Mechanism of chronic aristolochic acid nephropathy: role of Smad3

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Zhou L, Fu P, Huang XR, Liu F, Chung AC, Lai KN, Lan HY. Mechanism of chronic aristolochic acid nephropathy: role of Smad3. Am J Physiol Renal Physiol 298: F1006–F1017, 2010. First published January 20, 2010; doi:10.1152/ajprenal.00675.2009.—Aristolochic acid nephropathy (AAN) has become a worldwide disease and is the most severe complication related to the use of traditional Chinese medicine. However, the pathogenic mechanisms of AAN remain unclear and therapies are limited. The present study tested the hypothesis that transforming growth factor (TGF)-β/Smad3 may be a key pathway leading to chronic AAN. This was examined in vivo in Smad3 wild-type/knockout (WT/KO) mice and in vitro in tubular epithelial cells with knockdown of Smad2 or Smad3. Results revealed that chronic administration of aristolochic acid (AA) resulted in a severe AAN characterized by progressive renal dysfunction and tubulointerstitial fibrosis including epithelial-mesenchymal transition (EMT) in Smad3 WT mice, but not in Smad3 KO mice, suggesting a critical role for Smad3 in the development of AAN. This was further tested in vitro. We found that AA was able to activate Smad signaling to mediate EMT and renal fibrosis via both TGF-β-dependent and JNK/MAP kinase-dependent mechanisms because blockade of JNK and specific knockdown of Smad3, but not Smad2, were able to attenuate AA-stimulated collagen matrix expression and EMT. In conclusion, TGF-β/Smad3 may be an essential mediator for chronic AAN. Results from this study indicate that specific blockade of the TGF-β/Smad3 signaling pathway may have therapeutic potential for chronic AAN.

TGF-β signaling; renal fibrosis; herbal kidney disease

Hernal therapy has been used worldwide. However, the potential side effects of herbs on the kidney and other organs have been recently reported and caused much attention (1). Of them, the most severe one may be aristolochic acid nephropathy (AAN), which was originally called Chinese herb nephropathy before aristolochic acid was known as a cause of AAN (10, 23). Since the first report of Chinese herb nephropathy in Belgium in 1993 (24), AAN has been reported as a worldwide disease because it also shares many similarities to Balkan endemic nephropathy (5).

Clinically, patients with AAN exhibit a rapidly progressive renal function deterioration and kidney scarring, resulting in end-stage renal disease (5, 24, 29). This is also found in experimental animal models induced by aristolochic acid (AA) (6). Pathologically, chronic AAN is characterized by extensive tubulointerstitial fibrosis with atrophy and loss of the tubules in both patients and animal models of AAN (5, 6, 24, 29). The lesions of AAN are located in the cortex, primarily involving proximal tubular epithelial cells (5, 6, 24, 29). However, the precise mechanisms leading to the pathogenesis of chronic AAN remain largely unexplored, which has hampered the development of effective therapies for patients with AAN.

It is now clear that transforming growth factor (TGF)-β1 is a key mediator of tissue scarring and acts by stimulating its downstream mediator Smad3 to induce fibrosis under a variety of disease conditions (20), including radiation-associated skin injury (9), bleomycin-induced lung fibrosis (33), cardiac fibrosis after acute myocardial infarction (2), obstructive kidney disease (22), and liver and colon fibrosis (12, 13). Although upregulation of TGF-β1 and activation of its downstream signaling pathway Smad2/3 have been observed in the fibrotic tissues of chronic AAN (14, 18, 19, 27, 31), the functional role of TGF-β/Smad signaling in the pathogenesis of AAN remains unknown. Thus the present study tested the hypothesis that AA may mediate AAN via the TGF-β/Smad3-dependent mechanism. This was examined in a mouse model of chronic AA induced in Smad3 wild-type/knockout (WT/KO) mice by chronic administration of AA. Furthermore, the signaling mechanisms by which AA induces the epithelial-mesenchymal transition (EMT) and renal fibrosis were examined in vitro in a normal rat renal tubular epithelial cell (TEC) line, NRK52E, because TECs are the major target cells of AA.

Materials and methods

Chemicals and antibodies. Aristolochic acid sodium salt (the mixture of 65% AAI and 27% AAII) was purchased from Sigma-Aldrich (St. Louis, MO). A JNK1/2 inhibitor (SP600125) and ERK1/2 inhibitors (PD98059 and U0126) were purchased from Calbiochem (Darmstadt, Germany). Lipofectamine 2000 used as transfection reagent was purchased from Invitrogen (Carlsbad, CA).

Primary antibodies used in this study included phospho-p38, p38, phospho-ERK1/2, phospho-JNK, JNK, Smad2, Smad3, phospho-Smad2/3, phospho-Smad2/3, E-cadherin, TGF-β1 (Santa Cruz Biotechnology, Santa Cruz, CA); ERK1/2, phospho-Smad2, phospho-Smad3 (Cell Signaling, Danvers, MA); collagen type I and III (Southern Biotech, Birmingham, AL); alkaline phosphatase-conjugated α-smooth muscle actin (SMA; Sigma-Aldrich); and GAPDH (Chemicon, Temecula, CA). Secondary antibodies included IRDye 800-conjugated goat anti-rabbit, donkey anti-goat, or rabbit anti-mouse antibody (Rockland, Gilbertsville, PA); and AP-conjugated rabbit anti-mouse polymer antibody, peroxidase-conjugated rabbit anti-mouse, goat anti-rabbit polymer antibodies, rabbit anti-goat, swine anti-rabbit antibodies (DAKO, Glostrup, Denmark).

Cell lines and cell culture. A normal rat tubular epithelial cell line (NRK52E) was maintained in a 5% CO2 atmosphere at 37°C with DMEM-F12 containing 4 mM L-glutamine, 5% (FBS), 60 μg/ml penicillin, and 100 μg/ml streptomycin. Stable cell lines with Smad2 or Smad3 knockdown were established as described previously (4, 30). In brief, Smad3 siRNA or Smad2 siRNA was cloned into the P-supcr1 plasmid and transfected into NRK52E with Lipofectamine
2000 following the manufacturer’s protocol (Invitrogen). The cells were then selected with G418 in 100 mg/ml for 1 mo and maintained in 50 mg/ml of G418. A stable NRK52E cell line transfected with P-super1 empty plasmid only was used as a control.

The cells were grown to subconfluence on six-well plates (Falcon, Franklin Lakes, NJ) and made quiescent by incubation in serum-free DMEM for 24 h. Cells were then stimulated with AA at doses of 0, 0.5, 1.0, and 2.0 μg/ml for 0, 5, 15, and 30 min and 1, 3, 6, 12, 24, 48, and 72 h. AA-induced EMT was determined by de novo expression of a mesenchymal marker α-SMA and a loss of an epithelial cell marker E-cadherin by real-time RT-PCR, Western blotting, and two-color immunohistochemical analyses.

To study the signaling mechanism involving the MAPK pathway, NRK52E cells were pretreated with specific inhibitors to ERK1/2 kinase (PD98059 and U0126, 10 μM) and JNK (SP600125, 10 μM) for 1 h before AA (2 μg/ml) stimulation. To examine AA-induced Smad2/3 activation via the TGF-β-dependent and -independent mechanisms, NRK52E cells were pretreated with or without an anti-TGF-β-neutralizing antibody (or control rabbit IgG1) at 10 μg/ml (R&D Systems, Minneapolis, MN) for 1 h before AA (2 μg/ml) stimulation. In addition, NRK52E cells that stably expressed either Smad2 or Smad3 siRNA were used for dissection of the specific role of Smad2 and Smad3 in AA-induced EMT and collagen I expression.

To study the TGF-β-independent pathway in AA-induced Smad2 and Smad3 activation, a NRK52E cell line that stably expresses dominant negative TβRII (DN-TβRII) was used (4). The DN-TβRII- or PcDNA (control plasmid)-expressing NRK52E cells were cultured with AA for 30 min, and phosphorylation of Smad2 and Smad3 was detected by Western blot analysis. TGF-β1 (2.5 ng/ml) was used as negative control for studies in DN-TβRII cells.

**Chronic mouse model of AAN induced in Smad3 WT/KO mice.** Mice null for Smad3 were used in this study (32). A chronic AAN model was induced in littermate Smad3 KO and WT mice (male, aged 8–10 wk) as described previously (19, 31). Briefly, groups of six male Smad3 WT or KO mice were intraperitoneally injected with AA at a dose of 5 mg/kg every other day for 4 wk. The control mice had the same protocol but were injected intraperitoneally with saline instead of AA. The experimental procedures were approved by the committee on the use of live animals for teaching and research at the University of Hong Kong.

**Fig. 1.** Histology and renal function analysis show that aristolic acid (AA)-induced chronic aristolic acid nephropathy (AAN) at 4 wk is inhibited in Smad3 knockout (KO) mice. A: histological changes. B: serum creatinine. C: proteinuria. Note that although mice null for Smad3 are unable to prevent the development of a typical bare-basement membrane pathology induced by massive tubular epithelial cell (TEC) apoptosis/necrosis in acute AAN, they are protected from severe tubulointerstitial fibrosis and the development of proteinuria induced by a chronic administration of AA. Values are means ± SE from 6 mice. Sections were stained with periodic acid-Schiff. Magnification: ×200. *P < 0.05, **P < 0.01, ***P < 0.001 compared with normal mice treated with saline. #P < 0.05, ###P < 0.001 compared with the Smad3 wild-type (WT) mice with chronic AAN.
Fig. 2. Immunohistochemistry and real-time PCR show that Smad3 KO mice are protected from collagen I and III expression in chronic AAN at 4 wk after AA administration. A: collagen I expression. B: collagen III expression. i–iv: Immunohistochemistry. v: Quantitative analysis of immunohistochemistry. vi: Quantitative analysis of collagen mRNA expression by real-time PCR. Values are means ± SE for groups of 6 animals. Magnification: ×200. ***P < 0.001 compared with normal mice treated with saline. ###P < 0.001 compared with Smad3 WT mice with chronic AAN.
Renal function and proteinuria. Serum creatinine was used to evaluate renal function. Creatinine in serum or urine was detected with a QuantiChrom Creatinine Assay Kit (BioAssay Systems, Hayward, CA) by the improved Jaffé method following the manufacturer's instructions.

Sixteen-hour urine samples were collected before and weekly after AA treatment and examined by Coomassie blue as previously described (34). Results were expressed as protein/urine creatinine (mg/mg).

Histology and immunohistochemistry. Histological study was performed in 3-μm methyl Carnoy’s-fixed paraffin sections stained with periodic acid-Schiff (PAS). Extracellular matrix (collagen I and collagen III) deposition, EMT (loss of E-cadherin while de novo expression of α-SMA), expression of TGF-β1, and phosphorylation of Smad2/3 were determined in 3-μm methyl Carnoy’s-fixed paraffin sections by immunohistochemistry using a microwave-based antigen retrieval technique (11). Briefly, after 10-min microwave treatment in 10 mM sodium citrate (pH 6.0) and inactivation of endogenous peroxidase in 3% H2O2 in methanol for 30 min, sections were stained with the designated antibody, followed by peroxidase-conjugated secondary antibody and developed with diaminobenzidine to give a brown product. For two-color immunostaining, after the first set of

![Fig. 3. Two-color immunohistochemistry and Western blot analysis show that mice null for Smad3 are protected from AA-induced epithelial-mesenchymal transition (EMT) and α-smooth muscle actin (SMA)+ myofibroblast accumulation in chronic AAN. EMT is identified by 2-color immunostaining with the anti-α-SMA Ab (blue) and E-cadherin (brown). A and B: Smad3 WT mice with chronic AAN. AA-induced EMT cells (α-SMA+ E-cadherin+) are identified in a dilated tubulus (arrow) in a Smad3 WT mouse kidney (B). C: Smad3 KO mouse with chronic AAN. D: quantitative analysis of α-SMA+ myofibroblast accumulation in the area of tubulointerstitium. E: Western blot and real-time PCR analysis show that deletion of Smad3 blocks upregulation of α-SMA expression in the diseased kidney of chronic AAN. Values are means ± SE for groups of 6 animals. Magnification: ×200 (A and C); ×400 (B). *P < 0.05, **P < 0.01, ***P < 0.001 compared with normal mice treated with saline. #P < 0.05, ##P < 0.01 compared with Smad3 WT mice with chronic AAN.](http://ajprenal.physiology.org/)
Fig. 4. Smad3 KO mice are protected against AA-induced transforming growth factor (TGF)-β1 expression and activation of Smad2/3 in chronic AAN at 4 wk.  

A: TGF-β1 expression. B: Smad2/3 activation. i–iv: Immunohistochemistry. v: Quantitative analysis of immunohistochemistry. vi: Quantitative analysis of TGF-β1 mRNA expression by real-time PCR. Note that AA causes a marked activation of phospho-Smad2/3 in chronic AAN in Smad3 WT (B, iii and v). Interestingly, AA also induces phosphorylation of Smad2/3 in Smad3 KO mice, presumably being the phospho-Smad2. Values are means ± SE for groups of 6 animals. Magnification: ×200. **P < 0.01, ***P < 0.001 compared with normal mice treated with saline. ###P < 0.001 compared with Smad3 WT mice with chronic AAN.
antibodies staining for E-cadherin, sections were treated for 10 min in a microwave oven again to block antibody cross-reactivity and inactivate endogenous alkaline phosphatase, incubated with the alkaline phosphatase-conjugated mouse anti-α-SMA antibody overnight at 4°C, and then developed with fast blue BB salt (Sigma) at 37°C for 10 min to produce a blue product. All slides were counterstained with hematoxylin except for those with two-color immunostaining and nuclear phospho-Smad2/3 immunodetection. Phospho-Smad2/3-positive cells were counted and expressed as positive cells per centimeters squared. EMT was determined by double positive for E-cadherin (brown) and α-SMA (blue) and was recorded as percent positive scored from 500 tubules in the cortex of the kidney. Quantitation of extracellular matrix and TGF-β1 was analyzed using the quantitative Image Analysis System (AxioVision 4, Carl Zeiss), and results were expressed as percent positive staining area examined.

ELISA for TGF-β1 in supernatant of cell culture. TGF-β1 in supernatant of cell culture was examined by an ELISA kit (R&D Systems) according to the manufacturer’s instructions. Samples were acidified before assay to determine the total levels of TGF-β1 in the supernatant.

Western blot analysis. Proteins from cultured cells or renal tissue were extracted with RIPA lysis buffer and analyzed by Western blotting as previously described (4, 30). Briefly, after blocking the nonspecific binding with 5% skimmed milk in Tris-buffered saline for 1 h, the membranes were incubated with primary antibodies (phospho-Smad2/3, Smad3, α-SMA, E-cadherin, TGF-β1) at 4°C overnight, followed by IRDye 800-conjugated goat anti-rabbit or rabbit anti mouse antibody (Rockland). The signals were detected with the Odyssey Infrared Imaging System (Li-COR Biosciences, Lincoln, NE) and quantitated with the Image J program (National Institutes of Health).

**Fig. 5.** AA activates the TGF-β/Smad signaling pathway in TECs via both TGF-β-dependent and -independent mechanisms. 

A: AA (2 μg/ml) induces TGF-β1 mRNA expression in a time-dependent manner. 

B: ELISA shows that AA (2 μg/ml) induces TGF-β1 production in the cultured medium at 24 h. 

C: Western blot analysis shows AA induces TGF-β1 expression in a dosage-dependent manner at 24 h. 

D: Western blot analysis detects that AA (2 μg/ml) induces phosphorylation of Smad2/3, peaking at 30 min and 24 h. 

E: Western blot analysis reveals that addition of a neutralizing TGF-β1 Ab (α-TGF-β1 Ab; 10 μg/ml), but not the control normal rabbit IgG (CTRL Ab; 10 μg/ml), is able to block AA (2 μg/ml)-induced phosphorylation of Smad2 and 3 at 24 h (TGF-β dependent), but not at 30 min (TGF-β independent). The AA-induced early TGF-β-independent mechanism is further confirmed in TECs that stably express DN-TβRII in which AA (2 μg/ml), but not TGF-β1 (2.5 ng/ml), is capable of activating Smad2 and Smad3 by phosphorylation at 30 min (right). Values are means ± SE for 3 independent experiments. *P < 0.05, **P < 0.01 compared with time 0 or dose 0.
Real-time PCR. RNA was collected from cells or renal tissues and purified by an RNeasy kit according to the manufacturer’s instructions (Qiagen, Valencia, CA), and real-time PCR was performed with Sybergreen on an Opticon real-time PCR machine (MJ Research, Waltham, MA) as previously described (4, 30). The primers used in this study included mouse and rat α-SMA, E-cadherin, collagen I, III, TGF-β1, and GAPDH were described previously (4, 30).

Statistical analysis. Data obtained from this study are expressed as means ± SE from at least three independent experiments or groups of six mice. Statistical analyses were performed using one-way analysis of variance followed by a Newman-Keuls posttest (Prism 3.0, GraphPad Software, San Diego, CA).

RESULTS

AA-induced chronic AAN is protected in Smad3 KO mice. Compared with the normal mice (Fig. 1Ai and ii), after intraperitoneal injection of AA for 4 wk, Smad3 WT mice developed chronic AAN as evident by severe tubulointerstitial fibrosis (Fig. 1Aiii). In contrast, Smad3 KO mice showed little or no evidence of tubulointerstitial fibrosis at 4 wk after AA injection (Fig. 1Aiv). Interestingly, although tubulointerstitial fibrosis was lacking in Smad3 KO mice, a typical pathological feature of AAN with the dilated and bared tubular basement membrane changes was apparent in Smad3 KO mice with chronic AAN (Fig. 1Aiv). This may be related to AA-induced massive tubular apoptosis and necrosis in acute AAN as described previously (34). In addition, AA also caused renal dysfunction such as a significant increase in serum creatinine, and severe proteinuria was also developed in Smad3 WT mice, but attenuated in Smad3 KO mice (Fig. 1, B and C).

Fig. 6. Western blot analysis shows that AA induces a rapid activation of Smad3 (p-Smad3) at 30 min via the JNK/MAPK mechanism. A: AA (2 μg/ml) induces activation of ERK1/2, JNK, but not p38, in a time-dependent manner. B: blockade of JNK1/2 with a JNK inhibitor (SP600125; 10 μM), but not ERK1/2 with ERK inhibitors (PD98059 and U0126; 10 μM each) inhibits AA-induced phospho-Smad3 at 30 min. Results represent 3 independent experiments.

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Effect of JNK inhibitor on AA-induced p-Smad3

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Effect of ERK inhibitor on AA-induced p-Smad3

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Smad3 KO mice are protected against renal fibrosis and EMT in chronic AAN. Immunochemistry and real-time PCR analysis demonstrated that severe renal fibrosis in Smad3 WT mice with chronic AAN was associated with a marked upregulation of collagen I and collagen III (Fig. 2). In contrast, mice null for Smad3 exhibited little or no collagen matrix accumulation after a 4-wk injection of AA. Moreover, two-color immunohistochemistry also detected that AA caused a tubular EMT as evident by a loss of E-cadherin and de novo expression of α-SMA, accounting for up to 5% of total tubules and resulting in a marked α-SMA+ myofibroblast accumulation in the fibrotic tubulointerstitium (Fig. 3, A, B, and D). In contrast, no EMT and little α-SMA+ myofibroblast accumulation appeared in Smad3 KO mice (Fig. 3, C and D). Western blot and real-time PCR analyses also revealed that a significant increase in α-SMA expression in the diseased kidney was detected in Smad3 WT mice, but not in Smad3 KO mice (Fig. 3, E–G).

Activation of TGF-β/Smad3 signaling is a mechanism of AA-induced chronic AAN. We next investigated the mechanism of chronic AAN. As shown in Fig. 4A, immunohistochemistry and real-time PCR demonstrated that expression of renal TGF-β1 was markedly upregulated in the AAN kidney in Smad3 WT mice, particularly in the damaged areas of tubulointerstitium of the renal cortex, but this was abrogated in Smad3 KO mice. Upregulation of renal TGF-β1 was associated with a strong activation of its downstream Smad proteins, as detected by a marked increase in the nucleated phospho-Smad2/3 in the diseased kidney of Smad3 WT mice (Fig. 4B). In contrast, mice lacking Smad3 had a few phospho-Smad2/3+ cells, presumably being the phospho-Smad2+ cells (Fig. 4B).

AA activates TGF-β1/Smad2/3 directly via a JNK/MAPK-Smad cross talk signaling pathway. Because mice null for Smad3 were protected against chronic AAN, we further investigated the signaling mechanisms by which AA activates the TGF-β/Smad pathway. This was performed in vitro in a normal rat TEC line (NRK52E) and in TEC stably expressing with or without DN-TβRII. We found that AA at a dose of 2 μg/ml induced TGF-β1 mRNA expression by TECs at 3 h with a further increase at 24 h (Fig. 5A). This resulted in a significant TGF-β1 protein production at 24 h (Fig. 5B). Western blot
analysis also revealed that AA-induced TGF-β1 expression in TECs was in a dosage-dependent manner (Fig. 5C). Interestingly, AA was able to induce a rapid activation of Smad2 and Smad3 at 15–30 min, followed by the second peak of Smad2 and Smad3 activation at 24 h (Fig. 5D). Further study showed that AA-induced early activation of phospho-Smad2 and 3 at 15–30 min occurred in the absence of TGF-β1 (Fig. 5B), suggesting a TGF-β1-independent mechanism in AA-induced rapid activation of Smad2/3. However, a significant increase in TGF-β1 production at 24 h after AA stimulation may be attributed to the second peak of Smad2 and 3 activation (Fig. 5, B and D). The ability of addition of a TGF-β1-neutralizing antibody, but not a control antibody, to block AA-induced phospho-Smad3 at 24 h, but not at 30 min demonstrated the early TGF-β-independent and the late TGF-β-dependent Smad signaling in response to AA (Fig. 5E). The ability of AA to activate the Smad signaling independently of TGF-β was further confirmed in TECs that stably express DN-TβRII. As shown in Fig. 5E (right), addition of AA, but not TGF-β, was able to induce phosphorylation of Smad2 and Smad3 in DN-TβRII-expressing TECs at 30 min, confirming the early TGF-β-independent mechanism in AA-induced Smad activation.

To further explore the precise signaling mechanisms of AA-induced rapid, TGF-β-independent Smad2/3 activation at 30 min, we examined the hypothesis of whether AA activates Smad2/3 via the MAP kinase pathway, because many stress factors including angiotensin II and advanced glycation end products (AGE) are able to activate the Smad pathway via the MAP kinase-Smad cross talk mechanisms (28, 15). As shown in Fig. 6A, addition of AA (2 μg/ml) was capable of activating ERK1/2 and JNK, but not p38, at 15 min, which preceded or paralleled the early activation of Smad2/3 (Fig. 5D). Importantly, blockade of JNK with an inhibitor (SP600125; 10 μM), but not ERK with an ERK inhibitor (PD98059 or U0126; 10 μM), was able to inhibit AA-induced Smad3 phosphorylation (Fig. 6, B and C), suggesting that AA may activate Smad signaling via the JNK/MAP kinase pathway.

Activation of JNK/MAP kinase-Smad signaling pathway is a mechanism by which AA induces EMT and collagen matrix expression. After identifying the activation of the JNK-Smad crosstalk pathway in response to AA, we investigated whether the activation of this cross talk pathway is functional in the development of renal fibrosis induced by AA. As shown in Fig. 7A, real-time PCR detected that AA at a dose of 2 μg/ml was able to induce renal fibrosis such as upregulation of α-SMA and collagen I mRNA expression. Two-color immunohistochemistry also revealed that addition of AA (2 μg/ml) for 72 h caused EMT, as evident by de novo expression of a mesenchymal marker, α-SMA, with a loss of the epithelial phenotype such as E-cadherin (Fig. 7B). This phenotypic change was further demonstrated by Western blot analysis that AA induced EMT in a time- and dose-dependent manner (Fig. 7, C and D).

To investigate whether AA-induced EMT and renal fibrosis are through the ERK1/2- or JNK1/2-Smad-dependent mechanism, a JNK inhibitor (SP600125; 10 μM) or an ERK inhibitor (PD98059 or U0126; 10 μM) was added to the culture before AA stimulation. Western blot analysis showed that addition of a JNK inhibitor, but not ERK1/2 inhibitor, was able to block AA-induced α-SMA expression, although no effect on E-cadherin expression was observed (Fig. 8A). This finding demonstrated a critical role for the JNK-Smad crosstalk pathway in AA-induced renal fibrosis. This was further confirmed by real-time PCR that addition of a JNK inhibitor blocked AA-induced α-SMA and collagen I mRNA expression at 3 h after AA stimulation (Fig. 8B).

Activation of Smad3, not Smad2, is a mechanism by which AA induces EMT and fibrosis. We further dissected the specific role for Smad3 or Smad2 in AA-induced EMT and fibrosis in NRK52E cells with stable knockdown of Smad2 or Smad3 using the small interfering (si) RNA technique. As shown in Fig. 9A, NRK52E cells stably expressed Smad2 or Smad3 siRNA resulted in a specific knockdown of Smad2 or Smad3.
individually at the mRNA levels by real-time PCR and at the protein levels by Western blotting. Interestingly, Western blot analysis revealed that knockdown of Smad3, but not Smad2, inhibited AA-induced EMT including a significant reduction of α-SMA expression while preventing a loss of E-cadherin (Fig. 9B). This was further demonstrated by real-time PCR that knockdown of Smad3, but not Smad2, inhibited AA-induced α-SMA and collagen I mRNA expression (Fig. 9C). Further-
more, real-time PCR also showed that knockdown of Smad3, but not Smad2, produced a significant inhibition of AA-induced TGF-β1 mRNA expression by NRK52E cells (Fig. 9C), demonstrating a key role for Smad3 in the initiation and amplification of renal fibrosis during the development of AAN.

**DISCUSSION**

Increasing evidence has shown that AAN is a worldwide disease associated with the use of traditional Chinese herbal medications (5). In patients with chronic AAN, tubulointerstitial fibrosis is the most predominant feature of progressive renal injury and is a major cause of end-stage renal disease (5, 24, 29). However, mechanisms of chronic AAN remain largely unclear, which has hampered the development of effective strategies to prevent and treat patients with chronic AAN. Thus discovery of the mechanisms leading to AAN and the development of effective therapies are urgently needed.
In this study, we have examined the mechanism by which AA induces AAN in vivo and in vitro. A novel and significant finding was that TGF-β/Smad3 may be a key signaling pathway leading to the development of chronic AAN. This was supported by the novel findings that mice lacking Smad3 gene were protected against AA-induced chronic AAN in terms of prevention in renal dysfunction and severe renal fibrosis, including EMT. Thus results from this study demonstrated a new role for Smad3 in the pathogenesis of AAN.

Although it has been reported that TGF-β is upregulated in response to AA and may contribute to the development of AAN in both in vivo and in vitro (14, 18, 19, 27, 31), the functional role and molecular mechanisms as to how AA activates the TGF-β signaling pathway to mediate chronic AAN remain unexplored. It is now well accepted that TGF-β1 signals through its downstream mediators Smad2 and Smad3 to mediate fibrosis under a variety of disease conditions (2, 9, 12, 13, 20, 22, 33). In addition, mediators of fibrogenesis such as angiotensin II and advanced glycation end products are also able to induce renal and vascular fibrosis via both TGF-β-dependent and the ERK and p38-Smad cross talk pathways (15, 21, 28). In the present study, we also found that in addition to the classic TGF-β-dependent Smad signaling induced by AA, a TGF-β-independent pathway, the JNK-Smad3 cross talk signaling pathway, was also required for AA-mediated renal fibrosis. This was supported by the findings that addition of AA was also capable of activating the ERK1/2 and JNK, but not p38, MAP kinases at 15–30 min. However, only activation of JNK, but not ERK1/2, was able to cause phosphorylation of Smad3 and renal fibrosis because blockade of JNK with a JNK inhibitor, SP600125, but not ERK1/2 with the specific inhibitors was able to block AA-induced phosphorylation of Smad3, EMT, and extracellular matrix expression. This finding identified a critical role for the JNK-Smad3 cross talk pathway in renal fibrosis of chronic AAN. This is consistent with the previous finding that activation of the JNK-Smad3 pathway is responsible for TGF-β-induced EMT during peritonal fibrosis (16). Although it is also possible that activation of JNK might induce EMT and renal fibrosis directly, independently of Smad3 in response to AA, the ability of deleting Smad3 to prevent AA-induced EMT and renal fibrosis in vivo and in vitro supported the role for Smad3 signaling in the development of chronic AAN.

Emerging evidence has shown that TGF-β/Smad3 is a critical mediator in fibrosis under different disease conditions (2, 9, 12, 13, 20, 22, 33). However, the role of TGF-β/Smad signaling in the development of chronic AAN remains yet to be determined. The most significant and important finding in the present study is that TGF-β/Smad3 was a mediator of AA-induced chronic AAN. This was clearly demonstrated by the findings that Smad3 KO mice were protected from chronic AAN such as progressive tubulointerstitial fibrosis and EMT. This novel finding may also suggest the critical role for TGF-β/Smad3 signaling in the pathogenesis of progressive renal injury in patients with chronic AAN. It should be pointed out that deletion of Smad3 may not be able to prevent the development of acute AAN, which is mediated by AA-induced TEC apoptosis and necrosis through the Stat3-p53 dependent mechanism (34). The presence of a typical pathological feature of AAN with the dilated and denuded tubular basement membrane morphology noted in Smad3 KO with chronic AAN may be the result of the massive TEC death in response to AA in acute AAN (34). In contrast, this typical pathological change in AAN was lacking in Smad3 WT mice, which may be attributed to the collapse of bared tubular basement membrane due to the severe tubulointerstitial fibrosis.

In the present study, the finding of Smad3, not Smad2, in AA-mediated renal fibrosis in vitro provided further insight into the mechanism of chronic AAN. This result was consistent with the previous finding that conditional knockout of Smad3, not Smad2, inhibits vascular and renal fibrosis in response to angiotensin II and advanced glycation end products (4, 28, 30). However, it should be noted that results obtained in vitro may not imply directly in vivo and may not be conclusive because of the use of Smad2 siRNA and Smad3 siRNA knockdown cells, not their KO cells for studies. Nevertheless, although Smad2 and Smad3 are structurally similar and physically bind together, they may function differently under physiopathological conditions (7). In the context of fibrosis, many fibrogenic genes including TGF-β1 and those coded for COL1A1, COL1A2, COL3A1, COL5A1, COL6A1, COL6A3, COL7A1, and TIMP-1 have been shown to be Smad3 dependent (3, 25, 26). Furthermore, expression of Smad3 is critical in TGF-β augmentation of fibroblast-mediated collagen gel contraction (17). The distinct roles between Smad3 and Smad2 in fibrosis may be attributed to the ability of Smad3, but not Smad2, to bind the target genes directly (8). This may explain why there was no increase in renal TGF-β1 in the kidney with chronic AAN in the Smad3 KO mice, although Smad2 signaling remained activated. Taken together, the findings that Smad3 KO mice were protected from chronic AAN in vivo and that knockdown of Smad3, not Smad2, was able to block AA-induced EMT and fibrosis in vitro suggested that targeting Smad3 specifically may have effective therapeutic potential for prevention or treatment of chronic AAN.

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DISCLOSURES

No conflicts of interest are declared by the authors.

REFERENCES

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