Essential Role of Smad3 in Angiotensin II–Induced Vascular Fibrosis

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Abstract—Angiotensin II (Ang II) plays a pivotal role in vascular fibrosis, which leads to serious complications in hypertension and diabetes. However, the underlying signaling mechanisms are largely unclear. In hypertensive patients, we found that arteriosclerosis was associated with the activation of Smad2/3. This observation was further investigated in vitro by stimulating mouse primary aorta vascular smooth muscle cells (VSMCs) with Ang II. There were several novel findings. First, Ang II was able to activate an early Smad signaling pathway directly at 15 to 30 minutes. This was extracellular signal-regulated kinase 1/2 (ERK1/2) mitogen-activated protein kinase (MAPK) dependent but transforming growth factor-β (TGF-β) independent because Ang II–induced Smad signaling was blocked by addition of ERK1/2 inhibitor and by dominant-negative (DN) ERK1/2 but not by DN-TGF-β receptor II (TβRII) or conditional deletion of TβRII. Second, Ang II was also able to activate the late Smad2/3 signaling pathway at 24 hours, which was TGF-β dependent because it was blocked by the anti–TGF-β antibody and DN-TβRII. Finally, activation of Smad3 but not Smad2 was a key and necessary mechanism of Ang II–induced vascular fibrosis because Ang II induced Smad3/4 promoter activities and collagen matrix expression was abolished in VSMCs null for Smad3 but not Smad2. Thus, we concluded that Ang II induces vascular fibrosis via both TGF-β–dependent and ERK1/2 MAPK-dependent Smad signaling pathways. Activation of Smad3 but not Smad2 is a key mechanism by which Ang II mediates arteriosclerosis. **(*Circ Res*. 2006;98:1032-1039.)**

Key Words: angiotensin ■ TGF-β ■ Smads ■ vascular fibrosis

Increasing evidence shows that angiotensin II (Ang II) plays a critical role in cardiovascular disease and is a key mediator in the process of vascular fibrosis, characterized by reduced lumen diameter and arterial wall thickening attributable to excessive deposition of extracellular matrix (ECM). Vascular fibrosis is a major complication of hypertension and diabetic mellitus. It has been shown that upregulated tissue renin-angiotensin system is involved in development of vascular lesions in both human and experimental vascular diseases.[1,2] This observation is confirmed by the finding that infusion of Ang II is able to induce vascular fibrosis in rats.[3] The functional importance of Ang II in vascular fibrosis is further supported by the evidence that blockade of Ang II inhibits vascular fibrosis in diabetic and subtotal nephrectomy rats and NO-deficient mice.[4–6] However, the signaling mechanisms underlying the fibrotic effect of Ang II remain largely unknown.

Beyond its hemodynamic effects, Ang II is recognized as a cytokine with an active role in cardiovascular remodeling. It is well known that Ang II signals through its Ang II receptor 1 (AT1) receptor to exert most of its biological functions.[7] After binding to the AT1 receptor, Ang II activates multiple downstream intracellular signaling pathways, including tyrosine kinase, mitogen-activated protein kinase (MAPK), p38, and Janus family kinase (JNK).[8] Activation of these pathways leads to numerous heterogeneous downstream events that play essential roles in the biological activities of Ang II, such as cell growth and migration, ECM production, and apoptosis.[8]

It has long been thought that Ang II induces vascular fibrosis by stimulating transforming growth factor-β (TGF-β). According to this view, TGF-β induction is the initial step in promoting fibrosis during vascular remodeling in response to Ang II. The recent discovery of the TGFβ/Smad signaling pathway provides insights into the molecular signaling mechanisms of TGF-β in vascular fibrosis. TGF-β is known to mediate its fibrotic effects by activating receptor-associated Smads, including Smad2 and Smad3, which are counter-regulated by inducing inhibitory Smads (ie, Smad6 and Smad7).[9] A recent study indicates that Ang II is able to

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activate the TGF-β/Smad signaling via the p38 MAPK pathway.[10] This finding strongly suggests that an additional signaling mechanism may be required for the development of vascular fibrosis in response to Ang II. In the present study, we demonstrated that Ang II is able to activate an early Smad signaling pathway in vascular smooth muscle cells (VSMCs) directly via the extracellular signal-regulated kinase 1/2 (ERK1/2) MAPK signaling pathway in addition to the late classic TGF-β signaling pathway. More important, we also provide new evidence that Smad3 but not Smad2 is a key downstream mediator of TGF-β/Smad signaling in vascular fibrosis in response to Ang II.

Materials and Methods

Patients

Arterial tissues from 23 patients (10 males and 13 females; 37 to 80 years of age) with unequivocal hypertension (systolic 141±3.8 mm Hg) were obtained from the Department of Pathology, Baylor College of Medicine, according to the approved guidelines by the institutional review board. Of them, 21 patients were treated with either angiotensin-converting enzyme inhibitor or AT1 receptor blockers.

Immunohistochemistry

Immunohistochemistry was performed using a microwave-based antigen retrieval technique as described previously.[11] Briefly, paraffin-embedded tissue sections (4 μm) from patients with hypertensive nephropathy or periodate-lysine-paraformaldehyde (PLP)-fixed VSMCs after culturing in 8-chamber glass slides were stained with antibodies to TGF-β1, collagen I, and phosphorylated (p)-Smad2/3 (Santa Cruz Biotechnology) using a three-layer peroxidase antiperoxidase method.

Quantitative analyses of TGF-β1 and collagen I expression were performed using a quantitative image analysis system (Metamorph). Because the pattern of expression of TGF-β1 and collagen I are diffuse in nature, the percentage of positive staining in the vascular wall was quantified under a ×20 power field of microscope. Briefly, up to 10 random areas of renal arteries with the early stage (media:intima ≥1) and advanced stage (media:intima <1) were chosen from each tissue section and examined. The examined area was outlined, the positive staining patterns were identified, and the percent positive area in the examined area was then measured. Data were expressed as the percentage of mean±SEM. For analysis of Smad2/3 activation in human renal arteries, nucleus positive for p-Smad2/3 cells within the arterial walls were identified and counted under a ×40 power field of microscope in 10 random areas of renal arteries using a 0.02-mm^2 graticule fitted in the eyepiece of a microscope as described previously.[11] Data were expressed as cells/mm^2. The arterial lumen and periarterial areas were excluded from the study. For analysis of Smad2/3 activation in cultured VSMCs, nucleus positive stain for p-Smad2/3 was counted in 500 cells and expressed as percentage. All examinations were performed blindly on coded slides.

Cell Culture

Primary cultures of VSMCs at passages 2 or 3 were used for studies as described below. Cells were serum starved for 16 hours, followed by treatment with Ang II (1 μmol/L) for periods of 5, 15, 30, and 60 minutes and 2, 4, 12, 18, 20, 24, and 48 hours. To study the signaling mechanism involving AT1 and ERK1/2 MAPK signaling pathway, VSMCs that overexpressed adenovirus (Adv)-dominant-negative (DN)-ERK1/2 MAPK were stimulated with Ang II or were treated with AT1 receptor blocker (losartan; 1 μmol/L) and a specific inhibitor to ERK1/2 (PD98059; 20 μmol/L; R&D Systems Inc.) at 1 hour before Ang II stimulation. To study the signaling mechanism involving the TGF-β–dependent signaling pathway, VSMCs that overexpressed retrovirus (Rv)-DN-TGF-β receptor II (TβRII) or had conditional KO for TβRII were stimulated with Ang II as described above. In addition, a neutralizing rabbit anti–TGF-β antibody (Ab; 10 μg/mL; R&D Systems Inc.) was introduced into the medium 1 hour before Ang II stimulation. Furthermore, the functional role of Smad2 and Smad3 in Ang II–induced vascular fibrosis was determined in Smad3 wild-type (WT) and knockout (KO) VSMCs and in those with conditional KO for Smad2. The secretion of TGF-β by VSMCs was measured in the supernatant by using a commercial TGF-β quantitative ELISA kit (R&D Systems Inc.).

Western Blot Analysis

The Western blot was performed as described previously.[12] Briefly, samples were heated at 99°C for 5 minutes and then transferred to a polyvinylidene difluoride membrane. After blocking with 5% BSA, the membranes were then incubated overnight at 4°C with primary antibodies against collagen I (Southern Biotech Inc.), p-ERK1/2, ERK1/2 (Santa Cruz Biotechnology), or GAPDH (Chemicon Inc.). After washing, the membranes were incubated with a peroxidase-conjugated secondary Ab for 1 hour, and signals were visualized by an enhanced chemiluminescence system (Amersham).

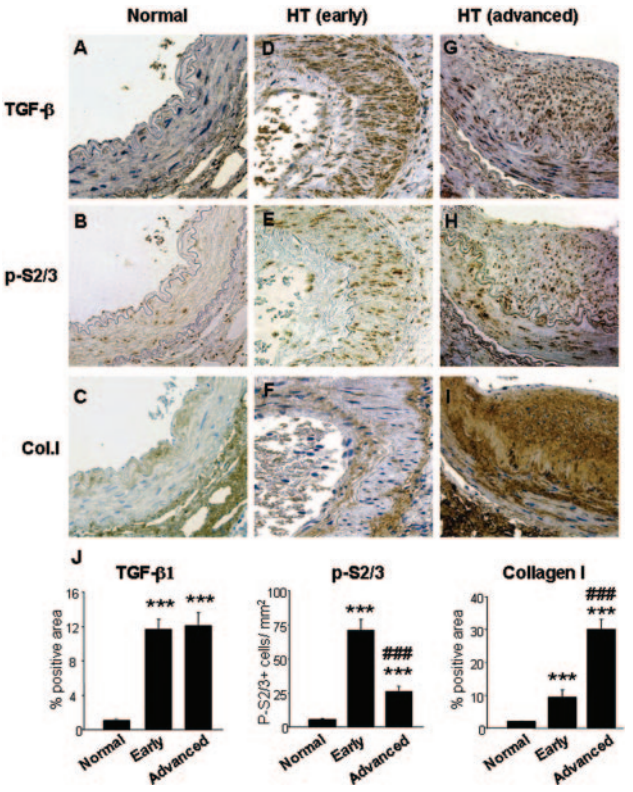

Figure 1. Immunohistochemistry shows that Smad2/3 is activated in human hypertensive arteriopathy. A through C, Serial sections of a normal human renal artery obtained from paratumor kidney tissues show that there are low levels of TGF-β, p-Smad2/3, and collagen I expression by VSMCs. D through F, Serial sections of a renal artery with the early stage of vascular fibrosis (media:intima >1.0) obtained from a patient with hypertension. Marked activation of p-Smad2/3 is noted in most medial VSMCs (E), which is associated with marked upregulation of TGF-β1 and some collagen I deposition (D and F). G through I, Serial sections of a renal artery with advanced arteriosclerosis (media:intima <1.0) obtained from a patient with hypertension. Note that activation of vascular p-Smad2/3 (H) is associated with upregulation of TGF-β1 and the development of marked accumulation of collagen I in the area of intimal thickening (G and I). J, Quantitative analysis: The results were expressed as mean±SEM. HT indicates hypertension. ***P<0.001 compared with normal; ###P<0.001 compared with the early stage of arteriosclerosis. Sections are counterstained with hematoxilin except B, E, and H. Magnification ×200.

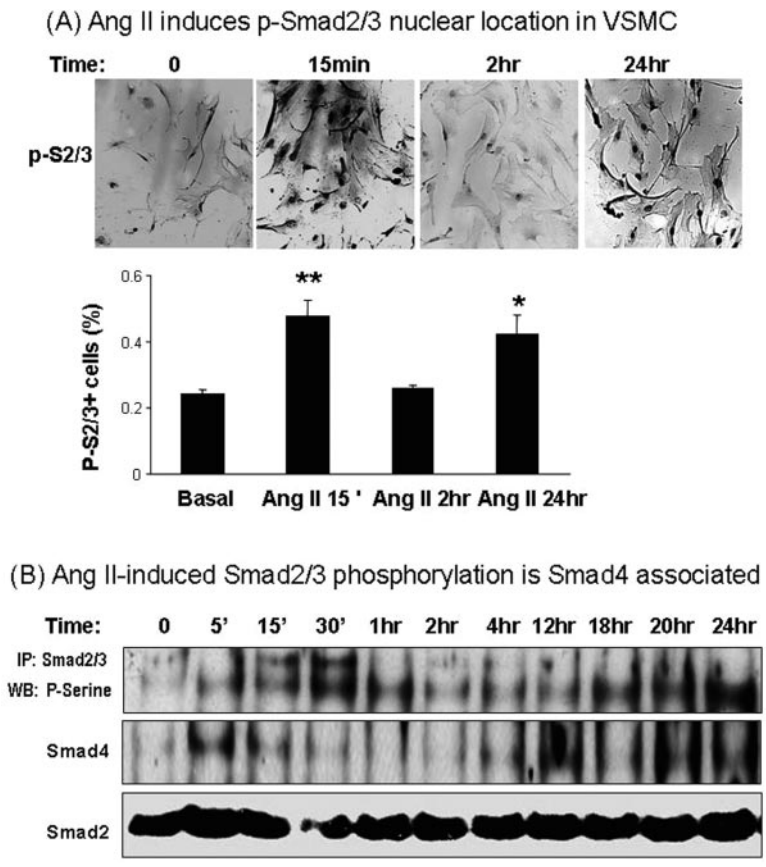

Figure 2. Ang II activates Smad2/3 in VSMCs in a biphasic manner. A, Immunohistochemistry shows that Ang II induces early (15 minutes) and a late (24 hours) p-Smad2/3 nuclear translocation (brown nuclei) in VSMCs. B, Immunoprecipitation shows that Ang II induced an early (5 minutes to 1 hour) and a late (18 to 24 hours) p-Smad2/3 in VSMCs, which is Smad4 associated. Data represent three independent experiments.

Immunoprecipitation

Cell lysates were immunoprecipitated at least 4 hours at 4°C with 0.4 μg of anti–Smad-2/3 (Santa Cruz Biotechnology), followed by precipitation with 20 μL of protein A/G Plus-Agarose (Santa Cruz Biotechnology) overnight at 4°C. After washing, the immunoprecipitates were boiled for 5 minutes in 2×sodium dodecyl sulfate sample buffer as described above. The resulting precipitated complexes were separated on sodium dodecyl sulfate–polyacrylamide gels and blotted with anti–Smad-4 (Santa Cruz Biotechnology), antiphosphoserine (EMD Biosciences), and anti-Smad2/3 (BD Bioscience Inc.), respectively.

Real-Time Polymerase Chain Reaction

Total RNA was isolated using the RNeasy kit, according to manufacturer instructions (Qiagen Inc.). The cDNA was synthesized as described previously.[13] Real-time polymerase chain reaction (PCR) was run with the Opticon real-time PCR machine as described previously (MJ Research Inc.).[12] The specificity of real-time PCR was confirmed via routine agarose gel electrophoresis and Melting-curve analysis. Housekeeping gene GAPDH was used as an internal standard. The primers used in this study are: collagen type I: forward 5′– TGCCGTGACCTCAAGATGTG, reverse 5′- CACAAGCGT-GCTGTAGGTGA; TGF-β1 forward 5′-CAACAATTCCTGGCGT-TACCTTGG, reverse 5′- GAAAGCCCTGTATTCCGTCTCCTT. GAPDH, forward 5′- CCTGGAGAAACCTGCCAAGTATGA, reverse 5′- TTGAAGTCACAGGAGACAACCTGG.

Transient Transfection and Promoter Activity Assay

Primary VSMCs were transiently transfected with a Smad3/4-responsive construct, $p(CAGA)_{12}$-Luc (a gift from Dr Peter ten Dijke, Ludwig Institute for Cancer Research, Uppsala, Sweden).[14] A control plasmid, pCMV–β-galactosidase (Clontech), was cotransfected into the cells for the control of transfection efficiency. The transfection procedure was performed using Lipofectamine (Invitrogen) according to manufacturer instruction. The luciferase and β-gal activities were analyzed by luciferase reporter gene assay kit and β-gal reporter gene assay kit, respectively (Roche Inc.) according to manufacturer instructions. The Smad3-responsive promoter activity was reported as the luciferase activity normalized to β-gal activity.

Statistical Analyses

Data obtained from this study are expressed as the mean±SEM. Statistical analyses were performed using one-way ANOVA from GraphPad Prism 3.0 (GraphPad Software, Inc.).

Results

Activation of Smad2/3 Is Associated With the Development of Arteriosclerosis in Patients With Hypertension

We first examined whether hypertensive vascular fibrosis is associated with the activation of TGF-β/Smad2/3 signaling. Extensive studies have shown that collagen I is an excellent index for the vascular fibrosis.[15] Thus, expression of collagen I was used as an index of the severity of vascular fibrosis. In normal human renal arteries, TGF-β1 was weakly expressed by vascular cells, and this was associated with few activated Smad2/3 cells, identified by the positive nuclear staining (Figure 1A and 1B) and minimal collagen I expression by VSMCs (Figure 1C). In contrast, marked upregulation of TGF-β was noted in the arterial wall, particularly by medial SMCs in the early stage of arterial fibrosis (Figure 1D), and this was closely associated with marked activation of Smad2/3 (Figure 1E). Interestingly, upregulation of TGF-β1

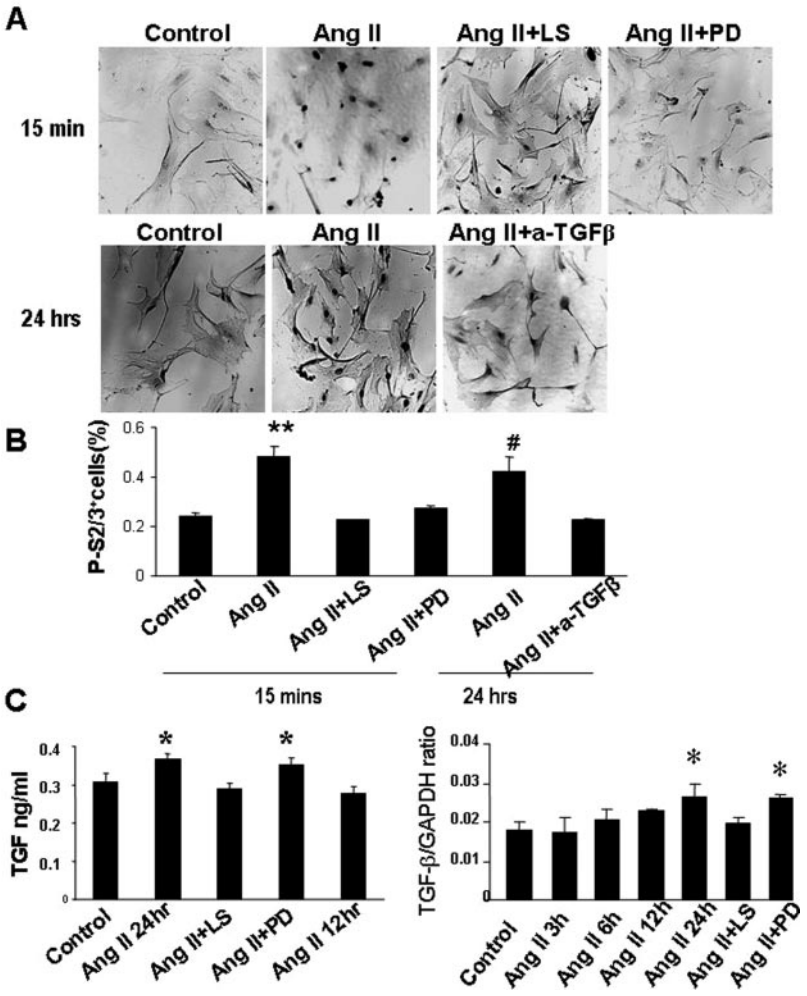

Figure 3. Signaling mechanisms of Ang II induce the early and late Smad activation in VSMCs shown by immunohistochemistry. A, Immunohistochemistry shows that Ang II (1 μmol/L)–induced early p-Smad2/3 nuclear translocation (brown nuclei) at 15 minutes is blocked by addition of losartan (1 μmol/L) and PD98059 (20 μmol/L). Ang II (1 μmol/L)–induced late p-Smad2/3 nuclear translocation is prevented by addition of a neutralizing TGF-β Ab (10 μg/mL). B, Quantitative analysis. Each bar represents the mean±SEM for three independent experiments. $^{**}P<0.01$ compared with the results from basal, Ang II +losartan, and Ang II + PD98059; $^{\#}P<0.05$ compared with Ang II + neutralizing TGF-β Ab. Magnifications ×250. C, ELISA (left) and real-time quantitative PCR (right) demonstrated that Ang II induced significant TGF-β expression at 24 hours. The increase of TGF-β expression by Ang II is AT1 dependent but not ERK1/2 dependent. Each bar represents the mean±SEM for three independent experiments. $^{*}P<0.05$ compared with the control.

and marked activation of Smad2/3 in medial SMC without significant collagen matrix deposition (Figure 1F) implies that activation of Smad signaling preceded vascular fibrosis, which may contribute to the pathogenesis of vascular scarring. In those with advanced vascular fibrosis, serial sections showed that marked upregulation of TGF-β1 and activation of p-Smad2/3 were associated with profound intimal thickening and severe vascular fibrosis as demonstrated by strong collagen I accumulation (Figure 1G through 1I). The specificity of this immunostaining was confirmed by a negative staining with an isotype control Ab in serial sections (data not shown). Quantitative analyses of immunohistochemical findings are shown in Figure 1J.

Ang II Is Able to Activate an Early and a Late TGF-β/Smad Signaling in VSMCs

We next examined the intracellular mechanisms by which Ang II activates Smad2/3 in VSMCs. Immunohistochemistry showed that Ang II was able to activate Smad2/3 (identified by nuclear location of p-Smad2/3) in VSMCs as early as 5 minutes, peaked at 15 to 30 minutes, and then declined to the basal level at 2 hours but formed the second peak at 24 hours (Figure 2A). Similar results were also demonstrated by immunoprecipitation (Figure 2B). Furthermore, Ang II–induced activation of Smad2/3 at the early (5 to 30 minutes) and late (18 to 24 hours) stage was physically associated with Smad4 (Figure 2B), indicating the formation of Smad2/3/4 complex, which is required for Smads entering to nuclear translation.[9]

Ang II Activates Smad Signaling via the AT1-Mediated, ERK MAPK-Dependent, and TGF-β–Dependent Pathways

We next dissected the signaling mechanisms whereby Ang II induces the early and late activation of Smad signaling. As shown in Figure 3A and 3B, immunohistochemistry showed that addition of losartan (1 μmol/L) and ERK1/2 MAPK inhibitor (PD98059; 20 μmol/L) almost completely blocked Ang II (1 μmol/L)–induced Smad2/3 phosphorylation and nuclear translocation at 15 minutes, whereas treatment with a neutralizing TGF-β Ab (10 μg/mL) prevented Ang II–induced Smad2/3 activation at 24 hours. Consistently, Ang II induced significant TGF-β1 expression until 24 hours, at both mRNA and protein levels, and the increased TGF-β1 expression was blocked by addition of losartan but not by ERK1/2 MAPK inhibitor (PD98059; Figure 3C). These observations suggest that Ang II may signal through the AT1 receptor to activate the early Smad signaling pathway via the ERK1/2 MAPK-dependent mechanism and the late TGF-β/Smad signaling pathway by the classic TGF-β–dependent Smad

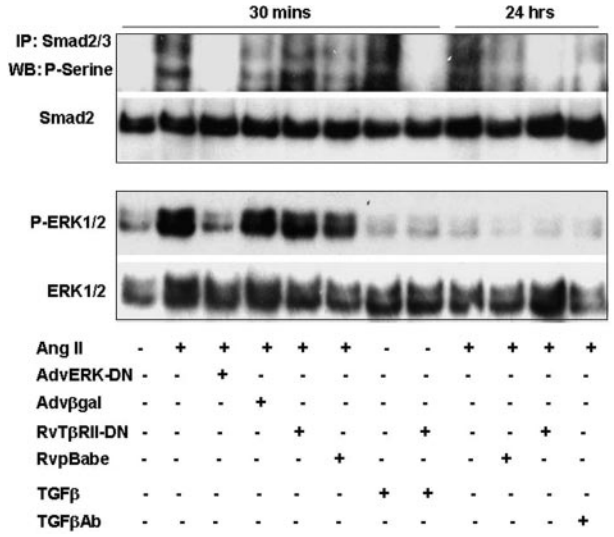

Figure 4. Signaling mechanisms of Ang II induce the early and late Smad activation in VSMCs shown by immunoprecipitation (IP) and Western blot (WB). Immunoprecipitation shows that Ang II (1 μmol/L)–induced early phosphorylation (p) of Smad2/3 at 30 minutes in VSMCs is associated with the activation of p-ERK1/2 MAPK, which is blocked by overexpressing Adv-ERK-DN (moi of 50) but not Rv-TβRII-DN, whereas Ang II–induced late activation of p-Smad2/3 at 24 hours is inhibited by overexpressing Rv-TβRII-DN and by neutralizing TGF-β Ab (10 μg/mL). The specificity of Adv-ERK-DN in inhibition of p-ERK1/2 and p-Smad2/3 is confirmed by the inability of a control Adv-β-gal to block activation of both ERK1/2 and Smad2/3. The specificity of Rv-TβRII-DN is also confirmed by a control Rv-pBabe vector and by the ability of Rv-TβRII-DN to block TGF-β (2.5 ng/mL)–induced p-Smad2/3 at 30 minutes. Results represent three independent experiments.

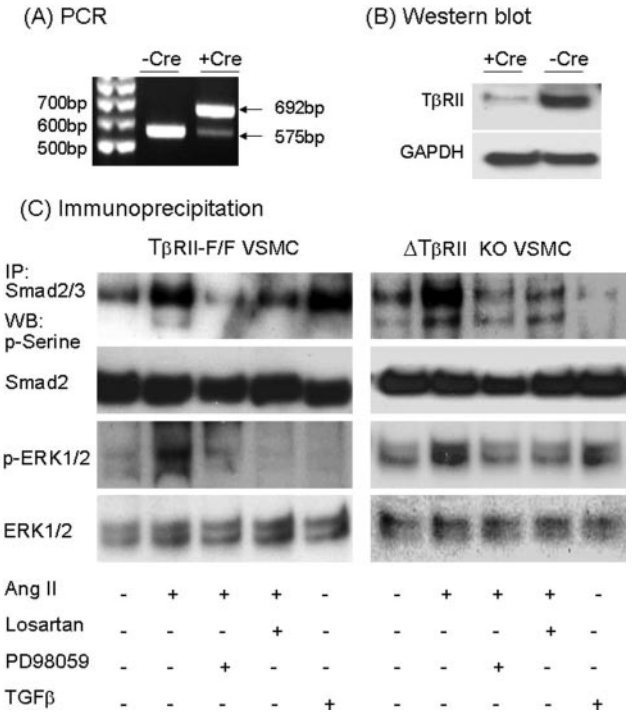

Figure 5. Conditional deletion of TβRII cannot prevent early Ang II–induced Smad2/3 phosphorylation in VSMCs, which is blocked by an ERK1/2 MAPK inhibitor. A, TβRII$^{f/f}$ VSMCs were treated with Cre-expressing adenovirus (moi of 50) as described in the Methods. PCR shows that Cre recombination substantially deletes the floxed TβRII gene (575 bp) as demonstrated by a high level of the deleted allele (692 bp) (primers sequences are: 1: 5′-TATGGACTGGCTGCTTTTGTATTC; 2: 5′-TGGGGATAGAGGTAGAAAGACATA; 3: 5′-TATTGGGTGTGGTTGTGGACTTTA).[27] B, Western blot analysis shows that infection of Adv-Cre causes a substantial lose of TβRII protein in TβRII$^{f/f}$ VSMCs. C, Immunoprecipitation demonstrates that compared with the TβRII$^{f/f}$ VSMC (left panel), conditional deletion of TβRII cannot prevent Ang II–induced p-ERK1/2 and p-Smad2/3 in VSMCs. In contrast, addition of losartan (1 μmol/L) and PD 98059 (20 μmol/L) is able to block Ang II–induced p-ERK1/2 and p-Smad2/3 in both TβRII$^{f/f}$ and conditional TβRII KO VSMCs. Effect of conditional deletion of TβRII on TGF-β–dependent Smad signaling in VSMCs is confirmed by the finding that conditional TβRII KO VSMCs are protected against TGF-β (2.5 ng/mL)–induced p-Smad2/3. Data represent three independent experiments.

signaling pathway. This was further confirmed by blocking the activity of ERK1/2 MAPK with Adv-ERK-DN and the TGF-β signaling pathway with overexpression of Rv-TβRII-DN or a neutralizing anti–TGF-β Ab. As shown in Figure 4, immunoprecipitation showed that Ang II–induced early activation of Smad2/3 at 30 minutes was completely blocked by overexpression of Adv-ERK-DN but not by Rv-TβRII-DN, whereas Ang II–induced late Smad2/3 activation at 24 hours was blocked by overexpression of Rv-TβRII-DN and a neutralizing TGF-β Ab. The specificity of Adv-ERK-DN in inhibition of Ang II–induced Smad2/3 activation at 30 minutes was confirmed by its ability to inhibit Ang II–induced ERK1/2 phosphorylation but not by the control Adv-β-gal. The specificity of Rv-TβRII-DN in inhibition of Ang II–induced late Smad2/3 activation at 24 hours was demonstrated by its ability to block TGF-β– but not Ang II–induced Smad2/3 activation at 30 minutes.

To further confirm that Ang II activated the early Smad signaling pathway via a TGF-β–independent mechanism, a genetic approach using conditional KO TβRII was applied because deletion of TβRII is embryonic lethal.[16] As shown in Figure 5A and 5B, VSMCs isolated from TβRII$^{f/f}$ mice were infected with Adv-Cre recombinase, and conditional KO TβRII VSMCs were generated. Deletion of TβRII produced no inhibitory effect on Ang II–induced ERK1/2 and Smad2/3 phosphorylation at 30 minutes, which was blocked by the AT1 blocker (losartan) and an inhibitor to ERK MAPK (PD98059; Figure 5C). In contrast, TGF-β signaling was defective and TGF-β–induced Smad2/3 phosphorylation at 24 hours was prevented in conditional KO TβRII VSMCs (Figure 5C).

The Early ERK MAPK–Smad Signaling Pathway Is Necessary for Ang II–Induced Vascular Fibrosis

Although it is well known that Ang II acts by stimulating TGF-β to induce collagen matrix synthesis in VSMCs,[1] it is not known whether the TGF-β–independent ERK1/2 MAPK–Smad signaling pathway is functionally important and contributes to vascular fibrosis in response to Ang II. This was examined in VSMCs that were conditionally deleted for TβRII. As shown in Figure 6, Ang II was able to induce a specific Smad3/4 promoter activity as well as collagen type I mRNA expression at 6 hours in both floxed and conditional KO VSMCs, which was blocked by addition of losartan and an ERK1/2 inhibitor. In contrast, deletion of TβRII prevented

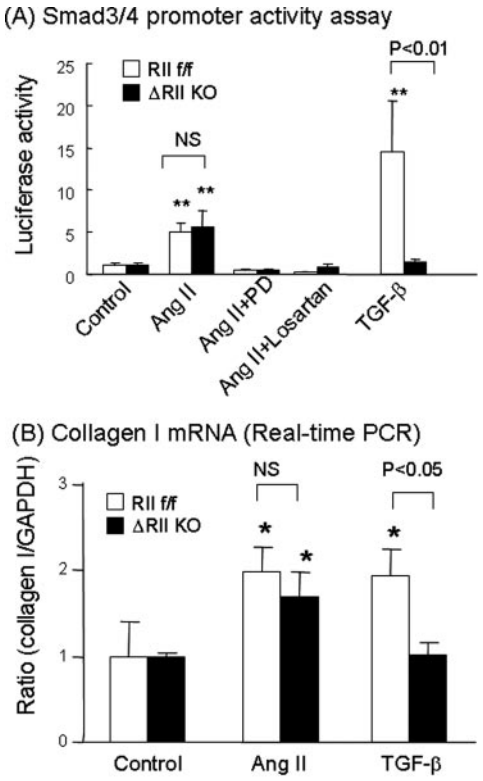

Figure 6. Functional role of TGF-β–independent ERK1/2 MAPK-dependent Smad signaling pathway in Ang II–induced Smad3/4 promoter activities and collagen I mRNA expression. A, Both TβRII$^{f/f}$ and conditional KO VSMCs were transiently transfected with p(CAGA)$_{12}$-Luc and pCMV-β-gal, followed by treatment with Ang II (1 μmol/L) in the presence of PD98059 (20 μmol/L), losartan (1 μmol/L), or TGF-β (1 ng/ ml) for 6 hours. The activity of luciferase and β-gal was measured, and results were expressed as the ratio of luciferase/β-gal (mean$\pm$SEM). Results show that conditional deletion of TβRII does not prevent Ang II–induced Smad3/4 promoter activities that are completely blocked by addition of losartan and PD98059. In contrast, conditional deletion of TβRII abrogates TGF-β–induced Smad3/4 promoter activities. B, Real-time PCR shows that conditional deletion of TβRII in VSMCs does not prevent Ang II–induced but is able to block TGF-β–induced collagen I mRNA expression. Results represent three independent experiments. *$P<0.05$; **$P<0.01$ compared with the normal control or as indicated. ns indicates not significant.

TGF-β–induced Smad3/4 promoter activity and collagen I mRNA expression (Figure 6A and 6B). These results demonstrate that the early ERK MAPK–Smad signaling pathway is necessary for Ang II–induced vascular fibrosis.

Smad3, But Not Smad2, Is a Critical Mediator of Smad Signaling for Vascular Sclerosis in Response to Ang II

After identifying the TGF-β–dependent and –independent Ang II–Smad signaling pathways in VSMCs, we next asked the question as to how important the Smad signaling pathway is in vascular fibrosis in response to Ang II. This was tested in VSMCs that lack Smad3 or conditional KO for Smad2. As shown in Figure 7, Ang II was able to induce collagen expression in a time-dependent manner in Smad3 WT VSMCs. Strikingly, Ang II– and TGF-β (positive control)–induced collagen I expression was completely abolished in

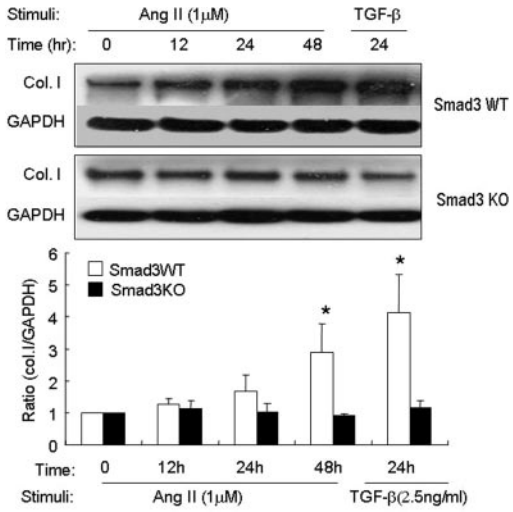

Figure 7. Essential role for Smad3 in Ang II–induced collagen matrix production. VSMCs were isolated from Smad3 WT and KO mice and stimulated with Ang II (1 μmol/L) for different time as described in the Methods. TGF-β (2.5 ng/mL) was used as a positive control. Western blot analysis shows that Ang II induces significant collagen I expression at 48 hours in Smad3 WT VSMCs, which is completely abrogated in Smad3 KO VSMCs. A similar result is seen in TGF-β stimulation. Results represent three independent experiments. *$P<0.05$ compared with time-matched Smad3 KO VSMCs.

VSMCs null for Smad3, demonstrating a critical role for Smad3 in vascular sclerosis in response to Ang II.

What is the specific role for Smad2 in Ang II–induced vascular fibrosis? Because mice lacking Smad2 is embryonic lethal,[17] we generated conditional KO Smad2 VSMCs by infecting Smad2$^{f/f}$ VSMCs with Cre-expressing adenovirus (Figure 8A and B). Interestingly, deletion of Smad2 produced no inhibitory effect on Ang II– and TGF-β–induced collagen I expression (Figure 8D) but was blocked by addition of losartan and an ERK1/2 inhibitor (PD98059), demonstrating that Smad2 may be not required in Ang II as well as TGF-β–mediated vascular sclerosis.

Discussion

Our results show that TGF-β/Smad signaling is activated in hypertensive arteriopathy and is associated with arteriosclerosis in patients with hypertension. The novel and significant finding in the present study is that Ang II is capable of activating the Smad signaling pathway to mediate vascular fibrosis directly via the AT1 receptor–mediated, through an early ERK MAPK–Smad signaling cross-talk pathway, in addition to a late TGF-β–dependent mechanism. The identification of the early, AT1 receptor, TGF-β–independent, but ERK MAPK-dependent Smad signaling pathway is demonstrated by the following findings: (1) Ang II is able to cause Smad2/3 phosphorylation and nuclear translocation in VSMCs as early as 5 minutes, peaked at 15 to 30 minutes, which precedes the synthesis of TGF-β at 24 hours and can be blocked by losartan (AT1 receptor antagonist); (2) blockade of TGF-β cannot prevent the early activation of Smad2/3 induced by Ang II because addition of a neutralizing anti–TGF-β Ab, overexpression of DN-TβRII, and conditional deletion of TβRII in VSMCs has no inhibitory effect on

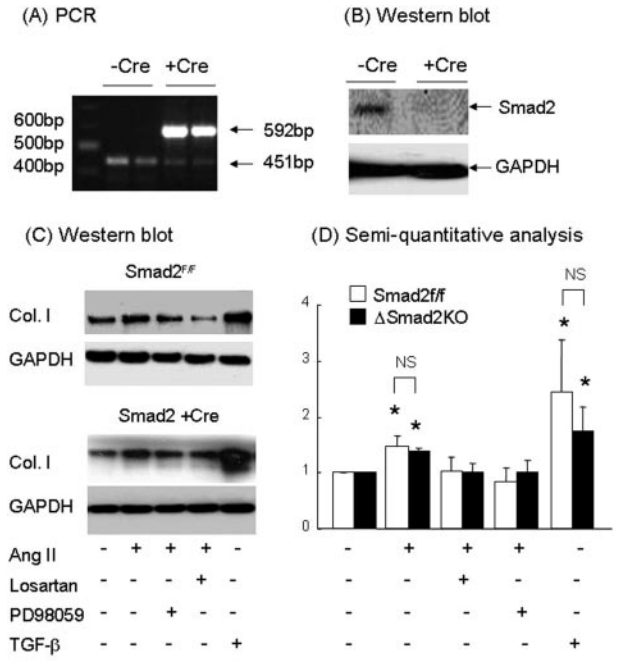

Figure 8. Smad2 is not necessary for Ang II–induced collagen type I expression. A, Smad2$^{f/f}$ VSMC was treated with Cre-adenovirus as described in the Methods. PCR shows that floxed Smad2 gene is clearly identified in Smad2$^{f/f}$ VSMC without Adv-Cre treatment (451 bp), which is substantially deleted by Cre recombination as identified by the deleted allele (592 bp; primers sequences are: 1: 5′-TTCCATCATCCTTCATGCAAT, 2: 5′-CTTGTGGCAAATGCCCTTAT, 3: 5′- 5′-GACCAAGGCGAAAGGAAACT).[32] B, Western blot analysis shows that addition of Adv-Cre causes a loss of Smad2 at the protein level. C, Growth-arrested Smad2$^{f/f}$ and conditional Smad2 KO VSMCs were treated with Ang II (1 μmol/L) in the presence of PD98059 (20 μmol/L), losartan (1 μmol/L), or positive control TGF-β (1 ng/ mL) for 24 hours. Western blot analysis shows that Ang II as well as TGF-β is able to induce collagen I protein expression in Smad2$^{f/f}$ VSMCs. However, conditional deletion of Smad2 does not prevent Ang II– and TGF-β–induced collagen I expression in VSMCs. In contrast, addition of losartan and PD98059 block Ang II–induced collagen I expression in both Smad2$^{f/f}$ and conditional KO VSMCs. D, Semiquantitative analysis. Each bar represents the mean±SEM from three independent experiments. *$P<0.05$ compared with control and treatment with losartan and PD98059. ns indicates not significant.

Smad2/3 activation at 15 to 30 minutes in response to Ang II; and (3) Ang II–induced early activation of Smad2/3 is ERK1/2 MAPK dependent because Ang II–stimulated Smad2/3 phosphorylation is associated with the phosphorylation of ERK1/2 and because blockade of ERK1/2 activation with PD98059 and by overexpressing Adv-DN-ERK1/2 inhibits the early activation of Smad 2/3 in VSMCs induced by Ang II. In addition, Ang II is also able to activate Smad2/3 at 24 hours, which is TGF-β dependent because the late activation of Smad2/3 is blocked by either overexpressing Rv-DN-TβRII or by a neutralizing TGF-β Ab.

It is known that Ang II signals through the AT1 receptor to activate ERK1/2, p38, and JNK MAPK to exert its biological effects in VSMCs via transactivation of the epithelial growth factor receptor.[18] In the present study, the identification of Ang II–induced ERK1/2 MAPK–Smad signaling cross-talk pathway in VSMCs provides a new signaling mechanism by which Ang II mediates vascular remodeling in pathophysiologic conditions. It has been shown that activation of ERK1/2 is involved in TGF-β–induced Smad2 phosphorylation and aggrecanin and furin gene expression,[19] implying that Smad proteins can act as signal integrators. This is further confirmed by the finding that Smads can be phosphorylated by other signaling pathways, including MAPKs and the calmodulin-dependent protein kinase II.[20–24] Our results show that the ERK1/2-Smad signaling cross-talk pathway appears to be a critical mechanism of Ang II–mediated vascular fibrosis. However, discrepancy exists in the role of ERK–Smad cross-talk pathway between our study and Rodriguez-Vita's study.[10] This discrepancy might be attributable to the differences of species of VSMCs or strategies to block the ERK1/2 activities. Nevertheless, our findings using the combination of pharmaceutical ERK1/2 inhibitor, molecular blockade by overexpressing DN-ERK1/2 or DN-TβRII, and genetic deletion of TβRII to indicate that Ang II can activate Smads directly via the ERK MAPK-dependent mechanism.

Another significant finding in the present study is that Smad3 but not Smad2 is necessary for the Ang II–induced collagen matrix production in VSMCs. Although Smad2 and Smad3 have >90% homology in their amino acid sequences and both are mediators of the functions of TGF-β, Smad3 but not Smad2 has binding sequences for COL1A2, COL2A1, COL3A1, COL5A1, COL6A1, and COL6A3 genes[25] and can bind directly to DNA to regulate gene transcription.[26] These differences may lead to a distinctive role for Smad2 and Smad3 in Ang II–mediated vascular fibrosis. Developmentally, deletion of Smad2 leads to early embryonic death,[17] whereas Smad3 KO mice are viable.[27] Pathophysiologically, Smad3 is responsible for the induction of c-fos, Smad7, TGF-β, and tissue inhibitor of metalloproteinase-1 (TIMP-1), whereas induction of matrix metalloproteinase-2 by TGF-β is Smad2 dependent.[28–30] All these findings suggest that Smad3 may be a key mediator in the process of fibrosis. This is consistent with Kobayashi et al's report that lack of Smad3 decreased ECM deposition in Ang II–induced vascular injury while enhancing neointimal hyperplasia.[31] Indeed, TGF-β has multiple functions. TGF-β can counteract proliferative effect of Ang II on VSMCs, another deleterious effect of Ang II in arteriosclerosis. It seems that Smad3 plays a critical role in the antiproliferation effect of TGF-β.

In summary, Smad signaling is activated in association with arteriosclerosis in patients with hypertension. Ang II is able to activate an early Smad signaling directly via the AT1 receptor–mediated ERK1/2 MAPK pathway, in addition to the late TGF-β–dependent mechanism. Activation of Smad3 but not Smad2 was a key mechanism of arteriosclerosis in response to Ang II. Findings from this study indicate that inhibition of Smad signaling may be one mechanism by which blockade of Ang II angiotensin-converting enzyme inhibitor or AT1 receptor blockers can prevent or slow the progression of chronic cardiovascular diseases.

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