% of the cortex. The overall mean scores corresponding to tubulointerstitial lesions in each rat were calculated based on individual values, which were determined on at least 10 randomly chosen non-overlapping fields at a magnification of 200×. The degree of TIF was assessed by Masson’s trichrome staining and collagen quantification. Digital images of renal tissues were acquired using an optical microscope (Olympus) equipped with image analysis software (Image-Pro Plus version 6.0; Media Cybernetics, Bethesda, MD, USA). For each experimental group, 5 fields from each of 5 different Masson’s trichrome-stained sections per rat were analyzed at 200× magnification. The areas of fibrotic lesion in the cortical interstitium were calculated in arbitrary fields and expressed as a percentage of fibrotic area relative to the entire area. All semiquantitative analysis was performed in a blinded fashion.

Measurement of renal HYP content

The HYP content in renal tissues was measured using a commercial detection kit (Nanjing Jincheng Bioengineering Institute, Nanjing, China) according to the manufacturer’s instructions. The results are expressed as mg of HYP per gram of tissue.

Immunohistochemical analysis

Kidney paraffin sections were deparaffinized in xylene and rehydrated. Non-enzymatic antigen retrieval was performed by heating the sections to 98°C in citrate buffer (10 mM, pH 6.0) for 10 min. The sections were immersed in 3% (v/v) H₂O₂ in methanol for 20 min at room temperature to quench potential endogenous peroxidase activity. Following three washes with phosphate buffered saline (PBS; pH 7.4) after blocking, the sections were subsequently incubated with primary antibodies overnight at 4°C in a humidity chamber. The primary antibodies were as follows: rabbit monoclonal antibody against α-SMA (1:100 dilution); rabbit polyclonal antibody against E-cadherin, vimentin, p-Smad2/3, CD68, TGF-β1, and TGF-β1 receptors (1:100 dilution); or mouse monoclonal antibody against fibronectin (1:100 dilution). The final incubation was carried out for 30 min at 37°C with peroxidase-labeled polymer conjugated to goat anti-rabbit or anti-mouse immunoglobulin (Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd., Beijing, China). Immunostaining was visualized with hydrogen peroxide and 3, 3’-diaminobenzidine (DAB) as the chromogen. The sections were lightly counterstained with hematoxylin and mounted on a coverslip. Negative controls were created by substituting the primary antibodies mentioned above with non-immune mouse or rabbit serum. Photomicrographs of the sections (n = 5 per animal) at ×200 magnification were taken using an optical microscope (Olympus) and analyzed using Image-Pro Plus image analysis software. Staining intensity was computed as the integrated optical density (IOD). For semi-quantitative analysis, the IOD was measured counting 3 randomly collected fields with the same area for each section. In addition, the number of interstitial CD68-positive macrophages and phosphorylated Smad2/3-positive cells were assessed in 3 fields of each of 5 sections (×200 magnification).

RNA reverse transcription and quantitative real-time PCR (RT-qPCR)

The total RNA was isolated from each renal cortical tissue sample using RNApure high-purity total RNA rapid extraction kit (spin-column, BioTeke, Beijing, China) according to the manufacturer’s instruction. The first strand complementary DNA (cDNA) was synthesized from equal amounts of total RNA (2μg) using M-MuLV reverse transcriptase with Oligo(dT) primers (BioTeke) in a 20 μl reaction volume. Real-time quantitative PCR analysis was performed using SYBR FAST qPCR Master Mix (Kapa Biosystems, Woburn, MA, USA) and primers (Table 1). These were specific for rat MCP-1, TNF-α, IL-1β, IFN-γ, IL-10 and β-actin. A standard curve from consecutive 5-fold dilutions of a cDNA pool representative of all samples was generated in each experiment to verify qPCR efficiency. The threshold cycle (Ct) is defined as the number of cycles required for the fluorescent signal to become detectable above background. The Ct levels are inversely proportional to the
amount of the initial template number. Fold changes in mRNA expression were calculated according to the comparative Ct method ($2^{-\Delta\Delta Ct}$) as described in our previous report (Li et al., 2015).

### Western blotting analysis

The separation of cytoplasmic and nuclear fractions using a NE-PER nuclear and cytoplasmic extraction kit (Pierce Biotechnology, Rockford, IL, USA) was performed according to the manufacturer's recommendations. The appropriate fractionation was confirmed by Western blotting for β-actin (a cytoplasmic protein) and lamin B1 (a nuclear protein). Tissues were lysed with a lysis buffer (150 mM NaCl, 50 mM Tris pH 8.0, 0.5% NP-40, 5 mM EDTA, 1 M DTT, 100 mM PMSF, 10 mg/ml aprotinin and leupeptin) and sonicated briefly to produce whole renal cortical homogenates. Protein concentration was quantified using the BCA assay method and equal amounts of protein extract (50 μg/lane) were separated by 8–12% sodium dodecysulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The separated proteins were electro-transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, USA). These membranes were blocked for 1 h at room temperature with 5% nonfat milk followed by incubation with primary antibodies at 4 °C overnight. After being washed four times in TBS-T, each membrane was incubated for 1 h at room temperature with a 1:3000 dilution of a horseradish peroxidase (HRP)-conjugated secondary anti-rabbit or anti-mouse immunoglobulin (Bio-world Technology). Immunoreactive bands were visualized with an enhanced chemiluminescence (ECL) kit (Millipore). Images were captured using a Gel Doc/Chemie Doc Imaging System and the intensity of each band was quantified by Quantity One software (Bio-Rad, Hercules, CA, USA).

### Measurement of antioxidant enzyme and lipid peroxidation

Renal cortical tissues were washed with saline to remove red blood and clots, and were then weighed and homogenized in cold Tris–HCl (5 mM containing 2 mM EDTA, pH 7.4). After the homogenate was centrifuged at 1500 g for 20 min at 4 °C, aliquots of the supernatants were analyzed for lipid peroxidation and antioxidant enzymes. Kidney lipid peroxidation was determined by measuring the level of MDA according to the manufacturer's protocol. The level of MDA was expressed as μM/g tissue. Antioxidant enzymes were assayed by measuring the activities of GPx, GR, CAT, and SOD. GPx and GR activities were expressed as μM NADPH oxidized/min/g protein. The CAT activity was expressed as μM HzO$_2$ consumed/min/mg protein. The hydroxylamine method was used to determine SOD activity, which was expressed as units (U)/mg protein. One unit of SOD activity was defined as the amount of enzyme that inhibited the oxidation reaction of hydroxylamine by 50%.

### Molecular docking

The crystal structure of rat SOD is unavailable in the Protein Data Bank (PDB), and thus homology models of rat SOD were built with the macromolecules module in Discovery Studio 3.0 software suite (Accelrys Software, http://.accelrys.com/). The amino acid sequences of rat SOD were retrieved from Uniprot database (http://www.uniprot.org/), SOD1: Q6LDS4, SOD2: P07895). Three-dimensional structures of human SOD were downloaded from PDB (SOD1: 4B3E; SOD2: 1N0J). The template sequence was identified through rat SOD sequence on NCBI &PDB using BLAST homology searching with default parameters in the Discovery Studio version 3.0. The target and the selected template sequences were aligned with ClustalW. Next, molecular docking was used to predict the modes of interactions of ATG with the rat SOD protein. The three-dimensional structure of ATG was then energy minimized with a default force field, and partial charges were calculated using MMFF94 method in SYBYL version 2.0 (Tripos Int., USA). Molecular docking studies used the docking module in LigandFit implemented in the Receptor-Ligand Interaction package of the Discovery Studio version 3.0 software suite. The docking parameters were the default values.

### Statistical analysis

Data are expressed as mean ± standard deviation (SD). Comparisons of the ranked data between different groups were performed using nonparametric Kruskal-Wallis one-way analysis of variance (ANOVA); when the differences were significant by this test ($P < 0.05$), the Mann-Whitney U test was subsequently performed to identify significant differences by pairwise comparison of each group. For other data, their statistical significance was evaluated by one-way ANOVA followed by Tukey post hoc test. All analysis was completed with SPSS version 16.0 Statistical Software (IBM, Chicago, IL, USA), and $P < 0.05$ was considered to be statistically significant.

### Results

**ATG attenuated progressive fibrosis in the obstructed kidneys induced by UUO**

To investigate whether ATG can suppress renal TIF in vivo, we examined interstitial fibrotic lesions and collagen accumulation in UUO kidneys. As shown in Fig. 1A, compared with sham-operated controls, the UUO caused obvious tubulointerstitial injury consisting of tubular dilatation and epithelial atrophy, interstitial inflammatory cell infiltration, and a marked dilation of interstitial spaces. This interstitial expansion was accompanied by a noticeable increase in collagen deposition as shown by a collagen-specific Masson's trichrome staining (Fig. 1B). However, treatment with
ATG and losartan substantially reduced the interstitial volume expansion and collagen deposition. Semi-quantitative morphometric analysis revealed that ATG reduced the scores of tubulointerstitial injury in the obstructed kidneys at day 14 after UUO (Fig. 1C). The administration of 1 or 3 mg/kg/d ATG led to renal interstitial fibrotic space that was only 47% or 14% of the levels in the UUO rats (Fig. 1D). Quantitative determination of renal HYP content also confirmed a reduced collagen deposition in the cortical interstitium after ATG or losartan treatment (Fig. 1E). There was no significant difference between ATG and losartan groups. Together, these findings suggest that ATG ameliorates UUO-induced renal fibrosis and ECM production.

Neither ATG nor UUO treatment significantly influenced the serum levels of creatinine, BUN, and urinary protein excretion (Table 2). This may be attributed to the traits of the UUO model in which the contralateral kidney is mostly normal and sufficient for maintaining renal function.

**ATG relieved inflammatory responses in the obstructed kidneys**

The recruitment of macrophages to the tubulointerstitium is a dominant event in the initiation of TIF. Immunohistochemical staining of macrophages used anti-CD68 antibody and confirmed these findings. Fig. 2A shows few CD68-positive macrophages in the sham-operated kidneys; 14 days after UUO, a greater number of macrophages infiltrated into the tubular interstitium of obstructed kidneys than sham-operated counterparts. Treatment with ATG or losartan prevented renal interstitial infiltration of macrophages in the obstructed kidneys. Quantification of immunostaining is shown in Fig. 2B indicating that the number of CD68-positive macrophages in the tubulointerstitium was significantly elevated 14 days after UUO, and the increase was alleviated by ATG treatment in a dose-dependent manner.

We next examined whether ATG had an effect on the pro-inflammatory mediator gene expression in macrophages. The RT-qPCR results showed that the mRNA levels of several pro-inflammatory mediators such as MCP-1 (Fig. 2C), TNF-α (Fig. 2D), IL-1β (Fig. 2E) and INF-γ (Fig. 2F) were significantly up-regulated in the kidneys after UUO, and their increases were significantly attenuated by ATG or losartan treatment. Interestingly, we found that UUO kidneys displayed marked induction of IL-10 mRNA versus sham controls (Fig. 2G), while ATG or losartan treatment did not alter UUO-induced IL-10 mRNA expression.

In resting cells, NF-κB is sequestered in the cytoplasm in an inactive state through its interaction with inhibitory protein IκB (Sanz et al., 2010). Upon fibrogenic stimuli, the IκB pro-
teins are phosphorylated, ubiquitinated, and degraded, thereby releasing NF-κB to enter the nucleus and trigger transcription of inflammation-related cytokine and chemokine genes. To clarify whether ATG or losartan inhibited activation of NF-κB, we measured relative levels of κBα in the cytoplasm and NF-κB p65 in the nucleus by Western blotting, respectively (Fig. 2H and I). In the kidneys of sham-operated rats, there were very high levels of cytoplasmic κBα and basal levels of nuclear NF-κB p65 proteins. In response to UUO, the NF-κB was significantly activated, as reflected by a simultaneous decrease in cytoplasmic κBα and an increase in nuclear NF-κB p65. Conversely, after treatment with ATG or losartan, the κBα was retained in the cytoplasm, and almost no NF-κB p65 signal was found in the nuclear fraction suggesting that ATG or losartan notably enhanced stability of κBα and blocked nuclear translocation of NF-κB.

These results collectively describe how ATG inhibits UUO-induced renal inflammatory responses—the compound inhibits UUO-induced macrophage infiltration, NF-κB activation and nuclear translocation. This prevents pro-inflammatory mediator expression in macrophages.

ATG inhibited tubular EMT in the obstructed kidneys

Whether renal inflammation progresses to TIF depends in part on whether TECs undergo a process of EMT. To determine the effects of ATG on UUO-mediated changes related to EMT after inflammatory responses, the expression of EMT markers in the obstructed kidneys was assessed using immunohistochemical and Western blotting analysis. In kidney sections from sham-operated rats, epithelial marker E-cadherin displayed moderate or strong complete membranous staining in all tubular epithelial cells (Fig. 3A). On the other hand, the mesenchymal marker α-SMA was found exclusively in the vascular smooth muscle cells (Fig. 3B). Two other markers of mesenchymal cells—fibronectin (Fig. 3C) and vimentin (Fig. 3D)—were almost not detected by immunostaining. Compared with sham-operated controls, expression of E-cadherin protein decreased in renal cortical areas of the UUO rats (Fig. 3E), especially around the renal tubules; conversely, expression levels of α-SMA (Fig. 3F), fibronectin (Fig. 3G) and vimentin (Fig. 3H) proteins were significantly elevated in the UUO rats suggesting that the TECs were undergoing EMT. The ATG administration largely blocked induction of α-SMA, fibronectin and vimentin expression in kidney tissues of UUO rats while markedly inhibiting a UUO-induced decrease in E-cadherin expression. Losartan had the same effects. Consistent with the immunohistochemistry studies, Western blotting analysis also showed that the UUO model resulted in a marked decrease in the E-cadherin/α-SMA ratio (Fig. 3I and G) concomitant with an increase in fibronectin and vimentin protein levels (Fig. 3I and K). In contrast, the expression of α-SMA, fibronectin and vimentin was down-regulated with ATG or losartan treatment in the obstructed kidneys of rats with UUO for 14 days, but the expression of E-cadherin increased in response to ATG or losartan treatment. These data suggest that ATG exerts its anti-fibrotic effect through inhibition of EMT-like phenotypic changes and preservation of the normal tubular epithelial phenotype.

ATG regulated TGF-β1/Smad signaling pathway in the obstructed kidneys

TGF-β1-induced EMT in a variety of cells is mediated by activation of serine/threonine kinase receptors and canonical Smad signaling pathway (Lan and Chung, 2011). We first evaluated the effect of ATG on renal TGF-β1, TβR-I and TβR-II abundance after obstructive injury. Immunohistochemical staining for TGF-β1 localized the expression of TGF-β1 on kidney sections. In kidneys at day 14 after UUO, clear tubular expression of TGF-β1 was observed (Fig. 4A and D) in addition to its expression in interstitial areas. In contrast, ATG or losartan treatment significantly inhibited TGF-β1 protein expression in the treatment groups 14 days after UUO. We also examined the expression of TβR-I and TβR-II in the obstructed kidneys. Renal expression of TβR-I (Fig. 4B and D) and TβR-II (Fig. 4C and D) was markedly up-regulated in the obstructed kidneys at day 14 versus sham-operated kidneys. Interestingly, the expression of TβR-I and TβR-II protein was primarily induced in renal tubules. After ATG or losartan treatment, the up-regulated tubular expression of TβR-I in diseased kidneys was reduced, whereas protein expression of TβR-II was almost unchanged. Consistent with the immunohistochemistry, Western blotting analysis indicated that TGF-β1 and TβR-I expression was significantly up-regulated in UUO rats compared with sham control group (Fig. 4E and F). These changes were significantly attenuated in ATG or losartan-treated UUO rats.

We next investigated the effect of ATG on activation status and expression levels of Smad proteins in the obstructed kidneys. After obstructive injury, Smad2/3 signaling was highly activated as revealed by higher levels of phosphorylated and total Smad2/3 protein (Fig. 5A and B), but significantly lower levels of inhibitory Smad7 protein (Fig. 5A and C) than in sham-operated kidneys. By immunohistochemical staining, we examined the localization of p-Smad2/3 in kidneys of rats with UUO on day 14. As shown in Fig. 5D, p-Smad2/3 was predominantly localized in the nuclei of severely congested tubular epithelial cells but also interstitial cells. The quantification of immunostaining also showed an increasing number of p-Smad2/3-positive nuclei in renal cortical area after UUO (Fig. 5E) indicating Smad2/3 nuclear localization in the fibrotic kidneys. Treatment with ATG or losartan notably attenuated UUO-induced phosphorylation and nuclear location of Smad2/3, which was associated with a significant up-regulation of renal Smad7. These results indicate that ATG might suppress UUO-induced TIF by regulation the TGF-β1/Smad pathway.

ATG reduced MDA content, increased activity and expression of SOD2, but had no effect on activities of GPx, GR, CAT, and SOD1 in the obstructed kidneys

Oxidative stress was evaluated by determination of MDA levels and activities of the antioxidant enzymes in the obstructed kidneys. Fig. 6A shows a significant increase in MDA production in the UUO-injured kidneys accompanied by a corresponding decrease in GPx (Fig. 6B), GR (Fig. 6C), and CAT (Fig. 6D) activities. ATG or losartan administration significantly decreased the MDA content
Fig. 2. ATG regulated inflammatory signaling pathway in the kidney tissues of UUO rats. (A) Representative photomicrographs illustrating infiltration of CD68-positive macrophages (black color directed by arrows) in the kidney tissues of rats in different groups: (a) sham (sham operated), (b) sham + ATG (ATG 3 mg/kg/d treatment only), (c) UUO model (UUO operated), (d) UUO + ATG (UUO operated plus ATG 1 mg/kg/d), (e) UUO + ATG (UUO operated plus ATG 3 mg/kg/d), and (f) UUO + losartan (UUO operated plus 20 mg/kg/d losartan). Scale bar: 50 μm. (B) Quantification of the number of CD68-positive macrophages per high-power field in different groups. (C–G) RT-qPCR analysis of gene expression was performed for inflammatory mediators MCP-1, TNF-α, IL-1β, INF-γ, and IL-10. (H) Cytoplasmic and nuclear fractions of kidney cortex tissues were collected and analyzed by Western blotting to detect IκBα and NF-κB p65, respectively. The β-actin and lamin B1 protein levels served as internal controls, respectively, for cytoplasmic extracts (CE) and nuclear extracts (NE). (I) The protein levels of IκBα in cytoplasmic extracts and NF-κB p65 in nuclear extracts were converted to arbitrary densitometric units normalized by the value of the corresponding loading control and expressed relative to the protein level in sham-operated group (defined as 1-fold). Each bar represents mean ± SD of three independent experiments. n = 6 rats per group. *P < 0.05 versus sham group; †P < 0.05 versus UUO model group.

in kidney tissues, while UUO-induced reduction of renal GPx, GR, and CAT activities was not ameliorated by ATG or losartan treatment.

The activity of antioxidant enzyme SOD2 was significantly elevated in the obstructed kidneys of UUO rats (Fig. 6E), while SOD1 activity was markedly decreased. ATG or losartan treatment further enhanced the activity of SOD2 when compared with the UUO model group. Interestingly, treatment with ATG alone in rats without UUO operation increased the activity of SOD2. However, ATG had no effect on the activity of SOD1 in sham or UUO-
Fig. 3. ATG inhibited the EMT process in the kidney tissues of UUO rats. (A–D) The location and expression of E-cadherin, α-SMA, fibronectin and vimentin were determined by immunohistochemical staining in the kidney sections of rats from different groups: (a) sham (sham operated), (b) sham + ATG (ATG 3 mg/kg/d treatment only), (c) UUO model (UUO operated), (d) UUO + ATG (UUO operated plus ATG 1 mg/kg/d), (e) UUO + ATG (UUO operated plus ATG 3 mg/kg/d), and (f) UUO + losartan (UUO operated plus 20 mg/kg/d losartan). Scale bar: 50 μm. (E–H) Semi-quantitative immunohistochemical analysis of the EMT-related protein expression in different groups. (I) Kidney tissue lysates were studied by Western blotting analysis with specific antibodies against E-cadherin, α-SMA, fibronectin, vimentin or β-actin. β-Actin was used as an internal control. (G, K) The protein levels of E-cadherin, α-SMA, fibronectin and vimentin were expressed as arbitrary densitometric units and normalized by the value of β-actin and expressed relative to the protein levels and ratio of E-cadherin/α-SMA protein levels in the sham-operated group (defined as 1-fold). Each bar represents mean ± SD of three independent experiments. n = 6 rats per group. *P < 0.05 versus sham group; **P < 0.05 versus UUO model group.
Operated kidneys. Consistent with the results of SOD1 and SOD2 activities, Western blotting studies showed that ATG treatment further increased UUO-induced up-regulation of SOD2 expression at day 14 after surgery (Fig. 6F and G), but did not significantly change the renal expression of the SOD1.

**ATG interacted with SOD in docking study**

The ATG molecule was docked into the three-dimensional structures of SOD to envisage the potential interaction (Fig. 7A and B). It is interesting to note that ATG offered a relatively higher docking score for the binding pocket of rat SOD2 (dock_score: 81.688) than SOD1 (dock_score: 80.383) implying that more
binding contacts and/or lower energy and steric penalties are calculated. The results of the molecular dockings showed functional molecular interactions between ATG and SOD. As shown in **Fig. 7C**, ATG was predicted to interact with SOD1 at the following sites: Asp10; Gly 9, and 55; Ala 144; Arg 142; Cys 56, and 145; Thr 53, and 57; and Gln 54. The molecule was also found to bind with the following SOD2 amino acid residues: His 54, and 187; Trp 185; Tyr 190; and Leu 191 (**Fig. 7D**). Compared with SOD1, the SOD2/ATG complex had more hydrophobic interactions with aromatic residues. Furthermore, a hydrogen bonding interaction was predicted between the hydroxyl group of the lignans' 4-hydroxy-3-methoxyphenyl moiety and the residue at His 187 of SOD2 (**Fig. 7E**). There was no hydrogen bond formed between ATG and SOD1, which might account for the potential affinity of ATG for SOD2 but not SOD1. These data suggested that ATG could bind to SOD2 and confirmed that SOD2 could be a molecular target for ATG enhancement of antioxidant capacity.

**Discussion**

Renal TIF has emerged as a common feature of progressive CKD because it eventually progresses to end-stage renal failure requiring dialysis or kidney transplantation (Liu, 2006). The activation of inflammatory cascade is an early and characteristic feature of CKD. It is widely accepted that sustained inflammation initiates the progression of renal injury in CKD, which eventually leads to the destruction and collapse of the renal parenchyma replaced by fibrotic scar tissues. An intrinsic regulatory loop connecting inflammation and TIF is that damaged TECs recruit inflammatory cells into the renal interstitial compartments and, in turn, infiltrated inflammatory cells—especially macrophages—also produce a myriad of pro-fibrotic and pro-inflammatory cytokines, and then act on renal TECs and resident fibroblasts to facilitate TIF in a paracrine fashion (Leask and Abraham, 2004). This hypothesis is confirmed by the available literature and our own studies as reflected by macrophage infiltration found in the obstructed kidneys after UUO using CD68 staining; nuclear translocation of NF-κB and up-regulation of the recruitment signal MCP-1 was also seen in the UUO kidneys. Infiltrating macrophages in UUO typically correlate with the degree of renal fibrosis, and disruption of macrophage recruitment reduces fibrosis in several disease models. We found that ATG limited the extent of renal inflammation in UUO, which possibly resulted from inhibition of NF-κB activity, reduced ex-
pression of MCP-1, and the resulting amelioration of macrophage infiltration in the tubular interstitium.

Recent studies have demonstrated the diversity and plasticity of macrophage phenotypes and functionality (Duffield, 2011). The macrophage M1/M2 polarization shift may cause different functions and even opposite effects in the progression of kidney diseases. Macrophages are heterogeneous population of immune cells that may acquire distinct functional phenotypes in response to different stimuli. Lipopolysaccharide and T helper 1 cytokine IFN-γ polarize macrophages towards the M1 phenotype which produces large amounts of TNF-α, IL-1β, IL-6, IL-12, IL-23, and MCP-1, increased levels of reactive nitrogen, and reactive oxygen intermediates. However, exposure of macrophages to the T helper 2 cytokines IL-4/IL-13 produces a M2 phenotype that is characterized by efficient phagocytic activity, production of ornithine and polyamines through the arginase pathway, and up-regulated expression of the mannose and galactose receptors, IL-10 and TGF-β1. Increasing evidence suggests that classically activated M1...
macrophages are implicated in initiating and sustaining inflammation while M2 macrophages are associated with anti-inflammatory and homeostatic functions linked to inflammation resolution and tissue remodeling (Wang et al., 2007). This study showed that obstructed kidneys expressed high levels of M1 cytokines TNF-α, IL-1β and IFN-γ. The level of M2 macrophages cytokines IL-10 and TGF-β1 were also up-regulated simultaneously 14 days after UUO injury indicating that an adoptive M1 to M2 macrophage shift takes place at later stages of UUO.

Earlier studies suggested that adoptive transfer of macrophages ameliorated renal fibrosis (Nishida et al., 2005). However, more recent studies showed that M2 macrophage depletion specifically inhibited EMT and subsequent interstitial fibrosis (Pan et al., 2015). These results were consistent with recent reports suggesting that the macrophage subpopulations could inter-switch their phenotype over the course of the response to UUO. One to four days after UUO, macrophage activation was skewed to the M1 type but later on, M2 macrophages were the major class of macrophages, which deteriorated renal fibrosis (Fujiu et al., 2011). Our study found that in addition to its inhibitory properties against pro-inflammatory M1 cytokine expression, ATG dramatically inhibited expression of the M2 cytokine TGF-β1. Nevertheless, another M2 marker IL-10, whose mRNA was not differentially expressed in obstructed kidneys after ATG treatment. This discrepancy warrants additional investigation but may reflect the differential effect of ATG during a sequential course of macrophage phenotype transition. Thus, our observations provided a mechanistic explanation for ATG shifting...
the inflammatory milieu toward resolution by modulation of a macrophage subset polarization.

Over the past decade, many studies have identified tubular EMT as a critical event in the pathogenesis and progression of renal fibrosis. Therefore, targeting EMT has been proposed as an effective therapeutic strategy for preventing renal fibrosis. Our experiments demonstrate that E-cadherin—a key component of adhesion junctions and critical in the maintenance of epithelial integrity—was highly re-expressed by treatment with ATG, but expression of the mesenchymal markers α-SMA, fibronectin and vimentin was strongly down-regulated. These data suggest that ATG may inhibit myofibroblast and ECM formation, at least in part, through inhibition of TEC transdifferentiation.

We know that TGF-β1, the major isoform of the TGF-β family, can be secreted by all types of renal cells and infiltrated inflammatory cells. Consistent with the concept, we noted TGF-β1 in TECs and the renal interstitium. Interestingly, ATG treatment significantly down-regulated protein expression of TGF-β1 in the injured kidneys, and this is accompanied by attenuation of interstitial macrophage infiltration. Reduced TGF-β1 expression by ATG may be the indirect consequence of reduced fibrogenic cellular components including myofibroblasts that originate from TECs by EMT. Also, this decrease in the level of TGF-β1 by ATG may be attributed to fewer inflammatory cells such as macrophages in the interstitium of the UUO kidney; TGF-β1 itself is a potential chemoattractant for these cell types. It was shown recently that M2 macrophages, not myofibroblast, are the main source for this profibrogenic cytokine (Pan et al., 2015). Indeed, we found that ATG decreased the number of infiltrating and M2-polarized macrophages, which were identified by immunohistochemistry and quantitative analysis of RT-PCR.

The canonical signaling pathway induced by TGF-β1 involves activation of the cellular substrates Smad2 and Smad3 by serine/threonine phosphorylation through the TβR-I kinase (Lan and Chung, 2011). The activated Smad2/3 then binds to co-activator Smad4 to form a complex, which subsequently translocates into the nucleus where it regulates the transcription of EMT and fibrosis-related target genes. On the other hand, activated TβR-I also induces the expression of Smad7 via a Smad3-dependent mechanism. This inhibitory Smad7 serves as an antagonist of TGF-β1 signaling by binding to the activated receptor complex to prevent access and phosphorylation of Smad2/3. In addition, Smad7 functions as an adaptor protein to recruit E3 ligases Smurf2 and Arkidia to the TβR-I to promote its degradation through the ubiquitin proteasome pathway (Kavasak et al., 2000). At the same time, ubiquitin-degradation of Smad7 occurs simultaneously. Here, we provide evidence that UUO-induced renal fibrosis was associated with a significant phosphorylation and nuclear translocation of Smad2/3 in the obstructed kidneys, while Smad7 was reduced. In contrast, the ATG treatment effectively reversed these changes.

More importantly, other investigators have previously demonstrated that reduced levels of inhibitory Smad7 protein caused by Smurf2-dependent degradation may be the underlying mechanism required for promotion of TGF-β1/Smad-mediated renal fibrosis. In contrast, overexpression of renal Smad7 could directly induce the expression of 1α,25D3 to inhibit NF-κB activation and NF-κB-driven inflammatory responses (Wang et al., 2005). These results suggest that blockade of TGF-β1/Smad signaling by enhancing the expression level of Smad7, but not influence of Smad2/3, reduces activation of NF-κB and restores the imbalance between TGF-β1/Smad signaling. This may be a key mechanism by which ATG substantially inhibited the UUO-induced inflammatory responses, Smad2/3 activation, and the resulting EMT. Gene knockdown studies of Smad7 mRNA are therefore warranted to provide further evidence of the renoprotective properties of ATG by induction of Smad7 in the development of renal TIF.

A recent report indicates that ROS play an important role in UUO-induced inflammatory responses through activation of NF-κB pathway (Qin et al., 2016). ROS mediate TGF-β1-induced EMT through ERK-directed activation of Smad pathway in TECs (Rhyu et al., 2005). These results support a role for ROS as potent signaling molecules. Inhibition of ROS generation by activation of scavenging enzymes may prevent renal inflammation and EMT in obstructive kidneys. The exacerbated UUO-induced TGF is associated with overproduced ROS in the obstructed kidneys accompanied by decreased antioxidant enzyme activities including SOD and CAT (Ricardo et al., 1997). Here, we demonstrate for the first time that ATG exerted obvious renoprotective effects through decreasing levels of MDA—a known product of lipid peroxidation in UUO rats. This suggests that the anti-fibrogenic effect of ATG is associated with reduced oxidative stress. Notably, we found that treatment with ATG significantly increased SOD2 activity in sham- or UUO-operated rat kidneys. This effect was specific to SOD2 because ATG had no obvious effects on other antioxidant enzymes including GPx, GR, CAT and SOD1. Next, we further investigated whether the increase in SOD2 activity resulted from an up-regulation of SOD2 expression. Western blotting showed that the renal protein level of SOD2 remained unchanged in sham-manipulated rats treated with ATG alone despite the increased activity of SOD2 under the same conditions. This suggests that increased activity of SOD2 by ATG does not merely result from up-regulation of its expression.

Hence, we next employed molecular docking studies to predict the interactions between ATG and SOD2. Our current docking results revealed that ATG established a hydrogen bond with important active residue His 187 of SOD2. In addition to hydrogen bonding, several hydrophobic interactions between ATG and aromatic residues of SOD2 were observed, e.g., His 54, and 187; Trp 185; Tyr 190; and Leu 191. The hydrogen and hydrophobic interactions of ATG to the active site residues might anchor the molecule on the active sites of SOD2 leading to their conformational changes and subsequent increase in enzyme activity. Thus, our data provide valuable insight into the molecular basis of ATG. Further experiments are required to validate SOD2 as a direct molecular target for ATG.

In this study, we employed losartan, an angiotensin II receptor blocker, as a positive control. It is widely accepted that angiotensin II plays a central role in the initiation and progression of renal inflammation, TGF-β production and EMT (Esteban et al., 2004). Here, we found that losartan markedly mitigated the UUO-induced kidney fibrotic changes including interstitial infiltration of macrophages, tubular atrophy, tubulointerstitial space expansion and ECM protein accumulation (fibronectin, vimentin and type I collagen). It is noteworthy that the effect of ATG (3 mg/kg/d) and losartan (20 mg/kg/d) on major determinants of renal TIF are similar, albeit at different doses. In addition, no mortality and obvious treatment-related clinical signs of toxicity were observed in animals treated with ATG (3 mg/kg/d) alone during the assessment period (14 days) suggesting that ATG may be relatively safe when used to treat renal fibrosis.

Conclusion

For the first time, we evaluate the anti-fibrotic effect of ATG using the UUO model. We found that ATG could attenuate tubular injuries and accumulation of ECM proteins in the kidneys of UUO rats. We also noted that ATG could suppress the infiltration of macrophages via inactivation of NF-κB pathway in the progression of renal TIF while inhibiting pro-inflammatory cytokine (TNF-α, IL-1β, and INF-γ) and chemokine (MCP-1) release. Meanwhile, ATG could block the TGF-β1-induced tubular EMT process, which was likely mediated via regulation of the TGF-β1/Smad pathway. Our findings also revealed that ATG might protect against renal in-
juries by increasing the enzyme activity of SOD2 and subsequently reducing renal oxidative stress. Molecular docking investigations indicated that the SOD2 could be a target molecule for ATG effects. These findings might be used for further investigations to explore the therapeutic potential of ATG in the attenuation of progressive renal TIF.

Conflict of interest

The authors declare no competing financial interest.

Acknowledgments

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Supplementary materials

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References


