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Original Article

Inhibition of STAT3 acetylation is associated with attenuated renal fibrosis in the obstructed kidney

Jun NI[1], Yang SHEN[1], Zhen WANG[1], De-cui SHAO[1], Jia LIU[1], Lan-jun FU[2], Ya-li KONG[1], Li ZHOU[1], Hong XUE[1], Yu HUANG[3], Wei ZHANG[1], Chen YU[2, *], Li-min LU[1, *]

[1]Department of Physiology and Pathophysiology, Shanghai Medical College, Fudan University, Shanghai 200032, China; [2]Department of Nephrology, Tongji Hospital, Tongji University School of Medicine, Shanghai 200065, China; [3]School of Biomedical Sciences and Institute of Vascular Medicine, Chinese University of Hong Kong, Hong Kong, China

Aim: To explore the relationship between the signal transducer and activator of transcription 3 (STAT3) signaling and renal fibrosis. **Methods:** Rat renal tubular epithelial NRK-52E cells were treated with angiotensin II (Ang II), nicotinamide (an inhibitor of NAD^+-dependent class III protein deacetylases, SIRT1–7), or resveratrol (an activator of SIRT1). Mice underwent unilateral ureteral obstruction (UUO) were used for *in vivo* studies. Renal interstitial fibrosis was observed with HE and Masson's trichrome staining. STAT3 acetylation and phosphorylation, fibronectin, collagen I, collagen IV, and α-smooth muscle actin (α-SMA) levels were examined using Western blotting. **Results:** Nicotinamide (0.625–10 mmol/L) dose-dependently increased STAT3 acetylation on Lys685 and phosphorylation on Tyr705 in NRK-52E cells, accompanied by accumulation of fibronectin and collagen IV. Ang II increased STAT3 phosphorylation on Tyr705 and the expression of fibronectin, collagen IV and α-SMA in the cells. Pretreatment with resveratrol (12.5 µmol/L) blocked Ang II-induced effects in the cells. UUO induced marked STAT3 phosphorylation, fibronectin, collagen IV and α-SMA accumulation, and renal interstitial fibrosis in the obstructed kidneys, which were significantly attenuated by daily administration of resveratrol (100 mg/kg). **Conclusion:** STAT3 acetylation plays an important role in activation of STAT3 signaling pathway and consequent renal fibrosis.

Keywords: chronic kidney disease; renal fibrosis; unilateral ureteral obstruction (UUO); angiotensin II; resveratrol; nicotinamide; STAT3; acetylation; phosphorylation

Introduction

Renal fibrosis, which is characterized by glomerulosclerosis and tubulointerstitial fibrosis, has been considered as a typical pathological feature during the progression of chronic kidney disease (CKD)[1, 2]. Although there are various apparent causes of renal fibrosis, this pathological feature may induce irreversible dysfunction of the kidney and eventually leads to end-stage kidney disease. Despite the debate whether injured epithelial cells can be the direct precursor of myofibroblasts via processing type II epithelial-to-mesenchymal transition (EMT), renal tubular epithelial cell is known as a primary cell in which pro-fibrotic cellular changes occur, contributing to

the early development and progression of renal interstitial fibrosis[3–9]. These changes include decrease in tubular diameter, increase in expression of α-smooth muscle actin (α-SMA) and deposition of extracellular matrix, ultimately leading to tubular atrophy, loss of functional renal parenchyma, which contribute to renal interstitial fibrosis.

Although the exact mechanism of renal interstitial fibrosis is unclear, increasing evidence suggests that the excessive activation of the local renin-angiotensin system (RAS), which leads to a prominent elevation of angiotensin II (Ang II), is involved in the progression of CKD[10–12]. Previous *in vivo* studies have shown that the elevation of Ang II by chronic infusion results in renal fibrosis, which is associated with expression of proinflammatory cytokines and fibrosis-associated genes[13]. Ang II is one of the main pathogenic mediators of renal interstitial fibrosis, especially in obstructive nephropathy[10–12, 14]. Additionally, a number of previous studies have revealed that

the suppression of intrarenal RAS prevents the pro-fibrotic changes induced by unilateral ureteral obstruction (UUO)[15–17].

The Janus kinase family (JAK)/signal transducers and activators of transcription (STAT) signaling pathway constitutes an important cascade for a wide range of signal transduction cytokines and growth factors expression. The JAK2/STAT3 signaling pathway can be activated by Ang II in different organs, including the kidney[18–22]. STAT3 has previously been considered to be involved in promoting cell cycle progression and cellular transformation and in preventing apoptosis[23, 24]. Increasing evidence suggests that STAT3 can be activated and highly expressed during the progression of fibrogenesis in the obstructed kidney[25–27] and under other pathophysiological circumstances[9]. Despite being one of the major mediators of transforming growth factor-β1 (TGF-β1)-driven kidney dysfunction[27–29], the role of Ang II-induced STAT3 signaling in renal fibrogenesis remains uncertain.

Although the classical view of STAT3 activation focuses on the phosphorylation on tyrosine 705 (Tyr705) and serine 727 (ser727)[30], recent studies have shown that acetylation on lysine 685 (Lys685) of STAT3 also plays a role in transactivation of target genes[31, 32]. The reversible acetylation of STAT3 is regulated by the CREB-binding protein/p300 family of histone acetyltransferases and histone deacetylases (HDACs)[31, 33]. Several HDACs have been reported to be involved in deacetylation of STAT3[32, 34]. One of the best characterized deacetylases is sirtuin 1 (SIRT1), a member of nicotinamide adenine dinucleotide (NAD$^+$)-dependent class III protein deacetylases, which can be inhibited by nicotinamide[35, 36]. SIRT1, which mediates the deacetylation of STAT3 Lys685 site[37–41], can be activated by resveratrol (trans-3,4′,5-trihydroxystilbene)[42, 43], a natural polyphenol with renal protective effects that depend on deacetylation of Smad3[44], p53[45], and NF-κB p65[46]. However, the role of STAT3 acetylation in the antifibrotic activity of resveratrol has yet to be clarified.

In the present study, we investigated the relationship between the acetylation and phosphorylation of STAT3 in Ang II-induced pro-fibrotic responses in renal tubular epithelial cells and UUO-induced renal fibrosis to determine the role of STAT3 signaling in renal fibrogenesis both *in vitro* and *in vivo*.

Materials and methods
Materials
Dulbecco's modified Eagle's medium (DMEM), human angiotensin II, nicotinamide and resveratrol were purchased from Sigma-Aldrich (Saint Louis, MO, USA). Fetal bovine serum (FBS) was obtained from Tianhang Biotechnology Co (Hangzhou, China). The BCA Protein Assay Kit was from Shenergy Biocolor BioScience and Technology (Shanghai, China). Anti-fibronectin antibody was obtained from Sigma-Aldrich (Saint Louis, MO, USA), and anti-α-SMA antibody, from Santa Cruz Biotechnologies, Inc (Santa Cruz, CA, USA). Anti-collagen I antibody and anti-collagen IV antibody were procured from Abcam (Cambridge, MA, USA). Anti-phospho-STAT3 (Tyr705) antibody, anti-phospho-STAT3 (Ser727) antibody,

anti-acetyl-STAT3 (K685) antibody and anti-STAT3 antibody were obtained from Cell Signaling Technology (Beverly, MA, USA). Anti-GAPDH antibody, anti-β-actin antibody, horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence (ECL) detection kit were obtained from Beyotime Institute of Biotechnology (Haimen, China). Polyvinylidene difluoride membrane was obtained from Millipore (Billerica, MA, USA). Protease and phosphatase inhibitors were from Roche (Mannheim, Germany). All of the other chemicals and reagents used were of analytical grade.

Animals
Male C57BL/6J mice (20–25 g) were obtained from Shanghai SLAC Laboratory Animal Co Ltd (Shanghai, China). All animal experiments were performed according to the Criteria of the Medical Laboratory Animal Administrative Committee of Shanghai and the Guide for Care and Use of Laboratory Animals of Fudan University, and the protocols were approved by the Ethics Committee for Experimental Research, Shanghai Medical College, Fudan University. UUO surgery was performed under 10% chloral hydrate anesthesia via intraperitoneal injection (4 mL/kg body weight). The left ureter was visualized by a flank incision and ligated with 5–0 silk. Sham mice underwent the same surgery, except the left ureter ligation. Over the course of the study, six mice were included in each group and were euthanized 1, 3, or 7 d after surgery. In the following study, four mice were used in each group. Mice that underwent UUO surgery were treated either with vehicle (0.5% carboxymethyl cellulose) or resveratrol (100 mg/kg body weight) through oral gavage at 0.1 mL per day until they were euthanized at the end of the experiment.

Cell culture
The rat renal tubular epithelial cell line (NRK-52E) was purchased from the Institute of Biochemistry and Cell Biology (Shanghai, China) and cultured in DMEM media containing 10% FBS in an atmosphere of 5% CO$_2$ at 37 °C. Before the experiment, cells were starved in a medium containing 0.5% FBS for 24 h to obtain quiescent cells. Cells were treated with Ang II at different concentrations for a designated time period before they were harvested. When necessary, cells were pretreated with diluent (DMSO) or resveratrol (12.5 µmol/L) for 60 min before Ang II application. Each experiment was repeated at least three times.

Western blotting
Cell lysate homogenates were prepared as previously described[47]. In brief, the renal cortex or cultured cells were lysed in 1×sodium dodecyl sulfate (SDS) and supplemented with proteinase inhibitor and phosphatase inhibitor. Protein concentrations were determined using a BCA Protein Assay Kit according to the manufacturer's instructions. An equal amount (40 µg per sample) of total protein was loaded and separated by electrophoresis on a 10% SDS-PAGE gel and then transblotted onto a polyvinylidene difluoride (PVDF) mem-

brane. After the transfer, the membrane was blocked with 5% skim milk powder dissolved in Tris-buffered saline with 0.1% Tween (TBS/Tween) for 1 h at room temperature with gentle shaking. The membranes were then incubated with primary antibody overnight at 4 °C [anti-fibronectin antibody, 1:20000; anti-collagen I antibody, 1:1000; anti-collagen IV antibody, 1:1000; anti-α-SMA antibody, 1:5000; anti-phospho-STAT3 (Tyr705) antibody, 1:1000; anti-phospho-STAT3 (Ser727) antibody, 1:1000; anti-acetyl-STAT3 (K685) antibody, 1:500; anti-STAT3 antibody 1:2000; and anti-β-actin antibody, 1:5000]. On the next day, the membranes were washed 3 times with TBS/Tween and incubated with horseradish peroxidase-conjugated secondary antibodies for 2 h. After another 3 washes with TBS/Tween, the hybridizing bands were developed using an ECL detection kit according to the manufacturer's instructions. The signals were then imaged by chemiluminescence for 0.1–5 min to visualize signals. Graphs represent densitometric analyses of bands and were normalized to GAPDH or STAT3 signals, with relative protein levels expressed as fold inductions over controls.

Renal histology
Kidneys were fixed in 10% neutral buffered formalin and then embedded in paraffin. Sections were collected and prepared accordingly. Slides of paraffinized tissue sections were deparaffinized, rehydrated and washed in distilled water. Sections were stained with hematoxylin-eosin (HE) and Masson's trichrome. Histological changes, such as the degrees of tubular atrophy and interstitial fibrosis, were observed at 400×optical magnification.

Statistical analysis
Data are presented as the mean±SEM with statistical analysis performed using one-way analysis of variance with post hoc analysis using Tukey's multiple comparison test. The differences between two groups were compared using Student's t-test. P<0.05 was considered to indicate a statistically significant difference between mean values.

Results
Nicotinamide induced STAT3 activation and pro-fibrotic responses in cultured renal tubular epithelial cells
Western blot studies showed that nicotinamide, an inhibitor of SIRTs[35, 36], significantly enhanced STAT3 acetylation on Lys685 and the phosphorylation on Tyr705 in NRK-52E cells (48 h) (Figure 1A). These effects were accompanied by an accumulation of fibronectin and collagen IV (Figure 1B).

Ang II induced STAT3 activation and pro-fibrotic responses in cultured renal tubular epithelial cells
Western blot analysis showed that the Ang II treatment of NRK-52E cells for 48 h increased fibronectin, collagen IV and α-SMA protein levels in a dose-dependent manner (Figure 2A). A dose of 1 μmol/L Ang II, which can significantly upregulate the hallmarks of fibrogenesis and ECM, increased STAT3 phosphorylation on Tyr705, peaking at 60 min (Figure

2B). Ang II application did not affect STAT3 phosphorylation on ser727 or acetylation on Lys685 (Figure 2B).

Resveratrol prevented Ang II-induced STAT3 phosphorylation on Try705 and pro-fibrotic responses in tubular epithelial cells via inhibition of STAT3 acetylation on Lys685
Resveratrol, an activator of SIRT1[42, 43], reduced the basal STAT3 acetylation on Lys685 (Figure 3A). Resveratrol pretreatment of tubular epithelial cells prevented Ang II-induced upregulation of STAT3 phosphorylation on Tyr705 (Figure 3A). Neither resveratrol nor Ang II had an obvious effect on STAT3's ser727 phosphorylation (Figure 3A). Furthermore, resveratrol attenuated Ang II-induced upregulation of fibronectin, collagen I, collagen IV, and α-SMA (Figure 3B).

Resveratrol inhibited STAT3 acetylation, phosphorylation and renal fibrosis in the obstructed kidney
In UUO mice, a significant increase in STAT3 phosphorylation on Tyr705 was observed 1 d after surgery, whereas no obvious change in STAT3 acetylation on Lys685 was found (Figure 4A). The fibrotic associated proteins, including fibronectin, collagen IV and α-SMA were increased in UUO mice and showed statistical significance starting on the third day after surgery (Figure 4B).

Resveratrol treatment of UUO mice through gavage decreased STAT3 acetylation on Lys685 (Figure 5A). This effect was accompanied by a reduction in UUO-induced Tyr705 phosphorylation (1 d) (Figure 5A). Resveratrol also inhibited UUO-induced upregulation of fibronectin, collagen I, collagen IV, and α-SMA protein levels in the obstructed kidney (3 d) (Figure 5B).

HE staining was performed to observe the histological changes in the kidney. Compared with the vehicle and sham kidneys, the obstructed kidneys showed interstitial fibrosis at d 3. Resveratrol treatment appeared to ameliorate UUO-induced renal fibrosis (Figure 6A). Masson's trichrome staining showed that collagen accumulation (blue stains) was also attenuated by resveratrol treatment (Figure 6B). These results were consistent with the in vitro findings, which suggested that STAT3 acetylation was involved in the pro-fibrotic action of the obstructed kidney.

Discussion
The major STAT3 acetylation site that enhances its DNA binding activity and transactivation activity is located at its C-terminal position lysine 685[31]. The present study demonstrated a novel mechanism of fibrogenesis mediated by STAT3 acetylation that had not been previously reported in the kidney. STAT3 acetylation on Lys685 is necessary for STAT3 signaling and the transactivation of downstream pro-fibrotic genes.

The class III deacetylases, silent information regulator 2 (Sir2) family histone/protein deacetylases (sirtuins or SIRTs), catalyze the NAD^+-dependent deacetylation of histone and non-histone proteins[35, 48–50]. In this study, we used nicotinamide, an inhibitor of SIRTs[35, 36], to investigate the role of STAT3 acetylation in the progression of tubulointerstitial
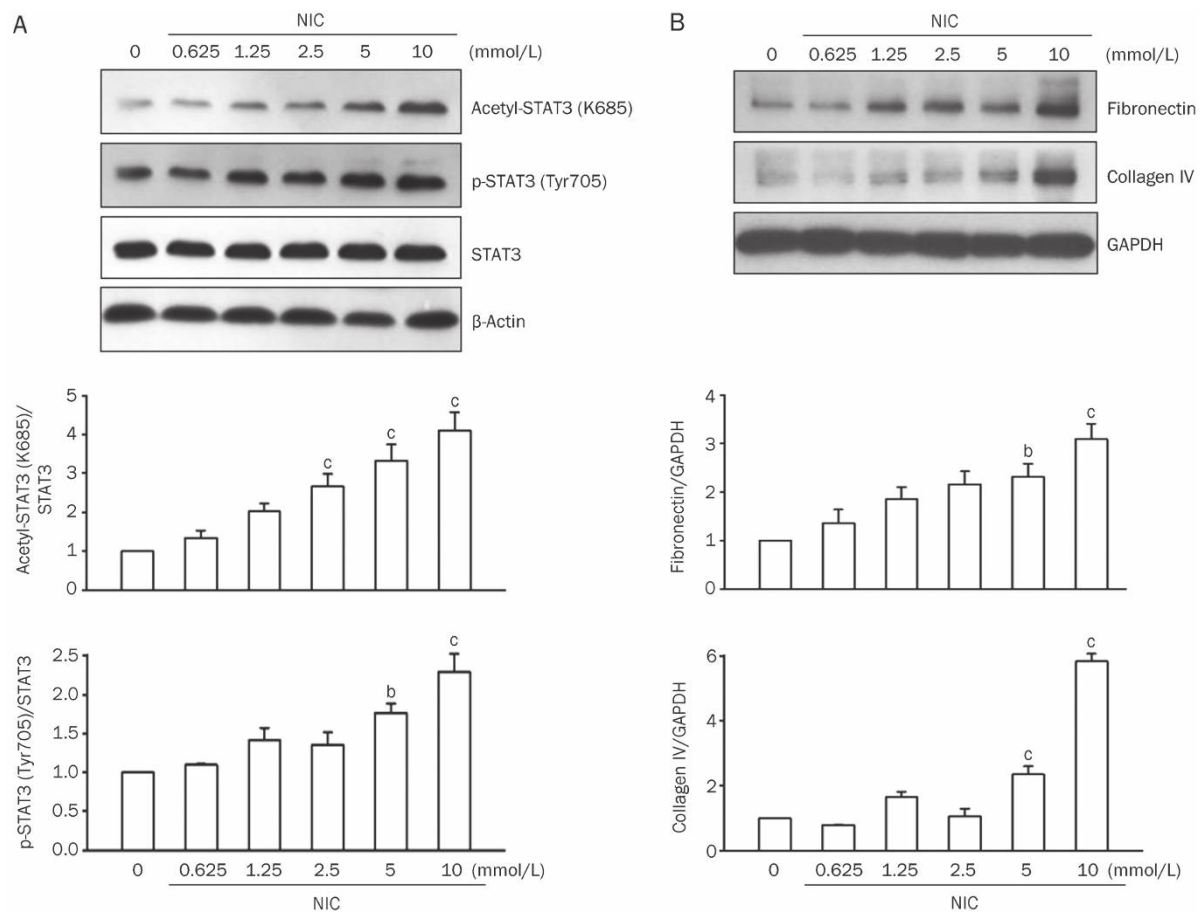
Figure 1. Effect of nicotinamide (NIC) on signal transducer and activator of transcription 3 (STAT3) activation and extracellular matrix (ECM) expression in NRK-52E cells. Cells were incubated with NIC at the indicated dose for 48 h. (A) Western blotting visualized the levels of acetyl-STAT3 (Lys685) and phospho-STAT3 (Tyr705). (B) Fibronectin and collagen IV levels were detected by Western blotting. Media containing NIC were changed every 24 h. Data are the mean±SEM of 3–6 experiments. [b]$P<0.05$, [c]$P<0.01$ compared with control [NIC (0 mmol/L)].

fibrosis. The present *in vitro* study showed that nicotinamide increased both STAT3 acetylation and phosphorylation, concomitant with the enhanced pro-fibrotic gene expression in tubular epithelial cells. These changes that had not been reported previously indicated the correlation between STAT3 signaling and renal fibrosis. Therefore, the most critical question remains as to the role of STAT3 acetylation and phosphorylation in pro-fibrotic responses.

To answer this question, we investigated the role of STAT3 activation in cellular pro-fibrotic responses by Ang II stimulation in cultured proximal tubular epithelial cells. Ang II has been demonstrated to be involved in multiple pathogenesis-induced renal fibrosis, while STAT3 signaling has been observed to be involved in Ang II-induced renal tubular epithelial cell dysfunction. For example, STAT3 is involved in the Ang II-induced expression of tissue inhibitor of metalloproteinase-1 (TIMP-1) in proximal tubular epithelial cells[22]. However, the exact role of STAT3 in Ang II-induced renal pro-fibrotic changes has not been thoroughly investigated. We observed that Ang II induced pro-fibrotic responses in a dose-dependent fashion, which was consistent with previ-

ous observations. Since nicotinamide can increase the basal level of STAT3 acetylation through the inhibition of SIRT1, we treated cells with nicotinamide for 48 h and detected a simultaneous increase of the phosphorylation on Tyr705. The long-lasting progress of nicotinamide was different from the effect of Ang II, which can upregulate STAT3 phosphorylation on Tyr705 by direct activation of JAK2, the upstream tyrosine kinase of STAT3. Thus, we detected Ang II/STAT3 signaling in a classical short time-dependent fashion within 2 h. Ang II treatment increased STAT3 Try705 phosphorylation, suggesting that STAT3 signaling might be involved in Ang II-induced functional changes in tubular epithelial cells. However, no obvious change in STAT3 Ser727 phosphorylation or Lys685 acetylation was shown. These findings indicated that the effect of Ang II on STAT3 signaling was selective.

SIRT1 has been proven to be the major enzyme that removes acetyl groups from STAT3 lysine residues[38, 39, 41]. As a SIRT1 activator[42, 43], resveratrol was used to identify whether acetylated STAT3 is involved in the activation of STAT3 signaling. The basal STAT3 acetylation on Lys685 was decreased by resveratrol, resulting in negation of Ang II-induced upregulation
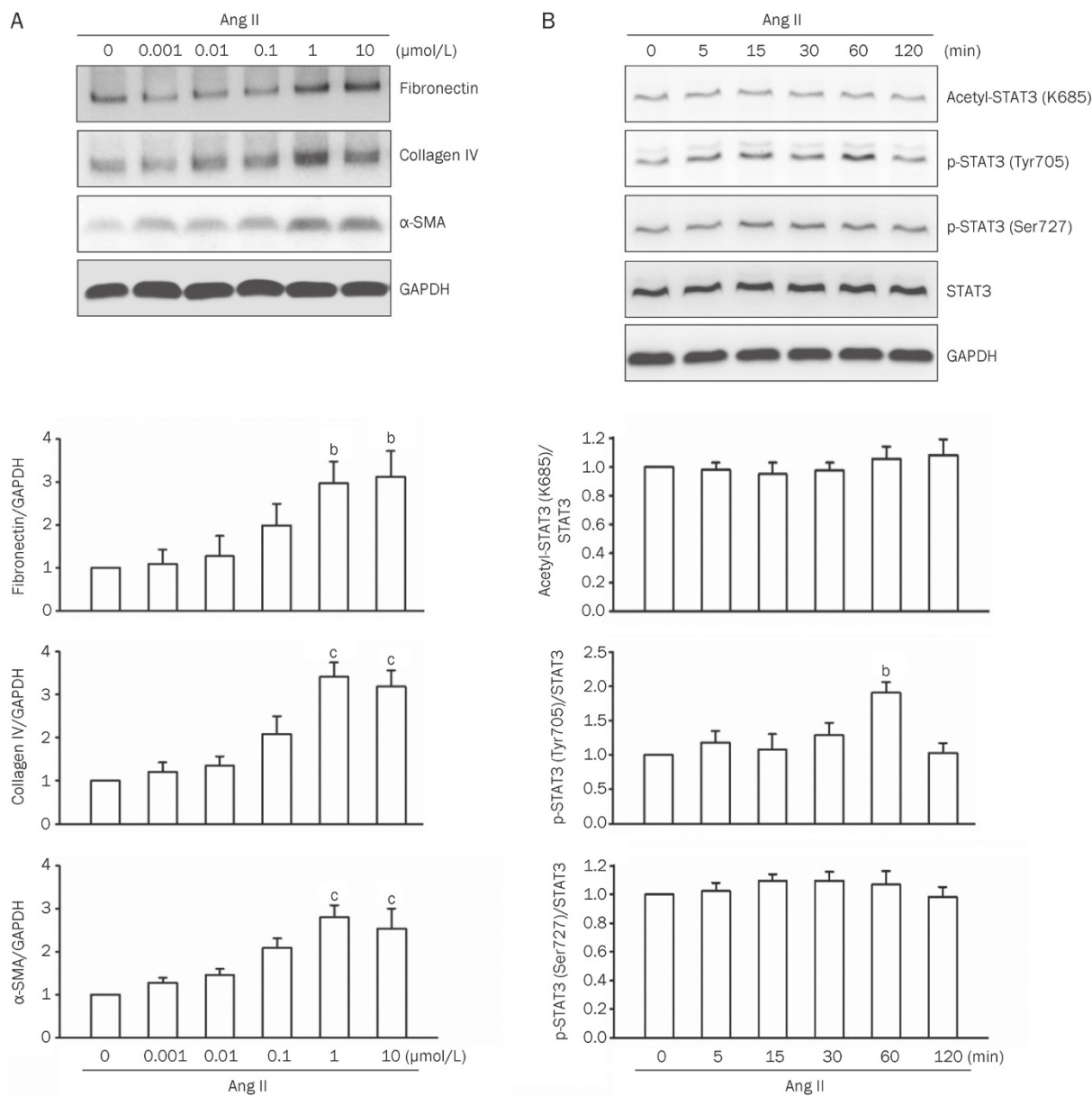
Figure 2. Effect of angiotensin II (Ang II) on STAT3 activation and expression of pro-fibrotic genes in NRK-52E cells. (A) Cells were treated with Ang II at the indicated concentrations for 48 h. Fibronectin, collagen IV and α-smooth muscle actin (α-SMA) were analyzed by Western blot analysis. Media containing Ang II were changed every 24 h. (B) Cells were stimulated with 1 μmol/L Ang II for 0–120 min. Acetyl-STAT3 (Lys685), phospho-STAT3 (Tyr705) and phospho-STAT3 (Ser727) were analyzed by Western blot analysis. Data are the mean±SEM of 4–5 experiments. [b]$P<0.05$, [c]$P<0.01$ compared with control [Ang II (0 μmol/L) or Ang II (0 min)].

of STAT3 Try705 phosphorylation and the downstream pro-fibrotic genes. Furthermore, resveratrol did not affect STAT3 phosphorylation on Ser727, indicating that the action of this treatment was selective specifically in the STAT3 signaling pathway. Thus, we speculate that STAT3 acetylation on Lys685 is necessary for activation of STAT3 signaling involved in Ang II-induced pro-fibrotic changes of tubular epithelial cells. However, the relationship between acetylation on Lys685 and phosphorylation on Try705 still requires further investigation.

In obstructed kidneys, Ang II levels have been proven to be markedly elevated[10, 11], whereas Ang II type 1 receptor blocker (ARB) and angiotensin-converting enzyme inhibitor (ACEI) demonstrate significant antifibrotic effects[15, 17]. Furthermore, the formation of renal cortical TGF-β1 and related fibrogenic factors can be considerably reduced by angiotensinogen antisense RNA treatment in early UUO[16]. Thus, the effect of STAT3 acetylation inhibition on antifibrotic actions was further determined in UUO mice. UUO significantly increased STAT3 phosphorylation on Try705 in the early stage of renal fibrosis, and this modification was partially inhibited by resveratrol. Moreover, resveratrol treatment exhibited an antifi-
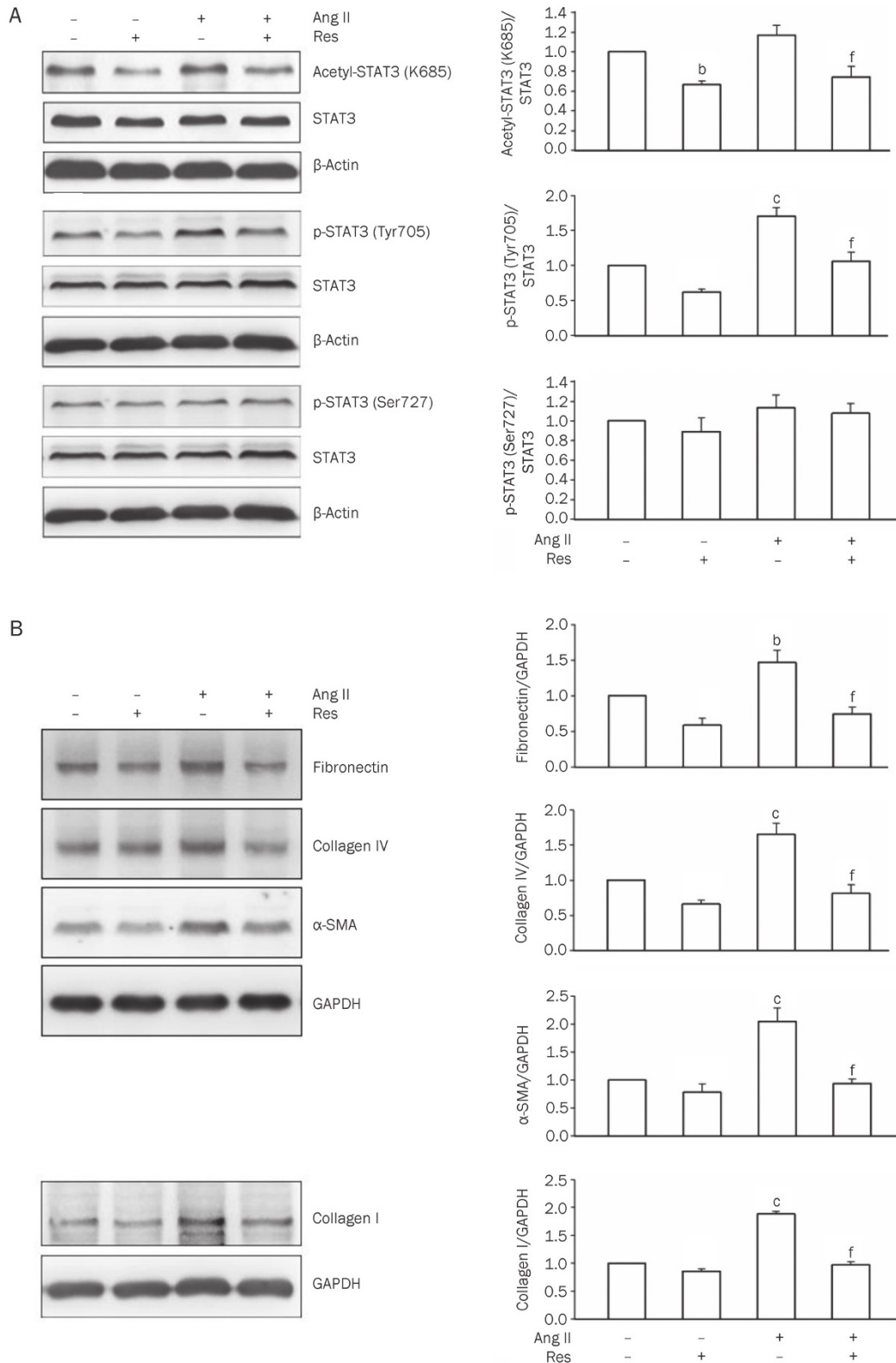
Figure 3. Effect of resveratrol (Res) on Ang II-induced STAT3 activation and expression of pro-fibrotic genes in NRK-52E cells. Cells were treated with Ang II in the presence or absence of Res. (A) After 1 h of Ang II treatment, with or without 1 h Res (12.5 μmol/L) pretreatment, acetyl-STAT3 (Lys685), phospho-STAT3 (Tyr705) and phospho-STAT3 (Ser727) were analyzed by Western blot analysis. (B) After 48 h of Ang II treatment, with or without 1 h Res (12.5 μmol/L) pretreatment, fibronectin, collagen I, collagen IV, and α-SMA were analyzed by Western blot analysis. Media containing Ang II or Res were changed every 24 h. Data are the mean±SEM of 4 experiments. $^{b}P<0.05$, $^{c}P<0.01$ compared with control [Ang II (−) and Res (−)]. $^{f}P<0.01$ compared with Ang II only.
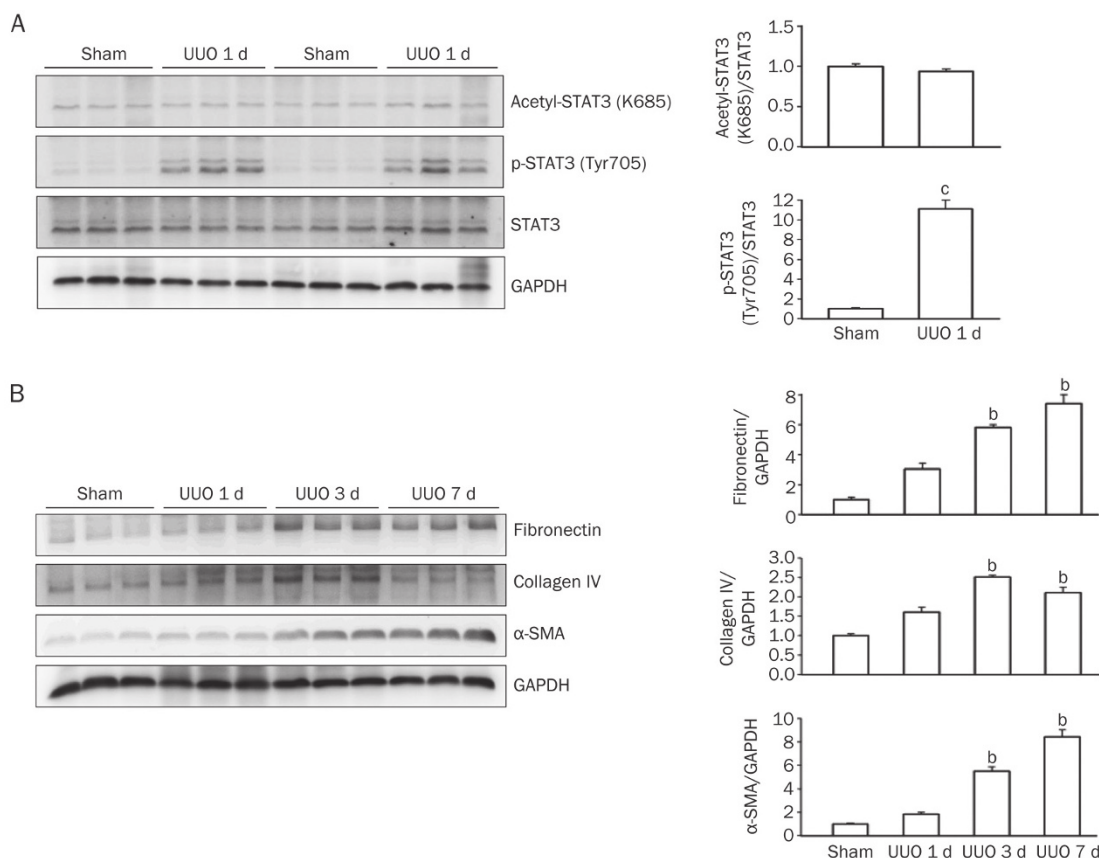
Figure 4. Unilateral ureteral obstruction (UUO) induced pro-fibrotic responses in the kidney. (A) Acetyl-STAT3 (Lys685) and phospho-STAT3 (Tyr705) were analyzed by Western blot analysis after UUO for 1 d. (B) Western blotting visualized fibronectin, collagen IV and α-SMA in a sample time course of UUO mice. Data are the mean±SEM of 6 animals. [b]$P<0.05$ compared with sham.

brotic effect that was similar to *in vitro* observations. To our knowledge, this is the first evidence suggesting that STAT3 acetylation is involved in renal fibrosis *in vivo*. Both *in vitro* and *in vivo* findings indicate that STAT3 acetylation facilitates phosphorylation at special sites, such as Try705. This conclusion is consistent with those of previous studies. For instance, it has been proven that SIRT1 opposes STAT3 phosphorylation and STAT3-dependent effects of IL-22 through STAT3 deacetylation in keratinocytes[39]. In addition, trichostatin A, a broad inhibitor of class I and class II HDACs[51, 52], can abolish the inhibition of STAT3 activation that is induced by class I HDAC overexpression in 293T cells, indicating that acetylation is critical for STAT3 activation[32]. The potential mechanism of the interaction between STAT3 acetylation and phosphorylation may be the sustained stabilization of STAT3 provided by acetylation resulting from the blockage of degradation via the ubiquitination-mediated proteasomal pathway[53].

The exact role that HDACs or acetylated STAT3 plays in renal fibrosis seems to be much more complicated than the features presented by the current study. Therefore, it is difficult to claim that the antifibrotic effect of resveratrol on the development of renal fibrosis is entirely due to the inhibition of STAT3 activation. Further studies using more selective approaches need to be performed to determine whether

antifibrotic actions are simply attributed to reducing STAT3 acetylation and the interaction between STAT3 acetylation and phosphorylation under physiological and pathophysiological circumstances. However, the similar molecular mechanisms of resveratrol's antifibrotic effects identified *in vitro* and *in vivo* suggest the essential role of STAT3 acetylation in renal profibrotic responses.

In this study, we demonstrated a constitutive STAT3 acetylation on Lys685 in the kidney that may be indispensable for STAT3 signaling-mediated fibrogenesis. These findings suggest that the inhibition of STAT3 acetylation may represent a novel therapeutic approach toward CKD associated with fibrosis.

Author contribution

Yu HUANG, Wei ZHANG, Chen YU, and Li-min LU designed the research project; Jun NI and Yang SHEN performed the experiments; Zhen WANG, De-cui SHAO, and Jia LIU ana-
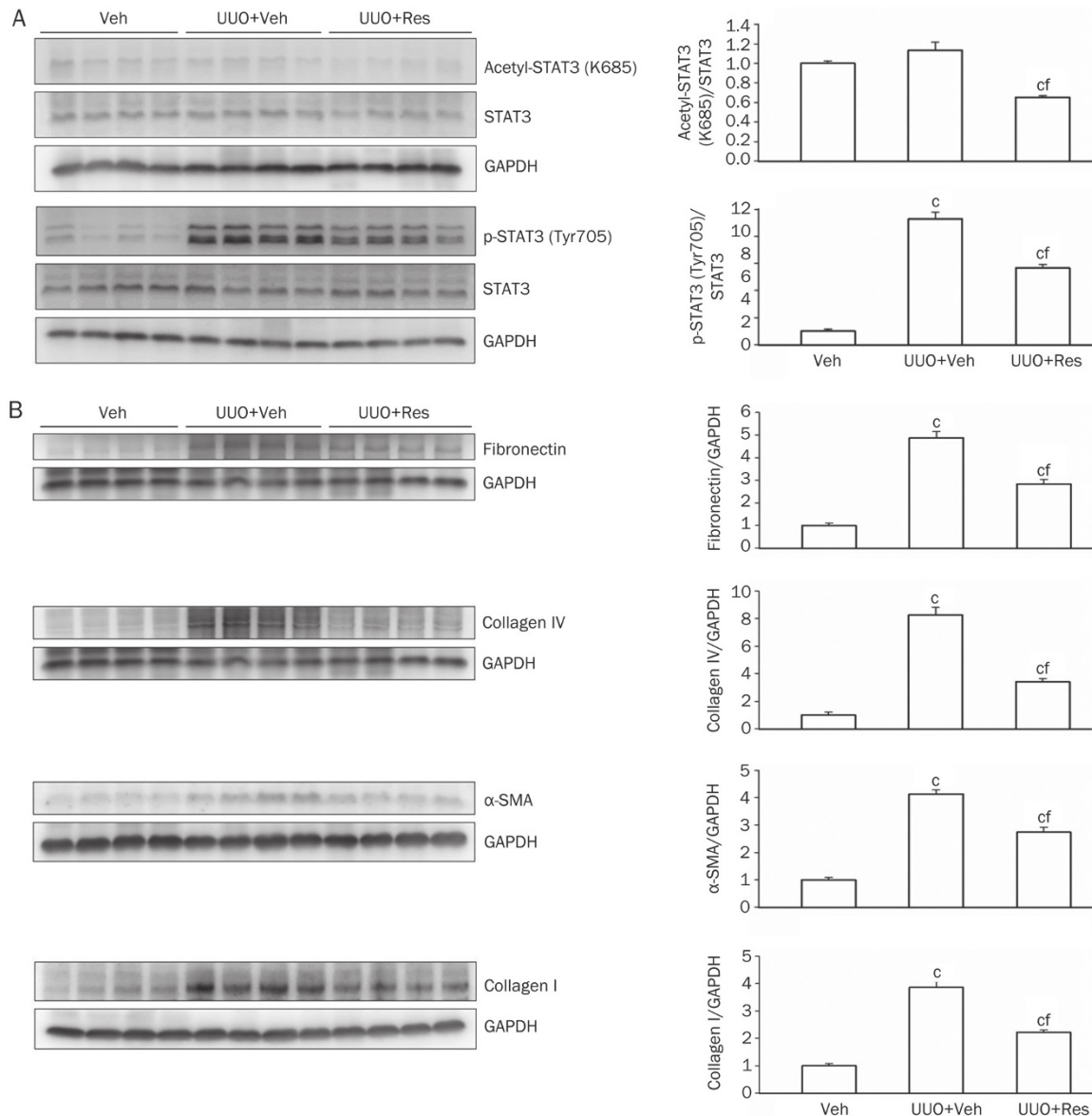
Figure 5. Effect of Res on UUO-induced STAT3 activation and expression of pro-fibrotic genes in the obstructed kidney. Control and UUO mice received vehicle or 100 mg/kg Res by oral gavage once a day for 1 d (A) or 3 d (B). (A) Acetyl-STAT3 (Lys685) and phospho-STAT3 (Tyr705) were analyzed by Western blot analysis. (B) Fibronectin, collagen IV, α-SMA, and collagen I were analyzed by Western blot analysis. Data are the mean±SEM of 4 animals. $^cP<0.01$ compared with vehicle (Veh). $^fP<0.01$ compared with UUO with vehicle (UUO+Veh).

lyzed the data; Lan-jun FU and Ya-li KONG contributed the reagents and materials; Jun NI, Li ZHOU, and Hong XUE wrote the manuscript.

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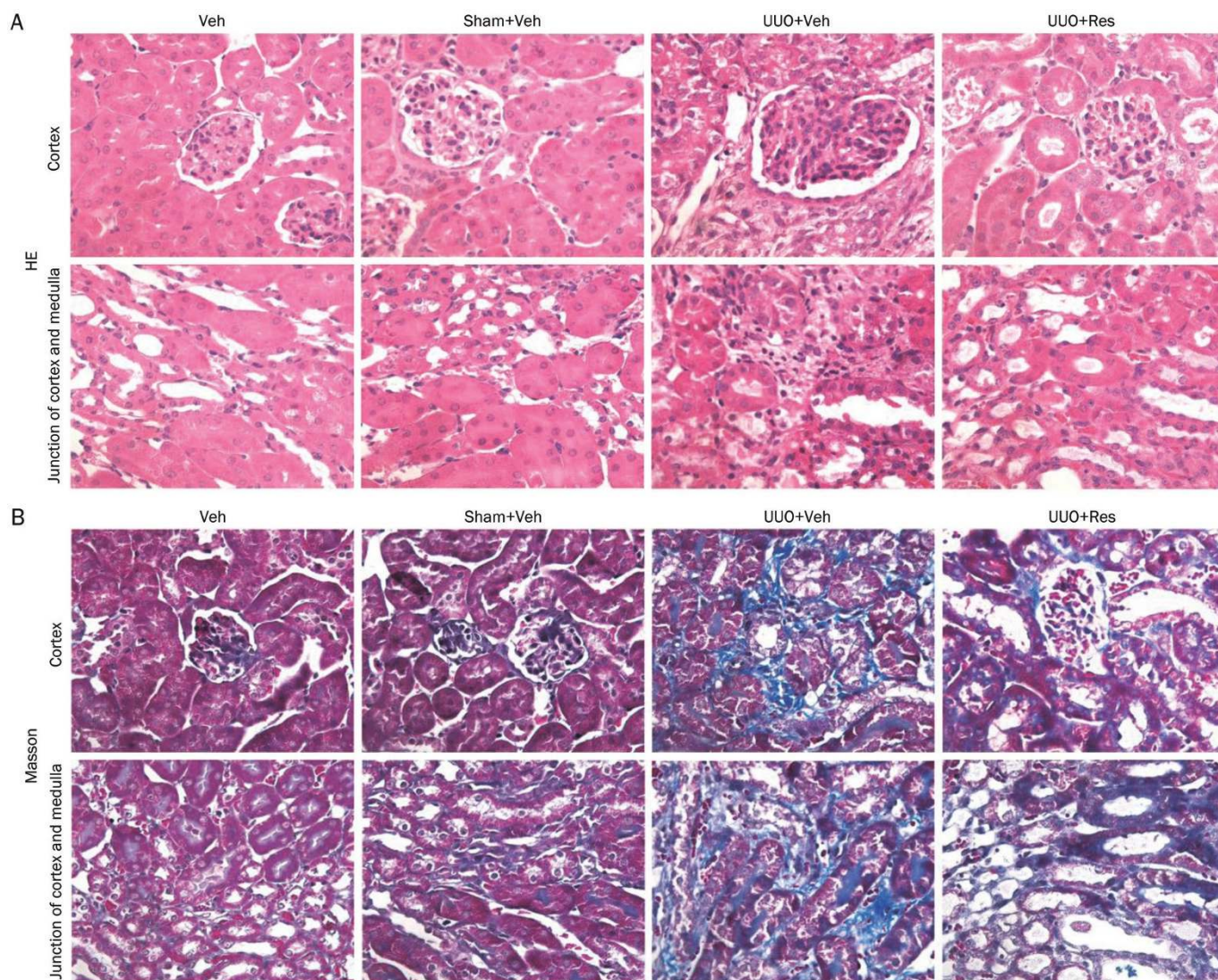
Figure 6. Effect of Res on tubulointerstitial fibrosis in the UUO kidney. Control, sham and UUO mice received vehicle or 100 mg/kg Res by oral gavage once a day for 3 d. (A) Representative sections of hematoxylin and eosin (HE)-stained kidneys. Original magnifications ×400. (B) Representative sections of Masson's trichrome-stained kidneys. Collagen was stained blue. Original magnifications ×400.

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