Tubastatin A suppresses renal fibrosis via regulation of epigenetic histone modification and Smad3-dependent fibrotic genes

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ABSTRACT

Inflammation and fibrosis are implicated in the pathogenesis of hypertensive kidney damage. We previously demonstrated that a non-specific histone deacetylase (HDAC) inhibitor attenuates cardiac fibrosis in deoxycorticosterone acetate-salt hypertensive rats, which induces HDAC6 protein and enzymatic activity. However, the HDAC inhibitor's effect and mechanism have not yet been demonstrated. We sought to determine whether an HDAC6-selective inhibitor could treat hypertension and kidney damage in angiotensin II-infused mice. Hypertension was induced by infusion of ANG in mice. Tubastatin A, an HDAC6 selective inhibitor, did not regulate blood pressure. Hypertensive stimuli enhanced the expression of HDAC6 in vivo and in vitro. We showed that the inhibition of HDAC6 prevents fibrosis and inflammation as determined by quantitative realtime PCR, western blot, and immunohistochemistry. Small interfering RNA (siRNA) against HDAC6 or Smad3 attenuated hypertensive stimuli-induced fibrosis and inflammation, whereas Smad2 siRNA failed to inhibit fibrosis. Interestingly, the combination of the HDAC6 inhibitor and Smad3 knockdown synergistically blocked transforming growth factor β (TGF-β) or ANG-induced fibrosis. We also demonstrated for the first time, to our knowledge, that acetylation of collagen type I can be regulated by HDAC6/p300 acetyltransferase. The chromatin immunoprecipitation assay revealed that the HDAC6 inhibitor suppressed TGF-β-induced acetylated histone H4 or phospho-Smad2/3 to Smad3 binding elements in the fibrosis-associated gene promoters including collagen type I. These results suggest that HDAC6 may be a valuable therapeutic target for the treatment of hypertension-induced kidney fibrosis and inflammation.

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1. Introduction

Gene expression is regulated by the post-translational modification of chromatin such as acetylation of histone and non-histone proteins. Acetylation/de-acetylation is balanced by histone acetyl transferases (HATs) and histone deacetylases (HDACs). The alteration of acetylation is closely involved in a variety of pathological diseases including neurological disorders, cancers, and cardiovascular, respiratory, and inflammatory diseases [7,28]. So far, several studies have shown that HDAC inhibitors decrease hypertrophy, inflammation, fibrosis, hypertension, proliferation, and atrial fibrillation [11,15,17,20,36,42,43]. Recently, we and a McKinsey research group have reported that HDAC6 is associated with chronic hypertension in certain animal models [15,21]. However, the exact molecular mechanism of HDACs on hypertension and kidney damage has not been demonstrated. Considering that 18 HDACs in mammals have different biological roles and tissue distribution, specific HDAC inhibitors need to be developed to treat diseases.

Hypertension is regarded as the most important risk factor for cardiovascular diseases, leading to heart failure. Hypertension is also a major mediator of renal disease and is common in patients with chronic kidney disease (CKD) [38]. CKD is characterized by the accumulation of fibroblasts and the deposition of extracellular matrix (ECM). Connective tissue growth factor (CTGF) expression is considered a molecular hallmark of renal fibrosis. CTGF is implicated in the formation of ECM including the ECM proteins fibronectin and collagen [39]. Angiotensin II (ANG), a potent vasoconstrictor, regulates blood pressure. Transforming growth factor β (TGF-β) expression can be stimulated by ANG. Recent reports have shown that TGF-β is a key regulator in kidney fibrosis [37]. TGF-β/Smad signaling participates in the process of fibrosis...
through activation of Smad2 and Smad3 phosphorylation. Recent studies indicated that Smad2 and Smad3 have opposing roles in regulating fibrosis, cell migration, and angiogenesis [8,27]. However, the exact molecular mechanism underlying Smad2/3 in kidney fibrosis remains unknown.

We hypothesized that HDAC6 inhibition or the combination with Smad3 siRNA suppresses fibrosis marker genes through epigenetic modification in cells or animal models. In this report, we demonstrate that angiotensin II or TGF-β induces expression of HDAC6, fibrosis, and inflammation markers, which were blunted by the HDAC6-selective inhibitor tubastatin A. In addition, to our knowledge, we show for the first time that collagen type 1 is a new non-histone protein substrate for p300/HDAC6. Interestingly, HDAC6 inhibition attenuated fibrosis through regulating the Smad3 binding activity to the Smad3 binding element in the fibrosis-associated gene promoters.

2. Materials and methods

2.1. Reagents

Tubastatin A HCl (S2627) was obtained from Selleckchem (USA). Angiotensin II (05-23-0101) was purchased from Calbiochem. Antibodies are presented in the Supplementary Materials.

2.2. Angiotensin II-induced hypertension and drug treatment

Male ICR mice weighing 30 g was purchased from the Samtako Company. All animal experiments were approved by the Animal Experimental Committee of the Chonnam National University Medical School. To establish the mouse hypertension model, angiotensin II (ANG, 1.3 mg/kg/day) was infused into mice with osmotic pump (Alzet) for 1.7 mg/kg/day) was infused into mice with osmotic pump (Alzet) for 1 week. Sham control received a 0.9% saline without ANG. One week after infusion of ANG, tubastatin A (10 mg/kg/day) or vehicle (4% DMSO in 0.9% saline) was injected intraperitoneally daily for an additional 1 week.

2.3. Blood pressure analysis

Blood pressures (systolic, diastolic, and mean BP) were measured weekly in awake mice by tail-cuff method (Visitech Systems, Brockton, MA). Blood pressure was measured approximately 10 repeated measurement values.

2.4. Cell cultures

Primary fibroblast cells were isolated from adult mouse kidney as described previously with modifications [39]. Briefly, kidneys were excised and minced into 1–2 mm pieces in 1× ADS buffer (116 mM NaCl, 20 mM Hepes, 10 mM NaH2PO4, 5.5 mM Glucose, 5 mM KCl, 0.8 mM MgSO4, pH 7.4). After digestion with collagenase type III (0.1%) and pancreatin (0.05%) for 30 min at 37 °C, kidneys were collected through the mesh (70 μm) and plated on cultured dishes. The primary kidney fibroblasts were cultured in high glucose Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Primary mouse adult kidney fibroblasts were used between passages 1 and 3. Primary mouse adult kidney fibroblasts cells were serum-starved overnight and pretreated with increasing concentrations of tubastatin A (10 nM–2 μM) for 2 h prior to the addition of ANG (100 nM) to determine the HDAC6, CTGF, collagen type I, and collagen type III mRNA levels and protein expression by qRT-PCR and western blot, respectively.

HK-2 cell normal kidney proximal tubular cell line was obtained from Korean Cell Line Bank (South Korea). HK-2 cells were maintained in RPMI 1640 medium supplemented with 10% FBS. HK2 cells were serum-starved overnight and incubated with ANG (100 nM) for 5 days and then treated the indicated doses of TubA for 24 h before cell harvesting. qRT-PCR and western blot analysis were performed.

2.5. siRNA transfection

Cells were transfected with 100 nM of control siRNA (Bioneer), human HDAC6 siRNA (Bioneer), human Smad2 siRNA (Bioneer), or human Smad3 siRNA (Bioneer) in serum-free medium using RNAiMAX reagent according to the manufacturer’s instructions. Six hours after transfection, transfection medium was replaced with fresh complete growth medium.

2.6. Western blot analysis and histone extraction

Kidney tissue lysates and cell lysates were prepared in RIPA buffer (150 mM NaCl, 1% Triton X-100, 1% Sodium deoxycholate, 0.1% SDS, 2 mM EDTA, 50 mM Tris–HCl, pH 7.5, protease inhibitors). Protein was separated on SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membrane. The membranes were blocked with 5% skim milk in TBST buffer at room temperature and indicated antibodies were used. HRP-conjugated secondary antibody was used and protein was detected by chemiluminescent HRP substrate (Immobilon Western WBKLS0500, Millipore). Protein expression levels were quantified by Bio-ID software (Vilber Lourmat, Germany).

For detection of acetylated histone H4, cells were harvested and lysed with triton extraction buffer (PBS containing 0.5% Triton X-100, 2 mM PMSF, protease inhibitor cocktail). Cell pellets were resuspended with 0.2 N HCl (final concentration) for 2 h with rigorous agitation. Protein was transferred to nitrocellulose membrane and probed with Histone H4 or acetyl histone H4.

2.7. Immunohistochemistry

Kidney tissues were fixed in 4% paraformaldehyde at 4 °C, embedded in paraffin, and sectioned at 4 μm intervals. For immunohistochemistry, paraffin-embedded sections were deparaffinized and permeabilized in 0.1% Triton X-100 for 10 min. Antigen retrieval was performed by microwaving the slides in citrate buffer for 20 min. Blocking buffer was used with horse serum for 1 h at room temperature. Kidney sections were immunostained with anti-CTGF, anti-HDAC6, anti-collagen type I, anti-S100A4 (FSP1) antibody at 4 °C for overnight. To reduce autofluorescence, kidney sections were treated with 0.1% Sudan Black B solution in 70% ethanol. Nuclei were counterstained with antifade reagent containing DAPI.

2.8. Immunoprecipitation (IP)

Kidney samples or cells were harvested with lysis buffer (0.5% v/v Igepal CA-630, 50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mM Na3VO4, protease inhibitors). For Dynabeads-antibody–antigen complex, acetylated lysine antibody (2–4 μg) was bounded to Dynabeads Protein G and cell lysates were incubated with antibody-conjugated Dynabeads. Dynabeads-antibody–antigen complexes were resuspended with SDS sample buffer and loaded to SDSPAGE. The indicated antibodies were used for western blot analysis.

2.9. Chromatin immunoprecipitation (ChIP) assay

ChIP was performed as described previously [18]. Briefly, HK2 cells were cross-linked with 1% formaldehyde for 10 min. Lysed cells were sonicated to shear DNA. The sonicated chromatin was pre-cleaned using salmon sperm DNA agarose A bead. Pre-cleaned cells were immunoprecipitated with acetyl histone H4 or phospho-Smad2/3 antibody and protein–DNA complexes were eluted. Normal rabbit IgG antibody was used as a negative control. After reversing cross-links, DNA was purified with PCR purification kit. DNA levels were measured by
SYBR green PCR kit with specific regions of CTGF (−824 to −631), fibronectin (−658 to −469), and collagen type I (−720 to −530) promoters. The primers were shown in supplementary Table 1.

2.10. Real-time reverse transcription polymerase chain reaction (qRT-PCR)

Quantitative real-time RT-PCR was performed as described previously [16]. Total RNA from mouse kidney tissues or cells was isolated with Trizol (Molecular Research Center, Inc). PCR primers were shown in Supplementary Table 2.

2.11. Statistics

Statistical analysis was performed with either the Student’s t-test or one-way ANOVA followed by a Bonferroni post hoc test using the GraphPad Prism software (version 5.0). Numbers of experiments are

![Graphs showing gene expression levels](image)

Fig. 1. Tubastatin A, an HDAC6-selective inhibitor, reduced HDAC6 expression and inflammation in angiotensin II-induced hypertensive mice. a. The transcript levels of HDAC6 were measured in mice (n = 7/7/7/7). b. Western blotting using an anti-HDAC6 antibody was performed on kidney tissues from Sham (n = 7), ANG (n = 7), and ANG+TubA mice (n = 7). c Immunohistochemistry for HDAC6. Representative photomicrographs are shown. Magnification, ×400. Scale bars: 50 μm. d–g. Quantitative transcript analysis of FGF 23, MCP-1, PAI-1, and TGF-β in sham-operated kidneys and ANG-infused kidneys of mice treated with vehicle or TubA (n = 7/7/7/7). The gene transcript levels are presented as the relative ratio to GAPDH. h. Western blots of TNF-α and TGF-β in kidney tissues of sham-operated and ANG-infused mice in the absence or presence of TubA.
shown in the figure legend. Quantitative PCR data were analyzed using the ΔΔCt method.

3. Results

3.1. Tubastatin A, an HDAC6-selective inhibitor, reduced HDAC6 expression and inflammation in angiotensin II-induced hypertension mice

We and other groups have reported that the cardiac expression and catalytic activity of HDAC6 are increased in the deoxycorticosterone acetate-salt hypertensive rat model [15,21]. This finding prompted us to investigate whether an HDAC6-selective inhibitor can regulate hypertension. TubA failed to reduce the ANG-induced increase in blood pressure (Supplementary Fig. 1a–c). We determined whether HDAC6 expression could be induced in ANG-treated mouse kidney. TubA blocked the ANG infusion-induced HDAC6 mRNA and protein expression (Fig. 1a, b). HDAC6 staining was increased in tubular epithelial cells of corticomedullary junction after ANG treatment and decreased with TubA treatment (Fig. 1c). HDAC6 was mainly localized in the cytoplasm of renal tubules.

To identify whether TubA can treat ANG-induced kidney damage, we assessed the fibroblast growth factor 23 (FGF23) mRNA expression level by qRT-PCR. FGF23 is a marker of acute kidney injury [5]. FGF23 mRNA was increased in kidneys of ANG-infused mice compared with
that in vehicle-treated sham mice (Fig. 1d), TubA also suppressed the ANG-mediated upregulation of monocyte chemoattractant protein-1 (MCP-1) and plasminogen activator inhibitor-1 (PAI-1) in the kidneys (Fig. 1e and f). TubA suppressed ANG-induced TGF-β expression in the kidneys (Fig. 1g). TGF-β is an important upstream factor of CTGF and involved in fibrosis [30]. We found that TNF-α and TGF-β proteins were increased in ANG-treated mice and decreased with TubA treatment (Fig. 1h).

3.2. Tubastatin A inhibited kidney fibrosis-related genes in vivo and in vitro

The potential regulation of kidney fibrosis in hypertensive mice by TubA was assessed using quantitative RT-PCR. CTGF is a well-known pro-fibrotic factor [31]. The mRNA level of CTGF was significantly increased in the ANG-treated mice compared with control sham group, which was blocked by TubA (Fig. 2a). Fibronectin is an essential component of the ECM and is implicated in the development of fibrosis [34]. The increased fibronectin mRNA expression was blocked by TubA (Fig. 2b). Likewise, collagen type I was upregulated in ANG-infused mice and inhibited by the administration of TubA (Fig. 2c). We further assessed whether TubA could affect the expression and localization of CTGF in kidney tissue sections. CTGF expression was partially shown in glomeruli (data not shown). However, CTGF immunoreactivity was mostly observed in the renal tubules after ANG treatment, the increase was reduced by TubA treatment (Fig. 2d and e).

To determine whether HDAC6 inhibition affects the fibrosis, we performed histological assessment of collagen deposition. We found that collagen type I expression was increased in response to ANG stimulation and then was decreased by TubA treatment (Fig. 2f). Fibroblast-specific protein 1 (FSP1, S100A4), is associated with fibrosis [41] and regarded as a specific marker of myofibroblasts. Immunohistochemistry showed that FSP1 expression was increased in the interstitial region of tubule cells in response to ANG, which was decreased by TubA administration (Fig. 2g).

To further confirm whether the inhibitory effect of TubA on fibrosis in vivo was also observed in vitro, adult kidney fibroblasts from kidney tissue were isolated and pretreated with TubA before the addition of ANG. ANG upregulated HDAC6 in fibroblast cells (Fig. 2h). Collagen types I and III mRNA levels were upregulated by ANG and these increases were suppressed at different concentrations of TubA (Fig. 2i and j). Furthermore, we demonstrated that the increased levels of HDAC6, collagen type I, and collagen type III protein were downregulated by TubA treatments (Fig. 2k).

3.3. Inhibition of HDAC6 by siRNA decreased angiotensin II-induced fibrosis marker genes and inflammation in primary mouse adult kidney fibroblasts

To identify whether fibrosis can be mediated by HDAC6, mouse kidney fibroblasts were transfected with HDAC6 siRNA or control siRNA and treated with ANG. HDAC6 mRNA was markedly decreased by transfection with HDAC6 siRNA (Fig. 3a). Likewise, HDAC6 protein was downregulated in HDAC6 siRNA-transfected cells (Fig. 3a, bottom). Knockdown of HDAC6 reduced the ANG-induced increase in HDAC6 mRNA in mouse kidney fibroblasts (Fig. 3b). qRT-PCR was performed to determine whether silencing of HDAC6 mRNA can affect fibrosis markers and we found that HDAC6 siRNA ameliorated the ANG-mediated increase in CTGF and collagen type I mRNA levels (Fig. 3c and d). In addition, HDAC6 siRNA prevented ANG-induced gene expression of the pro-inflammatory markers MCP-1, TGF-β, and PAI-1 (Fig. 3e–g), demonstrating that knockdown of HDAC6 in primary kidney fibroblasts can regulate ANG-mediated fibrosis and inflammation.

3.4. Tubastatin A suppressed fibrosis-related genes in HK2 epithelial cells

Several cell types can contribute to renal fibrosis. Tubular epithelial cells are involved in the fibrosis. We determined whether fibrosis-associated genes in HK2 kidney epithelial cells could be affected by ANG treatment. qRT-PCR showed a similar effect on the transcript levels of fibrosis-associated markers including CTGF, collagen type I, and SMA...
TubA suppressed the ANG-induced increase in the S100A4 mRNA level (Fig. 4d). Furthermore, kidney S100A4 expression was increased in ANG-treated mice and this increase was inhibited by TubA (Supplementary Fig. 2a, b). Interestingly, ANG upregulated HDAC6 mRNA in HK2 epithelial cells as well as fibroblast cells (Fig. 4e). TubA decreased the levels of the fibrosis-related proteins CTGF, collagen type I and SMA as well as HDAC6 protein (Fig. 4f, g). These data indicate that TubA is an anti-fibrotic agent in HK2 epithelial cells as well as in kidney fibroblasts.

3.5. Tubastatin A increased acetylation of histone/non-histone proteins and regulated acetylated histone H4 binding activity of collagen type I promoters

Gene expression is regulated by the post-translational modification of histone and non-histone proteins. HDAC inhibitors can increase the acetylation of histone and non-histone proteins such as transcription factors. First, we confirmed that TubA treatment increased acetylated histone H4 (Fig. 5a, b). We sought to determine whether TubA could affect the acetylation of fibrosis-related genes. Acetylation was assessed in the presence and absence of TubA and performed using an acetyl-lysine antibody. Unexpectedly, we found that collagen type I was acetylated in HK2 cells without any stimuli (Fig. 5c). The acetylation was further enhanced after treatment with TubA (Fig. 5c, d). However, fibronectin and CTGF were not acetylated (data not shown). We determined whether acetyltransferases are involved in the acetylation of collagen type I. HK2 cells were transfected with p300 siRNA or control siRNA. P300 siRNA reduced the endogenous p300 mRNA level (Fig. 5e). In contrast with TubA, knockdown of p300 diminished the acetylation of collagen type I (Fig. 5f, g). To elucidate the regulatory mechanism of TubA in fibrosis-related gene expression, we performed a ChIP assay in HK2 cells. The binding activity of acetylated histone H4 in the promoter of collagen type I was increased in response to TGF-β stimuli (Fig. 5h) but was attenuated with TubA treatment.
3.6. Tubastatin A regulated angiotensin II and TGF-β-induced fibrosis-related gene promoter activity through phosphorylated Smad2/3 in vitro and in vivo

TGF-β is a key mediator of renal fibrosis [25]. Based on our results about induction of TGF-β by ANG, we supposed that TGF-β may mediate renal fibrosis.

In light of the relevance of TGF-β/Smad signaling in fibrosis, we examined the role of Smad2 and Smad3 in fibrosis. We found that phosphorylated Smad3 protein was increased in the ANG–treated mice, which was blocked by treatment with TubA (Fig. 6a, b). Interestingly, Smad3, Smad2, and Smad4 expression was not changed. To identify the role of Smad2 and Smad3, either Smad2 or Smad3 was knocked down and the response to TGF-β was examined. Smad3 siRNA reduced the endogenous Smad3 level. TGF-β increased fibronectin, collagen, and CTGF, which was significantly decreased by Smad3 knockdown (Supplementary Fig. 3). However, knockdown of Smad2 failed to reduce the expression of fibrosis-related genes including fibronectin, collagen type I, and CTGF (Supplementary Fig. 4). Rather, Smad2 siRNA showed a tendency to accelerate the response to TGF-β stimuli. Interestingly, we found that Smad3 siRNA reduced TGF-β-induced phosphorylated Smad3, which was further blocked by treatment with TubA (Fig. 6c). In addition, co-treatment with TubA and Smad3 siRNA synergistically reduced TGF-β-induced fibronectin and collagen type I protein levels (Fig. 6c, d). Like TGF-β stimuli, ANG treatment had similar effects on the inhibition of fibrosis-related genes and phosphorylated Smad3 expression (Fig. 6e).

Fig. 4. Tubastatin A suppressed angiotensin II-induced fibrosis markers and HDAC6 expression in HK2 cells. a–c. Transcript levels of CTGF, collagen type I, and SMA were determined by qRT-PCR. d. The S100A4/FSP-1 mRNA level was examined by qRT-PCR. e. TubA suppressed ANG-induced HDAC6 mRNA levels. Data are the means ± SD of 4 independent experiments. f. Representative images of western blots. g. Quantification of 3 experiments.
To investigate whether Smad2/3 activation affects the downstream target gene promoter against TGF-β, we performed a ChIP assay. We searched for a putative Smad3 binding site (SBE) of the fibronectin, collagen type I, and CTGF promoter following previous reports[22]. Stimulation of the cells with TGF-β elevated the binding activity of phospho-Smad2/3 in the fibronectin promoter as compared with that in untreated cells (Fig. 6f). This increase was diminished by treatment with TubA. Similar effects were observed for collagen type I and the CTGF promoter (Fig. 6g, h).

4. Discussion

A schematic diagram shows ANG/TGFβ/HDAC6 pathway and activated Smad2/3 is involved in the regulation of fibrosis (Fig. 6i). In summary, we demonstrate that HDAC6 is involved in fibrosis and inflammation in two different types of kidney cells and in kidneys of the ANG-induced hypertension mouse model. Both the pharmacological HDAC6 inhibitor TubA and knockdown of HDAC6 suppressed the hypertensive stress-induced fibrosis-associated genes. We suggest a regulatory mechanism for TubA reduction of acetylated histone H4 and phosphorylated Smad2/3 binding activity in the fibrosis-related gene promoters.

It has been reported that HDAC inhibitors such as trichostatin A (TSA), valproic acid (VPA), and suberoylanilide hydroxamic acid (SAHA) reduce blood pressure in spontaneous or DOCA-salt hypertensive rats [4,13,15,35]. Recent reports indicate that HDAC3, HDAC4, HDAC6, and HDAC8 may be involved in the pathogenesis of hypertension [15,20,21,35]. However, which HDACs play critical roles in the development of hypertension is still unknown.
pressure increase in mice, it prevented kidney fibrosis both in vitro and in vivo. TubA and HDAC6 siRNA decreased ANG-induced CTGF, fibronectin, collagen type I, and collagen type III expression. TubA also reduced the HDAC6 mRNA and protein levels.

Hypertension can affect various organs and tissues such as the heart, the kidneys, and the vasculature [29]. Kidney damage is shown to be due to hypertension. ANG is involved in renal pathology including inflammation, oxidative stress, and tissue fibrosis [14]. Inflammation is characterized by hypertension-induced target organ damage [1]. In the present study, we did not examine the recruitment of immune cells to target organs. We observed that ANG caused inflammation via the upregulation of FGF23, MCP-1, PAI-1, TGF-β, TNF-α in vivo and in vitro. This increase was suppressed by knockdown or inhibition of HDAC6. Our data suggest that HDAC6 selective inhibitor attenuates kidney inflammation.

Inflammation can promote renal fibrosis [24]. CTGF acts as a downstream target of pro-fibrotic genes. Our results showed that HDAC6 inhibition attenuated ANG-induced CTGF and the expression of the ECM.
proteins collagen type I, collagen type III, and fibronectin in vivo and in vitro. CTGF expression was strikingly enhanced in the renal tubules. Indeed, CTGF antisense oligodeoxynucleotide blocked CTGF expression in the proximal tubular kidney epithelial cells [26]. We also observed that FSP/S100A4 mRNA and protein expression was increased by ANG, which was blocked by HDAC6 inhibition.

TGF-β has been regarded a key regulator in renal fibrosis and can be stimulated by ANG [6]. Smad2 and Smad3 are known to act as a downstream mediator of TGF-β in renal fibrosis [19]. In the present study, we observed that phosphorylation of Smad3 was markedly increased in ANG-infused mice, which was inhibited by TubA. This result suggests that Smad3 activation is essential in kidney fibrosis of hypertension. Furthermore, we observed that Smad3 and Smad2 showed opposite actions in response to TGF-β stimuli. Smad3 siRNA suppressed the TGF-β-induced expression of fibrosis genes, whereas Smad2 siRNA failed to reduce the response and rather exaggerated fibrosis. In accordance with our results, Smad3 was found to promote fibrosis, whereas Smad2 and Smad7 protected against renal fibrosis [23]. Yang et al. reported that Smad3, but not Smad2, plays an important role in ANG-induced CTGF and collagen 1 expression in tubular epithelial cells [40]. Pentoxifylline is used to inhibit the Smad2, Smad3, and Smad4 cascade [33]. Our results suggest that Smad3 is a therapeutic candidate gene to treat fibrosis. The development of a Smad3-selective inhibitor could therefore be helpful for treating hypertension-induced fibrosis since co-treatment with TubA and Smad3 siRNA blocked TGF-β-induced fibrosis and phospho-Smad3 activation.

HDACs and HATs contribute to gene expression through the regulation of the acetylation of histone and non-histone proteins [32]. HDAC6 deacetylates several genes including peroxiredoxins, heat shock protein 90 (Hsp90), α-tubulin, and cortactin [12]. In this study, we demonstrated that collagen type 1 is a new acetylation and deacetylation substrate, which are activated by p300 and HDAC6, respectively. The endogenous acetylation of collagen type 1 may be attributed to the action of p300 HAT activity. However, until now, little is known about the acetylation of collagen type 1 in regulating gene expression. Asano and Trojanowska reported that HDAC1/p300 occupied the Fli1 binding site in the promoter of the collagen gene [2]. Ghosh et al. demonstrated that collagen expression may be modulated by a p300 transcriptional coactivator [10].

It is of great interest to investigate whether HDAC6 inhibitor-mediated fibrosis is associated with epigenetic regulation. It is well known that increased histone acetylation can change the chromatin structure and affect transcription. The chromatin immunoprecipitation (ChIP) assay showed that HDAC6 inhibitor-mediated histone remodeling is associated with transcriptional activity. The HDAC6 inhibitor TubA highly increased the acetylation of histone H4. This increased acetylation also enhanced the binding activity in collagen type I promoter. However, of particular note is the finding that TubA partially inhibited the TGF-β-induced acetyl histone H4 bound to collagen type I promoter. Until now, little is known about how HDAC6 inhibitors differentially regulate the binding activity in response to the absence or presence of TGF-β. Understanding its regulatory mechanism could provide new insight into the up- or down-regulation of target genes by HDAC inhibition. Considering the importance of TGF-β/Smad signaling in renal fibrosis, Smad3 activation and its binding activity may contribute to fibrosis. Indeed, TubA blocked TGF-β-induced phospho-Smad2/3 bound to the fibronectin, collagen type I and CTGF promoter.

In conclusion, we provide evidence that HDAC6, which is induced by ANG and TGF-β, may be a therapeutic target gene for treating or preventing kidney fibrosis and inflammation in hypertension. Although the exact mechanism by which acetylation regulates collagen type I function or by which HDAC6 inhibition suppresses fibrosis-related genes is not clear, our results support the fact that attenuation of fibrosis by a pharmacological HDAC6 inhibitor or by a genetic deficiency of HDAC6 is correlated with chromatin remodeling. An HDAC6-selective inhibitor alone or in combination with a Smad3-selective inhibitor would be a valuable therapeutic intervention for preventing or treating hypertension-induced fibrosis.

Conflict of interest
None.

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Appendix A. Supplementary data
Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.vph.2015.04.006.

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