

Orphan nuclear receptor small heterodimer partner inhibits angiotensin II-stimulated PAI-1 expression in vascular smooth muscle cells

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Abbreviations: PAI-1, plasminogen activator inhibitor-1; SHP, small heterodimer partner; VSMCs, vascular smooth muscle cells

Abstract

Angiotensin II is a major effector molecule in the development of cardiovascular disease. In vascular smooth muscle cells (VSMCs), angiotensin II promotes cellular

proliferation and extracellular matrix accumulation through the upregulation of plasminogen activator inhibitor-1 (PAI-1) expression. Previously, we demonstrated that small heterodimer partner (SHP) represses PAI-1 expression in the liver through the inhibition of TGF- β signaling pathways. Here, we investigated whether SHP inhibited angiotensin II-stimulated PAI-1 expression in VSMCs. Adenovirus-mediated overexpression of SHP (Ad-SHP) in VSMCs inhibited angiotensin II- and TGF- β -stimulated PAI-1 expression. Ad-SHP also inhibited angiotensin II-, TGF- β - and Smad3-stimulated PAI-1 promoter activity, and angiotensin II-stimulated AP-1 activity. The level of PAI-1 expression was significantly higher in VSMCs of SHP^{-/-} mice than wild type mice. Moreover, loss of SHP increased PAI-1 mRNA expression after angiotensin II treatment. These results suggest that SHP inhibits PAI-1 expression in VSMCs through the suppression of TGF- β /Smad3 and AP-1 activity. Thus, agents that target the induction of SHP expression in VSMCs might help prevent the development and progression of atherosclerosis.

Keywords: angiotensin II; atherosclerosis; muscle, smooth, vascular; nuclear receptor subfamily 0, group B, member 2; plasminogen activator inhibitor 1; transforming growth factor β

Introduction

Plasminogen activator inhibitor-1 (PAI-1) was identified as a significant biomarker and predictor of cardiovascular disease-related death in the Framingham Heart Study (Wang *et al.*, 2006), and several studies have reported an important role for PAI-1 in the development of arteriosclerosis and perivascular fibrosis (Sobel *et al.*, 2003; Weisberg *et al.*, 2005; Vaughan *et al.*, 2006). Moreover, in addition to circulating PAI-1, arterial PAI-1 expression is increased by elevated angiotensin II in patients with hypertension (Vaughan *et al.*, 1995; Kaikita *et al.*, 2001).

Angiotensin II is a major effector molecule in the renin-angiotensin system. It regulates systemic blood pressure through a direct vasopressive effect, and through the induction of vasoactive

compounds (Ito *et al.*, 1995; Ferrario *et al.*, 1998; Weir and Dzau, 1999). Previous findings suggest that angiotensin II promotes cellular proliferation and extracellular matrix accumulation in vascular smooth muscle cells (VSMCs) through the upregulation of PAI-1 expression (Kato *et al.*, 1991; van Leeuwen *et al.*, 1994; Papakonstantinou *et al.*, 2001), and several transcription factors, including Smad3 and AP-1, have been implicated as important mediators of angiotensin II-stimulated PAI-1 expression in VSMCs (Ahn *et al.*, 2001; Binder *et al.*, 2002; Wang *et al.*, 2006). Thus, clarifying the signaling networks that underlie angiotensin II-induced PAI-1 expression might reveal novel, therapeutically useful targets for managing angiotensin II/PAI-1-dependent cardiovascular disorders.

The orphan nuclear receptor SHP is an atypical member of the orphan nuclear receptor superfamily because it lacks a conventional DNA binding domain (Seol *et al.*, 1996). SHP is a transcriptional repressor that exerts its regulatory effects through protein-protein interactions with other nuclear hormone receptors, and possibly other transcription factors, inhibiting or reversing transactivation (Seol *et al.*, 1998; Gobinet *et al.*, 2001; Brendel *et al.*, 2002; Lee and Moore, 2002; Sanyal *et al.*, 2002; Ourlin *et al.*, 2003; Yamagata *et al.*, 2004). Previously, our group demonstrated that SHP represses hepatic PAI-1 expression through the inhibition of TGF- β signaling pathways and the repression of Smad3 transactivation (Suh *et al.*, 2006). These results suggested that direct targeting of SHP might be a promising approach to the prevention of atherosclerosis, particularly in patients with hypertension. However, little is known about the role of SHP in vascular cell function, particularly in angiotensin II-stimulated PAI-1 gene expression. Here, we examined the effect of SHP on angiotensin II-stimulated PAI-1 expression in primary cultured rat VSMCs, and elucidated a putative molecular mechanism of action of SHP in these cells.

Results

SHP inhibits angiotensin II-stimulated PAI-1 expression in primary cultured rat VSMCs

To investigate whether SHP inhibited PAI-1 expression in primary cultured rat VSMCs, we measured the effect of adenovirus-mediated overexpression of SHP in VSMCs on angiotensin II-stimulated PAI-1 mRNA expression by Northern blot analysis. As shown in Figure 1A, Ad-SHP decreased angiotensin II-stimulated PAI-1 expression in a dose-de-

pendent manner. Transient transfection experiments using a luciferase reporter gene under the control of the PAI-1 promoter revealed that SHP expression decreased PAI-1 promoter activity in a dose-dependent manner (Figure 1B). Additionally, transient SHP expression decreased angiotensin II-stimulated PAI-1 promoter activity in a dose-dependent manner (Figure 1C). These data suggested that the inhibition of angiotensin II-stimulated PAI-1 gene expression by SHP is mediated at the transcriptional level.

SHP inhibits PAI-1 expression through the down-regulation of the TGF- β /Smad3 pathway

PAI-1 activity is tightly regulated at the transcriptional level (Binder *et al.*, 2002). Given that TGF- β is an important regulator of angiotensin II-induced PAI-1 transcription (Rodriguez-Vita *et al.*, 2005; Ruiz-Ortega *et al.*, 2007), we examined whether Ad-SHP inhibited TGF- β -stimulated PAI-1 transcription. Infection of cells with Ad-SHP significantly inhibited TGF- β -stimulated PAI-1 mRNA expression in a dose-dependent manner (Figure 2A). Similarly, transient expression of SHP in VSMCs significantly inhibited TGF- β -stimulated PAI-1 promoter activity in a dose-dependent manner (Figure 2B upper). Transient expression of SHP also significantly inhibited Smad3-stimulated PAI-1 promoter activity in a dose-dependent manner (Figure 2B lower). Taken together, these data suggested that SHP inhibits PAI-1 expression through the inhibition of the TGF- β /Smad3 signaling pathway.

SHP inhibits angiotensin-II-stimulated AP-1 activity

To determine whether SHP inhibited angiotensin II-stimulated PAI-1 transcription through a TGF- β /Smad3 independent pathway, VSMCs were co-transfected with a luciferase reporter gene under the control of a truncated form of the PAI-1 promoter, -240 PAI-1, in which two Smad binding sites were deleted (Figure 3A). Angiotensin II treatment stimulated the activity of the truncated promoter construct, although the level of activity was lower than that of the WT promoter construct (Figure 3A). These results suggested that in addition to Smad, other factors contribute to angiotensin II-stimulated PAI-1 expression. SHP inhibited angiotensin II-mediated -240 PAI-1 promoter activity, reinforcing the idea that SHP inhibits transcription factors other than Smad (Figure 3A). We also examined the effect of SHP on the activity of AP-1, which is the primary transcription factor involved in stimulating PAI-1 promoter activity in response to angiotensin II. AP-1 DNA binding was

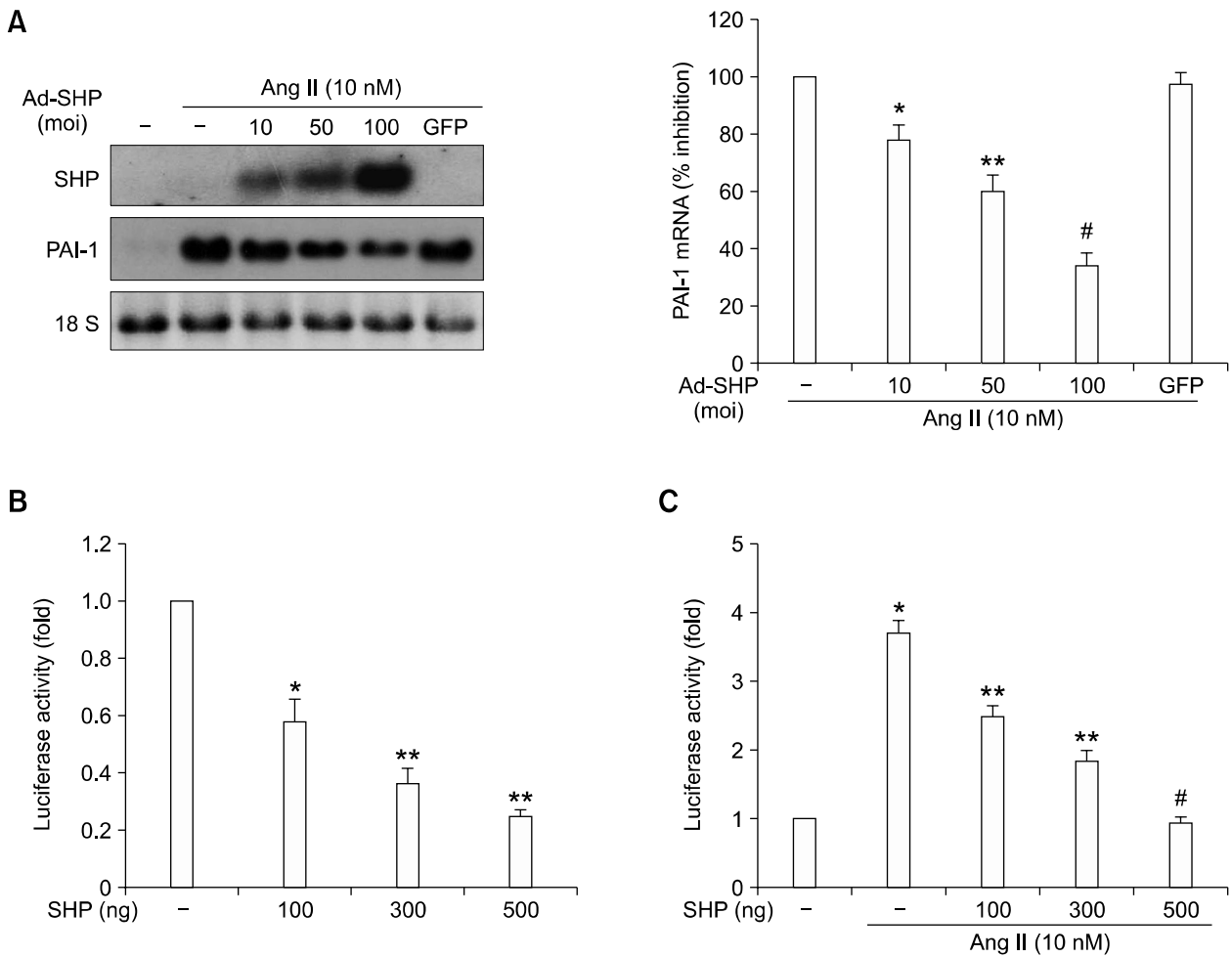


Figure 1. Effect of SHP on angiotensin II-stimulated PAI-1 mRNA expression and promoter activity. (A) Northern blot analysis of the effect of over-expression of SHP on PAI-1 mRNA expression. VSMCs were infected at the indicated multiplicity-of-infection (moi) with adenovirus expressing SHP (Ad-SHP), or green fluorescent protein (GFP) as a control, for 4 h. VSMCs were incubated in medium containing 0.5% FBS for 24 h, and then treated with angiotensin II (Ang II, 10 nmol/l) for 3 h before harvest. Data represent the means \pm SEM. * $P < 0.05$, ** $P < 0.01$, # $P < 0.001$ as compared to Ang II alone. (B and C) Luciferase gene reporter assay of the effect of SHP on PAI-1 promoter activity. (B) VSMCs were transfected with a reporter gene under the control of the PAI-1 promoter (-840 PAI-1 promoter Luc, 300 ng) together with the indicated amounts of an SHP expression vector (pcDNA3) for 5 h, and were then incubated in medium containing 10% FBS for 24 h. Data represent the means \pm SEM. * $P < 0.01$, ** $P < 0.001$ were as compared with the control. (C) VSMCs were transfected with a reporter gene under the control of the PAI-1 promoter (-840 PAI-1 promoter Luc, 300 ng) together with the indicated amounts of an SHP expression vector (pcDNA3) for 5 h. Cells were incubated in medium containing 0.5% FBS for 24 h, and then treated with angiotensin II (Ang II, 10 nM) for 1 h before harvest. Data represent the means \pm SEM. * $P < 0.001$ as compared to control. ** $P < 0.01$, # $P < 0.001$ as compared to Ang II alone.

stimulated by angiotensin II (10 nmol/l), and adenovirus-mediated overexpression of SHP inhibited angiotensin II-stimulated AP-1 binding activity in a dose-dependent manner (Figure 3B).

To see whether SHP inhibits angiotensin II-stimulated PAI-1 promoter activity independently from AP-1 inhibition, AP-1 decoy ODN, which inhibits the AP-1 binding to the PAI-1 promoter, was used. Angiotensin II stimulated the activity of the -840 PAI-1 promoter treated with AP-1 decoy ODN, although the level of activity was lower than that of the -840 PAI-1 promoter treated with mismatched

AP-1 ODN. SHP inhibited angiotensin II-stimulated PAI-1 promoter activity in a dose dependent manner. AP-1 decoy efficiency was confirmed by transient transfection with AP-1 proteins. c-jun and c-fos did not stimulate the activity of the -840 PAI-1 promoter treated with AP-1 decoy ODN (Figure 3C). Collectively, these data suggested that SHP inhibits PAI-1 expression in VSMCs through the downregulation of TGF- β /Smad3 and AP-1 activity.

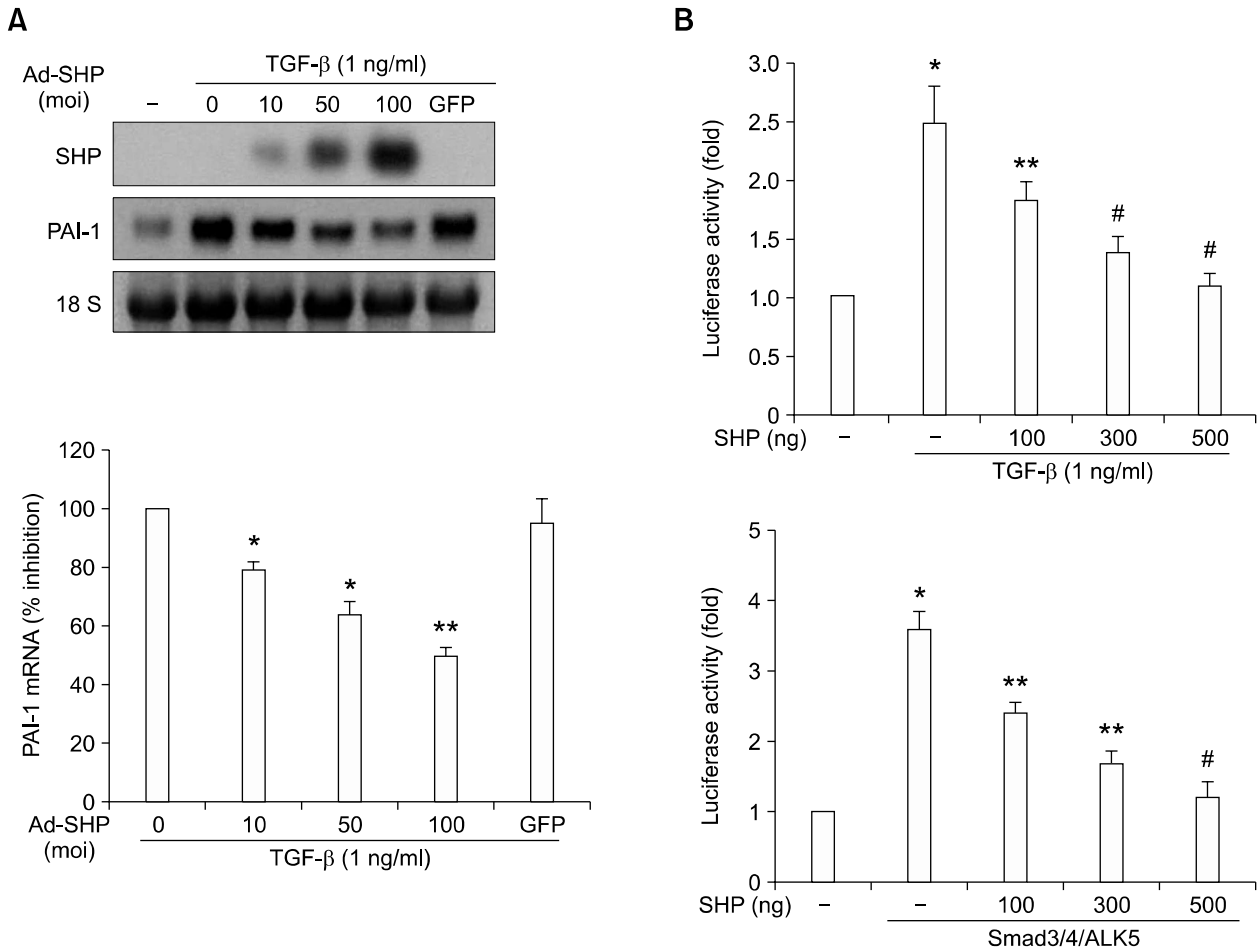


Figure 2. Effect of SHP on TGF-β-stimulated PAI-1 mRNA expression and Smad3 activity. (A) Northern blot analysis of the effect of overexpression of SHP on PAI-1 mRNA expression. VSMCs were infected at the indicated moi with Ad-SHP or GFP for 4 h. Cells were incubated in medium containing 0.5% FBS 24 h, and then treated with TGF-β (1 ng/ml) for 3 h before harvest. Data represents the means ± SEM. **P* < 0.05, ***P* < 0.01 as compared to TGF-β alone. (B) Luciferase gene reporter assay of the effect of SHP on PAI-1 promoter activity. (Upper) VSMCs were transfected with -840 PAI-1 promoter Luc (300 ng) together with the indicated amounts of an SHP expression vector (pcDNA3) for 5 h. Cells were incubated in medium containing 0.5% FBS for 24 h, and then treated with TGF-β (1 ng/ml) for 1 h before harvest. Data represents the means ± SEM. **P* < 0.01 as compared to the control. ***P* < 0.05, #*P* < 0.01 as compared to TGF-β alone. (Lower) VSMCs were co-transfected with -840 PAI-1 promoter Luc (300 ng) and expression vectors for Smad3 (100 ng), Smad4 (100 ng) and ALK5 (100 ng), together with the indicated amounts of SHP expression vector for 5 h. Cells were then incubated in medium containing 0.5% FBS for 24 h. Data represents the means ± SEM. **P* < 0.001 as compared to control. ***P* < 0.01, #*P* < 0.001 as compared to Smad3/4/ALK5 alone.

PAI-1 mRNA expression is upregulated in primary cultured VSMCs of SHP^{-/-} mice

We next examined whether the loss of SHP influenced the expression of PAI-1 in VSMCs in SHP^{-/-} mice. In primary cultured VSMCs from SHP^{-/-} mice, PAI-1 mRNA levels were increased as compared to VSMCs of WT mice. Moreover, loss of SHP increased PAI-1 mRNA expression after angiotensin II treatment (Figure 4). These data suggested that endogenous SHP acts as negative regulator of PAI-1 expression in VSMCs *in vivo*.

Discussion

In the present study, we demonstrated that adenovirus-mediated overexpression of SHP decreases angiotensin II- and TGF-β-stimulated PAI-1 mRNA expression in primary cultured rat VSMCs. We also elucidated a potential mechanism of downregulation of PAI-1 expression by SHP involving the inhibition of both TGF-β/Smad3 and AP-1 activity. PAI-1 plays a critical role in cardiovascular disease and is a key mediator in the process of vascular fibrosis (Lyon and Hsueh, 2003). It has long been thought that angiotensin II induces vascular fibrosis by stimulating TGF-β (Ruiz-Ortega *et al.*, 2003;

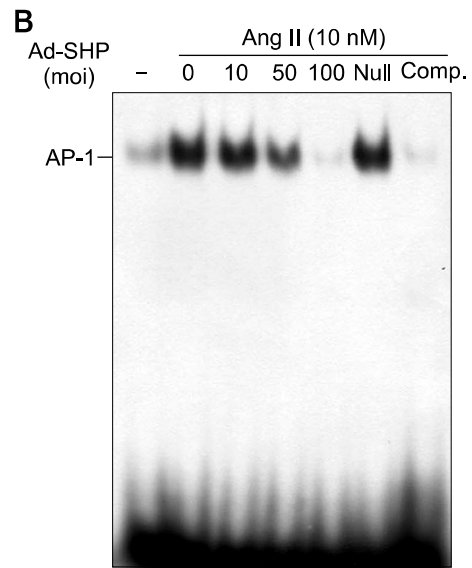
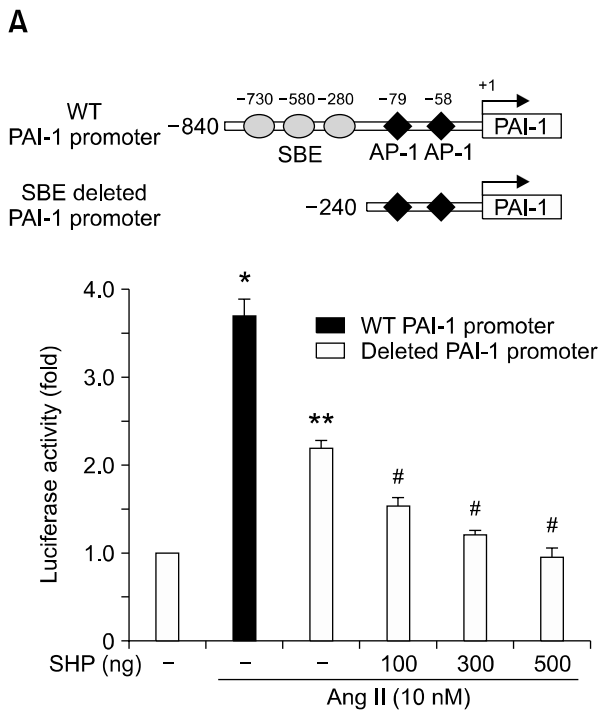
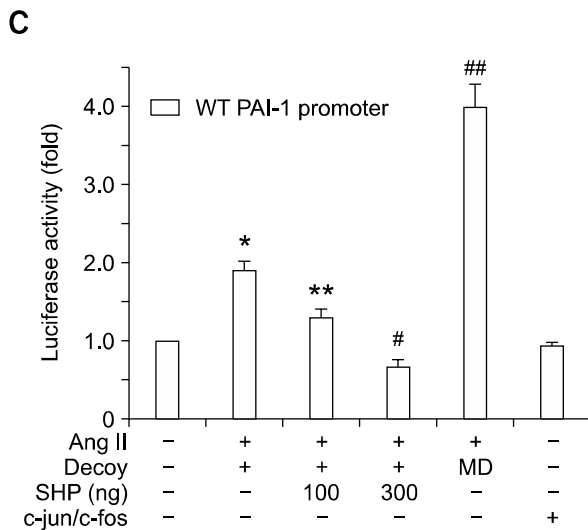


Figure 3. Effect of SHP on angiotensin II-stimulated AP-1 activity. (A) Luciferase gene reporter assay of the effect of SHP on Smad3-independent PAI-1 promoter activity. VSMCs were transfected with a luciferase reporter gene under the control of an SBE deleted PAI-1 promoter (-240 PAI-1 promoter Luc, 300 ng), together with the indicated amounts of SHP expression vector for 5 h. Cells were incubated in medium containing 0.5% FBS for 24 h, and then treated with angiotensin II (Ang II, 10 nM) for 1 h before harvest. Data represents the means \pm SEM. * P < 0.001 as compared to control. ** P < 0.01 as compared to Ang II alone (WT PAI-1 promoter), # P < 0.05 as compared to Ang II alone (SBE-deleted PAI-1 promoter). (B) Electrophoretic mobility shift assay of the effect of SHP on AP-1 DNA binding activity. VSMCs were infected at the indicated moi with Ad-SHP or GFP for 4 h. Cells were incubated in medium containing 0.5% FBS for 24 h, and then treated with angiotensin II (Ang II, 10 nmol/l) for 1 h before harvest. (C) VSMCs were cotransfected with the -840 PAI-1 promoter Luc (300 ng) and the AP-1 decoy ODN (10 nM) or mismatched AP-1 decoy ODN (MD, 10 nM) together with the indicated amounts of an SHP expression vector (pcDNA3) or c-jun/c-fos expression vector for 5 h. Cells were incubated in medium containing 0.5% FBS for 24 h, and were then treated with angiotensin II (Ang II, 10 nmol/l) for 1 h before harvesting. Data represents the means \pm SEM. * P < 0.05, ## P < 0.001 as compared to the control. ** P < 0.05, # P < 0.01 were as compared to Ang II.



Wang *et al.*, 2006). TGF- β mediates its fibrotic effects through the activation of receptor-associated Smads, including Smad2 and Smad3 (Dennler *et al.*, 1998; Javelaud *et al.*, 2004; Ruiz-Ortega *et al.*, 2007). Recent studies have shown that angiotensin II activates an early Smad signaling pathway in VSMCs directly through ERK1/2 MAPK, as well as the classic late TGF- β signaling pathway (Ruiz-Ortega *et al.*, 2003, 2007; Rodriguez-Vita *et al.*, 2005; Wang *et al.*, 2006). In the current study, adenovirus-mediated overexpression of SHP in

VSMCs inhibited angiotensin II-stimulated PAI-1 expression. SHP also inhibited TGF- β -stimulated PAI-1 expression and transactivation of the PAI-1 promoter by Smad3.

There is increasing evidence that, in addition to the TGF- β /Smad pathway, other transcription factors, such as AP-1, are also important in angiotensin II-induced PAI-1 expression (Ahn *et al.*, 2001). In the current study, angiotensin II stimulated the activity of a luciferase reporter gene under the control of an SBE-deleted PAI-1 promoter, in which

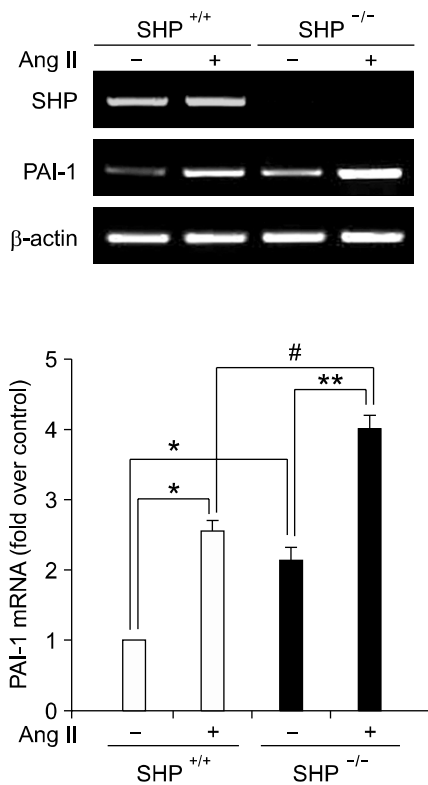


Figure 4. PAI-1 expression in primary cultured VSMCs of SHP^{-/-} mice. RT-PCR analysis of PAI-1 and SHP mRNA expression in primary cultured VSMCs from WT (SHP^{+/+}) and SHP-knockout (SHP^{-/-}) mice. Cells were incubated in medium containing 0.5% FBS for 24 h, and were then treated with angiotensin II (Ang II, 10 nM) for 3 h before harvesting. Data represent the means \pm SEM. * $P < 0.05$ was as compared to WT VSMC (SHP^{+/+}) without Ang II, ** $P < 0.05$ was as compared to KO VSMC (SHP^{-/-}) without Ang II, and # $P < 0.05$ was as compared to WT VSMC (SHP^{+/+}) with Ang II.

two Smad binding sites were deleted. These results suggest the involvement of transcription factors other than Smad3 in the action of angiotensin II. There is ample evidence that risk factors for atherosclerosis stimulate the transactivity of AP-1, a redox sensitive transcription factor, at the PAI-1 promoter. Previously, we and others have shown that angiotensin II increases PAI-1 expression through the upregulation of AP-1 activity in VSMCs (Ahn *et al.* 2001; Chen and Feener, 2004).

Previous reports have demonstrated the mechanisms by which SHP regulates target gene transcription without directly binding to target promoter sequences. SHP represses transcription factor-mediated transactivation by inhibiting DNA binding (Seol *et al.*, 1996; Ourlin *et al.*, 2003; Kim *et al.*, 2004), by recruiting unknown corepressors (Johansson *et al.*, 2000; Lee *et al.*, 2000, 2002) and by interfering with the interaction with coactivator (Suh *et al.*, 2006). Fiorucci *et al.* (2004) showed that the

inhibitory effect on AP-1 activity is mediated by a physical interaction between AP-1 and SHP that decreases AP-1 DNA binding. Our results, using an electrophoretic mobility shift assay, suggest that the inhibition of angiotensin II-stimulated PAI-1 expression by SHP involves in part the inhibition of AP-1-DNA binding at the PAI-1 promoter. Thus, the effect of SHP on vascular PAI-1 expression is likely to be multifactorial, involving the inhibition of Smad3 and AP-1 activity.

In conclusion, we have demonstrated that SHP inhibits PAI-1 expression by suppressing angiotensin II-stimulated transactivation of Smad3 and AP-1. Agents that increase SHP expression in vascular cells might help prevent the development and progression of atherosclerosis in patients with hypertension.

Methods

Materials

Recombinant human TGF- β 1 was purchased from R&D Systems (Minneapolis, MN). Recombinant human angiotensin II was purchased from Calbiochem (San Diego, CA). Radiochemicals ($[\alpha\text{-}^{32}\text{P}]$ dCTP, $[\gamma\text{-}^{32}\text{P}]$ ATP) were purchased from Perkin Elmer (Boston, MA).

Cell culture

VSMCs were isolated from the thoracic aorta of 4-week-old Sprague-Dawley (Samtako, Korea) rats weighing 100 g, and from SHP^{-/-} mice, using the explant method. SHP^{-/-} mice were a kind gift from Dr. David D. Moore (Baylor College of Medicine, Houston, TX). VSMCs culture was performed as described previously (Kim *et al.*, 2007). Briefly, VSMCs were cultured in DMEM (Gibco BRL, Grand Island, NY) containing 20% FBS (Hyclone, Logan, UT). VSMC purity was assessed by positive staining with smooth muscle specific α -actin monoclonal antibodies (Santa Cruz, Santa Cruz, CA). Cells from the third and fifth passages were used for all experiments.

Northern blot analysis

Total RNA was isolated from VSMCs using Trizol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Twenty-microgram aliquots of total RNA from each sample were used. The probes for SHP and PAI-1 were labeled with $[\alpha\text{-}^{32}\text{P}]$ dCTP using a random-primer DNA-labeling system (Amersham Biosciences, Little Chalfont, UK). Northern analysis was performed as described previously (Ahn *et al.*, 2001).

In vitro transient transfection and reporter assays

VSMCs were plated at a density 7×10^4 per well in a 12-well plate and cultured for 24 h. Cells were transiently transfected with a luciferase reporter gene under the

control of the wild-type (WT) PAI-1 (-840 PAI-1 promoter) promoter, or a Smad binding element (SBE)-deleted PAI-1 (-240 PAI-1 promoter) promoter (300 ng/well), along with the indicated expression vectors, using Lipofectamine Plus reagent (Invitrogen). Cells were co-transfected with an expression plasmid for β -galactosidase as an internal control. Cells were transfected for 5 h, then washed to remove unincorporated plasmids and cultured in conditioned culture medium. Cells were harvested approximately 24 h after transfection and assayed for luciferase and β -galactosidase activity. Cell lysate (20 μ l) was analyzed using the luciferase assay system, according to the manufacturer's instructions (Promega, Madison, WI), and luciferase activity was detected using a SIRUS Lumino-meter (Berthold, Pforzheim, Germany). Luciferase activity was normalized to β -galactosidase activity.

Construction of the AP-1 decoy

The construction of decoy oligodeoxynucleotides (ODN) was performed as described previously (Ahn *et al.*, 2002). The sequences of the dumbbell-shaped decoy ODN directed against the AP-1 binding site and its mismatched AP-1 decoy ODN were constructed as described (Ahn *et al.*, 2002) and were as follows (with consensus sequences underlined): AP-1 decoy ODN, 5'-GGATCCATGACTCAG-AAGACGACACACGTCTTCTGAGTCAT-3'; Mismatched AP-1 decoy ODN, 5'-GGATCCAAATCTCAGAAGACGACACA-CGTCTTCTGAGATTT-3'. The stability and cellular uptake of the decoy ODN were similar to those previously described (Ahn *et al.*, 2002).

Electrophoretic mobility shift assay

Nuclear extracts were isolated from cells using Nuc-Buster™ Protein Extraction kit (Calbiochem), according to the manufacturer's instructions. After centrifugation, supernatants (nuclear extracts) were collected and protein concentration was measured using a protein assay kit (Bio-Rad, Richmond, CA). Nuclear extracts (6 μ g) were incubated with approximately 60,000 cpm of a ³²P-labeled AP-1 binding-site oligomer, 5'-CGCTTGATGACTCAGCC-GGAA-3', for 20 min at 20°C.

RT-PCR

Total RNA was obtained from VSMCs using Trizol Reagent (Invitrogen). cDNA was synthesized using a first-strand cDNA synthesis kit (Fermentas, Hanover, MD) and 2 μ g of total RNA, according to the manufacturer's instructions. PCR was carried out using *Taq* polymerase (Takara, Japan) and the following thermal cycling parameters: 94°C for 5 min; 35 cycles of 94°C for 1 min; 70°C for 50 s; and 72°C for 1 min. The following primers and thermal cycling parameters were used for mSHP: 5'-CCGCACCGCAC-CTGCAGGGAGGCCCTT-3' (forward) and 5'-ACTCCAGGC-AGCGCTGCAGCCACTGAA-3' (reverse); 94°C for 5 min; and then 30 cycles of 94°C for 50 s, 67°C for 50 s and 72°C for 2 min. The following primers were used for standard PCR of mPAI-1 and actin: for mPAI-1, 5'-CCTCA-TCCTGGCCTGGTTCTGGTCT-3' (forward) and 5'-GGT-TTCCCCGCTGTGGTCATCTGC-3' (reverse); for actin,

5'-GGCATCGTCACCAACTGGGAC-3' (forward) and 5'-CGATTTCCCGCTCCGTGG-3' (reverse).

Statistical analysis

Results are expressed as the means \pm standard error of the mean (SEM). Variance analysis with a subsequent Duncan's test was used to determine significant differences in multiple comparisons. A *P* value of < 0.05 was considered statistically significant. All experiments were performed at least 3 times.

Acknowledgements

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