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Hypertensive effects of transforming growth factor-β1 in vascular smooth muscles cells from spontaneously hypertensive rats are mediated by sulfatase 2

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ABSTRACT

Extracellular sulfatases (sulfatase 1 and sulfatase 2) mediate up- or down-regulatory effects of cytokines on angiotensin II (Ang II)-induced expression of hypertensive mediators in hypertensive cells. The overproduction of transforming growth factor-β1 (TGF-β1) is associated with chronic hypertension. In this study, we examined the role of extracellular sulfatases on TGF-β1-induced effects associated with the expression of mediators related to hypertension in vascular smooth muscle cells (VSMCs) from spontaneously hypertensive rats (SHR). First, TGF-β1 increased the expression of 12-lipoxygenase (12-LO) and endothelin-1 (ET-1), inhibited dimethylarginine dimethylaminohydrolase-1 (DDAH-1) expression and showed additive effects on Ang II-induced 12-LO and ET-1 expression as well as Ang II-induced inhibition of DDAH-1 expression in SHR VSMCs. However, it had no effect on the expression of 12-LO, ET-1, and DDAH-1 in VSMCs from normotensive Wistar Kyoto rats. Downregulation of sulfatase 2 (Sulf2) inhibited all of these hypertensive effects caused by TGF-β1, while sulfatase 1 (Sulf1) had no effect on these events in SHR VSMCs. All these hypertensive effects of TGF-β1 were dependent on the Ang II subtype 1 receptor (AT_1 R) pathway, and not on Ang II subtype 2 receptor (AT_2 R). In addition, downregulation of Sulf2 inhibited the expression of TGF-β1-induced AT_1 R and the additive effect of TGF-β1 on Ang II-induced AT_1 R expression. Additionally, downregulation of Sulf2, but not Sulf1, abrogated TGF-β1-induced inhibition of AMP-activated protein kinase (AMPK) activation and the additive effect of TGF-β1 on Ang II-induced inhibition of AMPK activation via the AT_1 R pathway. Moreover, TGF-β1-induced VSMCs proliferation and the additive effect of TGF-β1 on Ang II-induced VSMCs proliferation were abrogated in Sulf2 siRNA-transfected SHR VSMCs, while these effects were maintained in Sulf1 siRNA-transfected SHR VSMCs. The hypertensive effects of TGF-β1 through the AT_1 R pathway were mainly dependent on Sulf2 activity in SHR VSMCs. Taken together, these results suggest that Sulf2, but not Sulf1, plays a major role in mediating the increased effects of TGF-β1 in hypertensive VSMCs.

1. Introduction

The extracellular sulfatases, sulfatase 1 (Sulf1) and sulfatase 2 (Sulf2), are known as heparin sulfate 6-*O*-endosulfatases [1,2]. Signaling pathways of growth factors are initiated through the removal of 6-*O*-sulfate from heparan sulfate proteoglycans (HSPGs) by extracellular sulfatases [1]. Therefore, extracellular sulfatases play an important role in cell signaling. In addition, they play active roles in tumorigenesis and cell growth [3–5]. In tumorigenesis, extracellular sulfatases exhibit opposite effects wherein Sulf1 inhibits cell growth and angiogenesis, while Sulf2 enhances these processes [3,5,6]. These extracellular sulfatases have opposite effects in hypertensive vascular smooth muscle cells (VSMCs) as well, where Sulf1 mediates anti-hypertensive effects of cytokines, whereas Sulf2 upregulates hypertensive effects [7–9].

Abbreviations: Sulf1, sulfatase 1; Sulf2, sulfatase 2; TGF-β1, transforming growth factor-β1; WKY, Wistar-Kyoto; SHR, spontaneously hypertensive rats; VSMCs, vascular smooth muscle cells; Ang II, angiotensin II; AT_1 R, Ang II type 1 receptor; AT_2 R, Ang II type 2 receptor; IL-10, interleukin-10; CCL5, CC-chemokine ligand 5; CXCL8, CXC-chemokine ligand 8; DDAH-1, dimethylarginine dimethylaminohydrolase-1; 12-LO, 12-lipoxygenase; ET-1, endothelin-1; AMPK, AMP-activated protein kinase; HSPGs, heparan sulfate proteoglycans; DMEM, Dulbecco's modified Eagle medium; ADMA, asymmetric (N^G,N^G) dimethylarginine; siRNA, small interfering RNA; PCR, polymerase chain reaction; SEM, standard error of the mean.

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However, the role of extracellular sulfatases associated with the expression of hypertension-related mediators induced by cytokines has not been fully investigated.

Transforming growth factor-β (TGF-β) is a crucial modulator of cell growth, differentiation, and homeostasis, including tolerance to self-antigens [10,11]. Among the three isoforms of the TGF-β family (TGF-β1, TGF-β2, and TGF-β3), TGF-β1 is the major isoform involved in the immune system [12]. TGF-β1 has pleiotropic functions with complex mechanisms of action in the immune response [10,11,13]. While TGF-β1 is a well-known inhibitory cytokine that plays a major role in immune homeostasis, it is also a pleiotropic cytokine [10,11]. In hypertension, elevated levels of TGF-β1 have been reported, there by suggesting its role in the development of this condition [13,14]. Furthermore, TGF-β1 is elevated in the peripheral blood in patients with hypertension [15–17]. In addition, increased TGF-β1 expression promotes VSMCs proliferation in spontaneously hypertensive rats (SHR), while it inhibits cell proliferation in most cell types [18,19].

Unlike TGF-β1, interleukin-10 (IL-10) plays a known downregulatory role in hypertension, including the potent inhibition of vascular inflammation; the plasma levels of IL-10 are reduced in experimental hypertensive rats [20–22]. In our previous studies on extracellular sulfatases, we observed that Sulf1 mediated anti-hypertensive effects of IL-10 and CC-chemokine ligand 5 (CCL5) in SHR VSMCs [7,9]. However, the upregulatory effect of CXC-chemokine ligand 8 (CXCL8) on Ang II-induced endothelin-1 (ET-1) expression was mediated by Sulf2 in SHR VSMCs [8]. Based on these previous studies, it is possible that Sulf1 and Sulf2 clearly have different effects on the expression levels of cytokine-induced hypertensive mediators in SHR VSMCs. However, few studies have investigated the functional role of extracellular sulfatases in cytokine-induced hypertensive effects in hypertensive cells.

Therefore, to evaluate the relationship between extracellular sulfatase activity and hypertensive or antihypertensive effects of cytokines in hypertensive cells, we examined whether extracellular sulfatases play a role in TGF-β1-induced hypertensive effects in SHR VSMCs.

2. Materials and methods

2.1. Reagents

Ang II was obtained from Calbiochem (San Diego, CA, USA), and TGF-β1 was purchased from R&D Systems (Minneapolis, MN, USA). Ang II type 1 receptor (AT_1 R) blocker (losartan), Ang II type 2 receptor (AT_2 R) blocker (PD123319), and anti-γ-tubulin antibody (dilution 1:2000; cat. no. T6557) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Total RNA extraction kit was supplied by iNtRON Biotechnology (Seoul, Korea). LightCycler FastStart DNA SYBR Green I Mix was supplied by Roche (Mannheim, Germany). The primers for 12-LO, ET-1, DDAH-1, AT_1 R, AT_2 R, and β-actin were supplied by Bionics (Daejeon, Korea). Rat Sulf1 small interfering RNA (siRNA), AT_1 R siRNA, and AT_2 R siRNA were supplied by Bioneer (Daejeon, Korea). Control siRNA and Lipofectamine 2000 were supplied by Invitrogen Life Technologies (Carlsbad, CA, USA). Rat Sulf2 siRNA and rat primary antibodies against Sulf1 (dilution 1:200; cat. no. sc-98325), and Sulf2 (dilution 1:200; cat. no. sc-271772), 12-LO (dilution 1:400; cat. no. sc-27357), ET-1 (dilution 1:400; cat. no. sc-21625), and goat anti-rabbit IgG-HRP (dilution 1:2000; cat. no. sc-2004), goat anti-mouse IgG-HRP (dilution 1:2000; cat. no. sc-2005), and donkey anti-goat IgG-HRP (dilution 1:2000; cat. no. sc-2020) secondary antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Primary antibodies against AT_1 R (dilution 1:400; cat. no. ab9391), and AT_2 R (dilution 1:800; cat. no. ab19134) were supplied by Abcam (Cambridge, UK). Primary antibodies against p-AMPK (dilution 1:2000; cat. no. 4188), and AMPK (dilution 1:1000; cat. no. 2532) were obtained from Cell Signaling Technology (Danvers, MA, USA).

Table 1
Real-time PCR primer sequences.

Primers	Sequences
AT_1 R (445 bp)	5′-cacctatgtaagatcgcttc-3′ (Forward) 5′-gcacaatcgccataattatcc-3′ (Reverse)
AT_2 R (65 bp)	5′-ccgtgaccaagtcttgaagatg-3′ (Forward) 5′-agggaagccagcaaatgatg-3′ (Reverse)
12-LO (312 bp)	5′-tggggcaactggaagg-3′ (Forward) 5′-agagcgcttcagcaccat-3′ (Reverse)
ET-1 (370 bp)	5′-ctcctccttgatggacaagg-3′ (Forward) 5′-cttgatgctgttgctcatgg-3′ (Reverse)
DDAH-1 (181 bp)	5′-cgcaatagggtccagtgaat-3′ (Forward) 5′-ttgcgctttctgggtactct-3′ (Reverse)
β-actin (101 bp)	5′-tactgccctggctcctagca-3′ (Forward) 5′-tggacagtgaggccaggatag-3′ (Reverse)

2.2. VSMCs preparation

Twenty-two-week-old, specific pathogen-free male inbred spontaneously hypertensive rats (SHR) and normotensive Wistar Kyoto (WKY) rats were obtained from Japan SLC (Shizuka, Japan). The rats were managed according to the guidelines of the Guide for the Care and Use of Experimental Animals of Yeungnam Medical Center.

VSMCs were obtained from the thoracic aortas of male SHR and WKY rats and cultured as described previously [23]. VSMCs in all experiments were used between passages 3 and 7. Prior to stimulation, VSMCs were cultured overnight in serum-starved Dulbecco's modified Eagle's medium (DMEM).

2.3. Real-time polymerase chain reaction

Total RNA from VSMCs was obtained using the easy-BLUE total RNA extraction kit (iNtRON Biotechnology, Seoul, Korea) according to the manufacturer's instructions. One microgram of total RNA was added to the reaction mixture (reaction volume, 20 μL) of the Maxime RT PreMix kit (iNtRON Biotechnology, Daejeon, Korea) according to the manufacturer's instructions. Real-time PCR amplification of 12-LO, ET-1, DDAH-1, AT_1 R, and AT_2 R was performed using the LightCycler system (Roche, Germany) as described previously [23]. Primer sequences are listed in Table 1. β-actin served as the housekeeping gene.

2.4. Measurement of DDAH activity

DDAH activity was measured as described previously by Ueda et al. [24]. Twenty micrograms of the protein sample was incubated in a total volume of 0.5 mL of 4 mmol/L ADMA–0.1 mol/L sodium phosphate buffer (pH 6.5) for 3 h at 37 °C. After the addition of an equal volume of 4% sulfosalicylic acid, the supernatant (100 μL) was stimulated with diacetyl monoxime (0.8% w/v in 5% acetic acid) and antipyrine (0.5% w/v in 50% sulfuric acid). The amount of L-citrulline formed was determined using a spectrophotometer at 466 nm (UV–visible spectrophotometer, Shimadzu UV-160, Kyoto, Japan).

2.5. Western blot analysis

Proteins were detected by western blot analysis according to a previously described method [23]. In brief, 20 μg of each protein sample was separated on 10% SDS-polyacrylamide gels. After transferring the proteins onto nitrocellulose membranes, the membranes were soaked in 5% non-fat dried milk with TBST (10 mmol/L Tris/HCl pH 7.5, 150 mmol NaCl, and 0.05% Tween-20) for 1 h and then incubated with primary antibodies against Sulf1, Sulf2, 12-LO, ET-1, AT_1 R, AT_2 R, AMPK, p-AMPK, and γ-tubulin for 16–18 h at 4 °C. Subsequently, the membranes were washed three times with TBST for 10 min and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h at room temperature. The immune complexes were visualized by enhanced chemiluminescence (LAS-3000, Fujifilm, Tokyo,

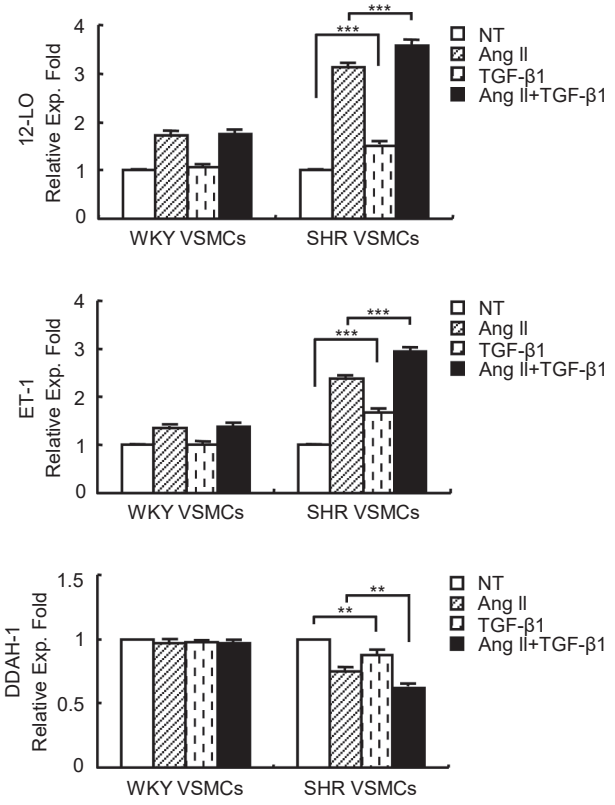

Fig. 1. Effects of TGF-β1 on the expression levels of 12-LO, ET-1 and DDAH-1 in SHR VSMCs. SHR or WKY rats VSMCs were untreated or treated with Ang II (0.1 μmol/L) and/or TGF-β1 (5 ng/mL) for 2 h. After total RNA isolation, real-time PCR assays were performed. The bars represent the mean ± SEM of three independent experiments. $^{**}p < 0.01$, $^{***}p < 0.001$.

Japan).

2.6. Small interfering RNA (siRNA) transfection

VSMCs seeded in 6-well plates were transfected with non-targeting control or sequence-specific siRNA using Lipofectamine 2000, according to the manufacturer's instructions. The siRNAs were used at a concentration of 50 nmol/L. The sequences used were as follows: Sulf1 siRNA – sense sequence, 5′-gugacuucaggaaugagau-3′ and antisense sequence, 5′-aucucauuccugaagucac-3′; Sulf2 siRNA – sense sequence, 5′-cacaucacaccgaguuaca-3′ and antisense sequence, 5′-uguaacucggugugaugug-3′; AT_1 R siRNA – sense sequence, 5′-gucacuguuacuacaccua-3′ and antisense sequence, 5′-uagguguaguaacagugac-3′; AT_2 R siRNA –sense sequence, 5′-gaguguugauagguaccaa-3′ and antisense sequence, 5′-uugguaccuaucaacacuc-3′.

2.7. VSMCs proliferation

SHR VSMCs plated in 24-well plates were cultured in serum-free medium for 24 h and then treated with the stimulants. [^{3}H]-thymidine (1 μCi/mL) (PerkinElmer, Boston, MA, USA) was added to the plates during the last 24 h of incubation. After washing three times with cold PBS, [^{3}H]-thymidine-labeled cells were collected with 0.1% SDS, and the amount of [^{3}H]-thymidine was measured using a Packard scintillation counter (Packard Instrument Company, Meriden, CT, USA).

2.8. Statistical analysis

All values are expressed as the mean ± standard error of the mean (SEM). SPSS version 25.0 software (IBM Co., Armonk, NY, USA) was used for statistical analysis. Statistical significance between the mean values was analyzed by Student's *t*-test or one-way analysis of variance (ANOVA) followed by Bonferroni or Dunnett's T3 post-hoc test. Results with p values < 0.05 were considered as statistically significant.

3. Results

3.1. Sulf2 mediates the hypertensive effects of TGF-β1 in SHR VSMCs

TGF-β1 and IL-10 mediate anti-inflammatory effects in vascular cells [20,25]. However, TGF-β1 exhibits different effects from IL to 10 on Ang II-induced chemokine expression in SHR VSMCs [26]. Therefore, we first examined the effect of TGF-β1 on the expression levels of mediators related to hypertension and on Ang II-induced expression levels of these mediators in SHR VSMCs and WKY rats VSMCs. TGF-β1 had no effect on the expression of 12-LO, ET-1, and DDAH-1 in WKY rats VSMCs. However, it increased 12-LO and ET-1 expression and upregulated Ang II-induced 12-LO and ET-1 expression in SHR VSMCs. Additionally, it inhibited DDAH-1 expression and increased Ang II-induced inhibition of DDAH-1 expression in SHR VSMCs (Fig. 1). Next, to compare the effects of extracellular sulfatases on TGF-β1-induced expression of 12-LO and ET-1 and inhibition of DDAH-1 in SHR VSMCs and WKY rat VSMCs, we performed real-time PCR after Sulf1 or Sulf2 siRNA transfection. Both, Sulf1 and Sulf2 did not affect the expression of 12-LO, ET-1, and DDAH-1 in response to TGF-β1 treatment in WKY rats VSMCs (Fig. 2A). However, downregulation of Sulf2 inhibited TGF-β1-induced 12-LO and ET-1 expression and abrogated the additive effect of TGF-β1 on Ang II-induced 12-LO and ET-1 in SHR VSMCs. Additionally, it abrogated the TGF-β1-induced inhibition of DDAH-1 and the additive effect of TGF-β1 on Ang II-induced inhibition of DDAH-1 in SHR VSMCs. In contrast, Sulf1 did not affect the hypertensive effects of TGF-β1 in SHR VSMCs (Fig. 2B). The disappearance of TGF-β1-induced effects with respect to 12-LO and ET-1 protein production and the additive effect of TGF-β1 on Ang II-induced 12-LO and ET-1 protein production was detected in Sulf2 siRNA-transfected SHR VSMCs using immunoblotting analysis (Fig. 2C). The levels of DDAH-1 activity following TGF-β1 and/or Ang II treatment in Sulf2 siRNA-transfected SHR VSMCs were correlated with the mRNA levels of DDAH-1 (Fig. 2D).

3.2. Sulf2 mediates TGF-β1-induced hypertensive effects via the AT_1 R pathway in SHR VSMCs

Upregulation of Ang II or CXCL8-induced hypertensive mediator expression is mainly dependent on the AT_1 R pathway, and the downregulation of hypertensive mediators by IL-10 or CCL5 is dependent on the AT_2 R pathway in SHR VSMCs [7–9,27]. Therefore, we examined whether the effect of Sulf2 on TGF-β1-induced hypertensive effects in SHR VSMCs is linked to Ang II receptors. First, we examined whether TGF-β1-induced hypertensive effects were mediated via the AT_1 R or AT_2 R pathway. We performed real-time PCR after treatment with losartan (AT_1 R inhibitor; 10 μmol/L) or PD123319 (AT_2 R inhibitor; 10 μmol/L) in control siRNA-transfected-SHR VSMCs. TGF-β1-induced 12-LO and ET-1 expression and inhibition of DDAH-1 in SHR VSMCs were mediated via the AT_1 R pathway, but not via the AT_2 R pathway (Fig. 3). The additive effect of TGF-β1 on Ang II-induced 12-LO and ET-1 expression disappeared in the control siRNA-transfected VSMCs treated with losartan (Fig. 3B,C). Additionally, TGF-β1-induced DDAH-1 inhibition and the additive effect of TGF-β1 on the Ang II-induced DDAH-1 inhibition were also reduced in the control siRNA-transfected VSMCs treated with an AT_1 R blocker (Fig. 3D). Next, we examined the effect of TGF-β1 on the expression levels of Ang II receptors and the effects of extracellular sulfatases on TGF-β1-induced Ang II receptor expression. TGF-β1 increased AT_1 R expression as well as Ang II-induced AT_1 R expression. In contrast, TGF-β1 had no effect on AT_2 R expression and Ang II-induced AT_2 R expression in SHR VSMCs. Furthermore, the downregulation of Sulf2 abrogated TGF-β1-induced AT_1 R expression

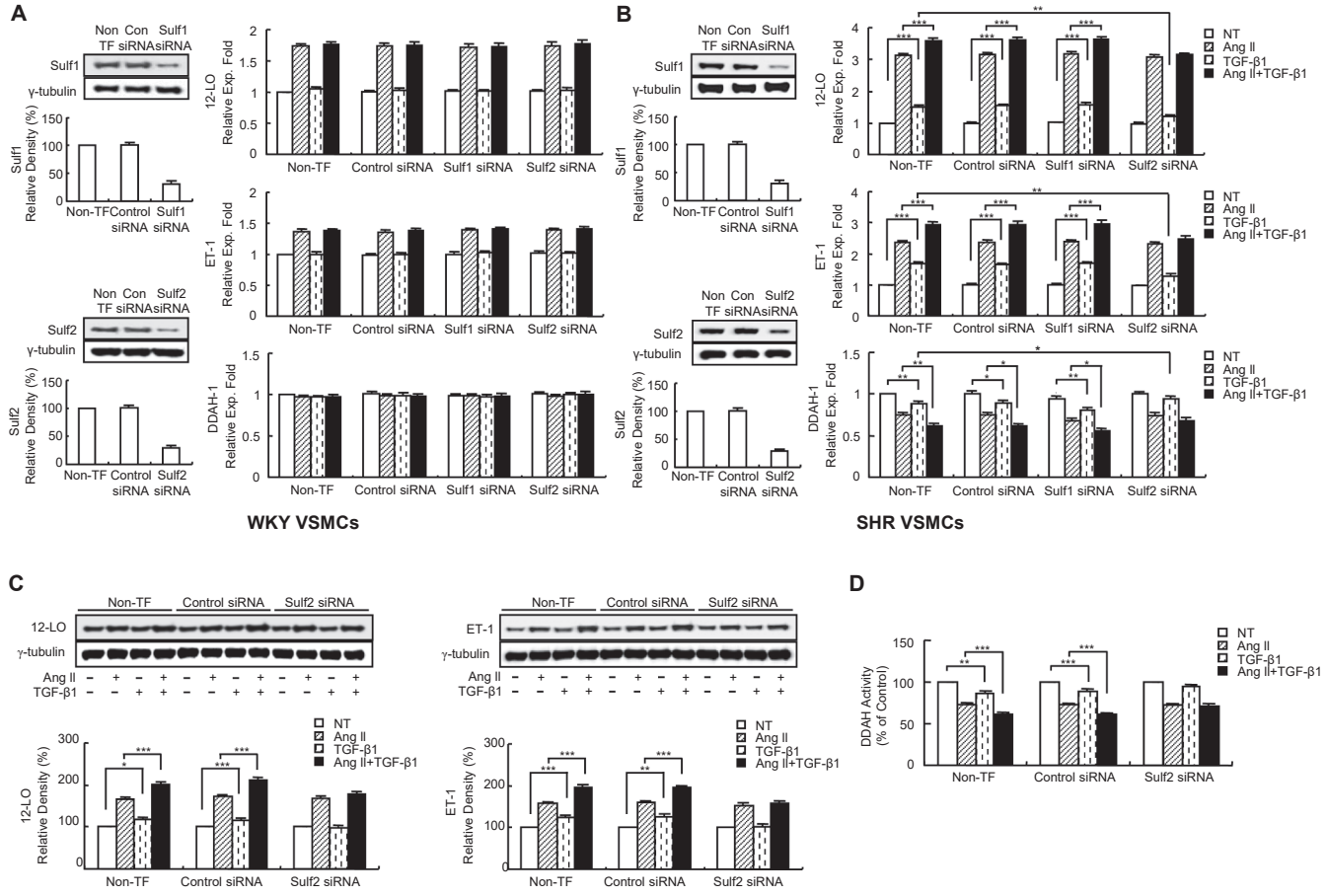

Fig. 2. TGF-β1-induced effects on the expression levels of 12-LO, ET-1 and DDAH-1 are mediated by Sulf2 in SHR VSMCs. SHR or WKY rats VSMCs were plated in 6-well plates, grown to 90% confluency, and transfected with Sulf1, Sulf2, or control siRNA oligomers (50 nmol/L). Successful transfection of Sulf1, Sulf2, or control siRNA oligomers in VSMCs was confirmed by immunoblotting analysis. Following transfection, (A,B) WKY or SHR rats VSMCs were untreated or treated with Ang II (0.1 μmol/L) and/or TGF-β1 (5 ng/mL) for 2 h. After total RNA isolation, real-time PCR assays were performed. (C,D) SHR VSMCs were untreated or treated with Ang II (0.1 μmol/L) and/or TGF-β1 (5 ng/mL) for 2 h. Cell lysates were prepared, and subsequently, immunoblotting analysis (C), and measurement of DDAH-1 activity (D) were performed. The activity of DDAH-1 was measured by converting ADMA to L-citrulline. Non-TF: non-transfected VSMCs. The data shown are representative of three independent experiments. The bars represent the mean ± SEM of three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

and the additive effect of TGF-β1 on Ang II-induced AT_1 R expression. However, the downregulation of Sulf1 did not influence these effects, and both Sulf1 and Sulf2 had no effect on AT_2 R expression (Fig. 4B). The protein levels of AT_1 R in Sulf2 siRNA-transfected SHR VSMCs treated with TGF-β1 and/or Ang II correlated with the mRNA levels presented in Fig. 4B (Fig. 4C).

Abnormal activation of adenosine monophosphate-activated protein kinase (AMPK) has been observed in hypertension [28]. Increased AMPK activation results in decreased levels of hypertensive mediators in SHR VSMCs [29]. Therefore, we examined the effect of TGF-β1 on AMPK activity and the involvement of extracellular sulfatases in TGF-β1-induced AMPK activity in SHR VSMCs using immunoblotting analysis. TGF-β1 inhibited AMPK phosphorylation and increased Ang II-induced inhibition of AMPK phosphorylation. Sulf1 did not affect TGF-β1-induced inhibition of AMPK phosphorylation and the additive effect of TGF-β1 on Ang II-induced inhibition of AMPK phosphorylation in SHR VSMCs (Fig. 5A). However, downregulation of Sulf2 abrogated TGF-β1-induced inhibition of AMPK phosphorylation and the additive effect of TGF-β1 on Ang II-induced inhibition of AMPK phosphorylation (Fig. 5B). We examined further whether the effects of TGF-β1 on AMPK activation and Ang II-induced inhibition of AMPK activation were associated with the Ang II receptor pathway in SHR VSMCs. TGF-β1-induced the inhibition of AMPK phosphorylation and its additive effect on Ang II-induced inhibition of AMPK phosphorylation disappeared in AT_1 R siRNA-transfected SHR VSMCs (Fig. 5C). However, these effects were sustained in AT_2 R siRNA-transfected SHR VSMCs (Fig. 5D). There were no significant differences between the abrogation of TGF-β1-induced inhibition of AMPK phosphorylation and the additive effect of TGF-β1 on the Ang II-induced downregulation of AMPK phosphorylation in AT_1 R siRNA-transfected SHR VSMCs and Sulf2 siRNA-transfected SHR VSMCs (Fig. 5E).

3.3. *Sulf2 mediates TGF-β1-induced SHR VSMCs proliferation via the AT_1 R pathway*

TGF-β1 has a proliferative effect on SHR VSMCs [19,30]. Thus, we examined whether extracellular sulfatases mediate TGF-β1-induced proliferation of SHR VSMCs. First, the additive proliferative effect of TGF-β1 on Ang II-induced SHR VSMCs proliferation was observed. Sulf1 neither affected TGF-β1-induced SHR VSMCs proliferation nor the additive effect of TGF-β1 on Ang II-induced SHR VSMCs proliferation. However, the downregulation of Sulf2 abrogated TGF-β1-induced SHR VSMCs proliferation and the additive effect of TGF-β1 on Ang II-induced SHR VSMCs proliferation (Fig. 6B). Additionally, TGF-β1-induced SHR VSMCs proliferation and the additive effect of TGF-β1 on Ang II-induced SHR VSMCs proliferation were mediated by the AT_1 R pathway, but not the AT_2 R pathway (Fig. 6C). The proliferation rate of AT_1 R siRNA-transfected SHR VSMCs was lower than that of Sulf2 siRNA-transfected SHR VSMCs. However, the proliferation rate of SHR VSMCs treated with Ang II alone did not differ significantly compared to

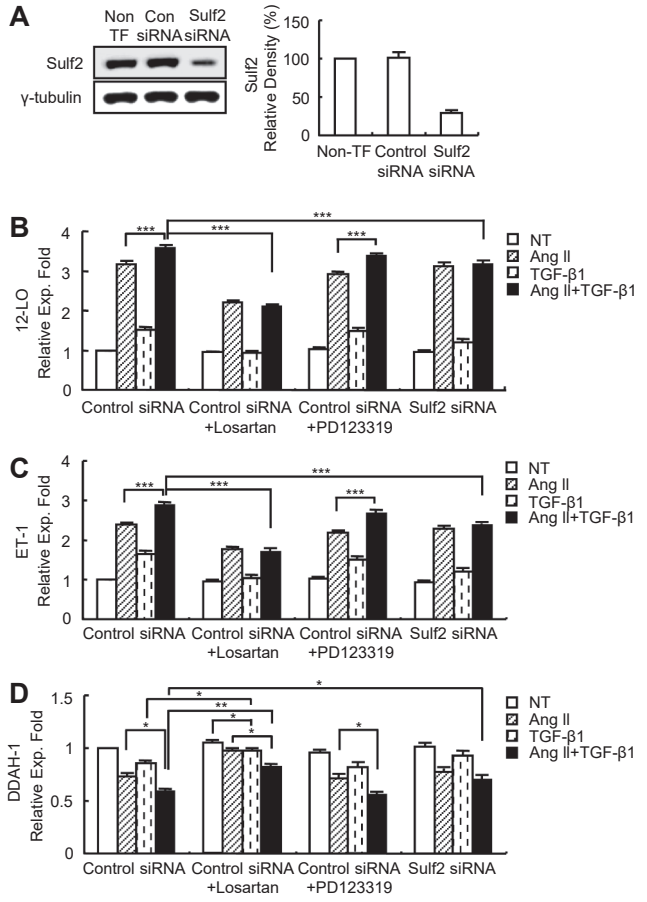

Fig. 3. TGF-β1-induced expression of 12-LO and ET-1 and inhibition of DDAH-1 is dependent on the AT_1 R pathway in SHR VSMCs. SHR VSMCs were plated in 6-well plates, grown to 90% confluency, and transfected with Sulf2 or control siRNA oligomers (50 nmol/L). Successful transfection of Sulf2 or control siRNA oligomers in SHR VSMCs was confirmed by immunoblotting analysis (A). Following transfection, control siRNA-transfected and Sulf2 siRNA-transfected VSMCs were untreated or treated with Ang II (0.1 μmol/L) and/or TGF-β1 (5 ng/mL) for 2 h. In addition, control siRNA-transfected SHR VSMCs were untreated or treated with Ang II (0.1 μmol/L) and/or TGF-β1 (5 ng/mL) in the presence of losartan (AT_1 R inhibitor, 10 μmol/L) or PD123319 (AT_2 R inhibitor, 10 μmol/L) for 2 h. Total RNA was isolated and 12-LO, ET-1, and DDAH-1 mRNA levels were measured by performing real-time PCR (B,C,D). The bars represent the mean ± SEM from three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

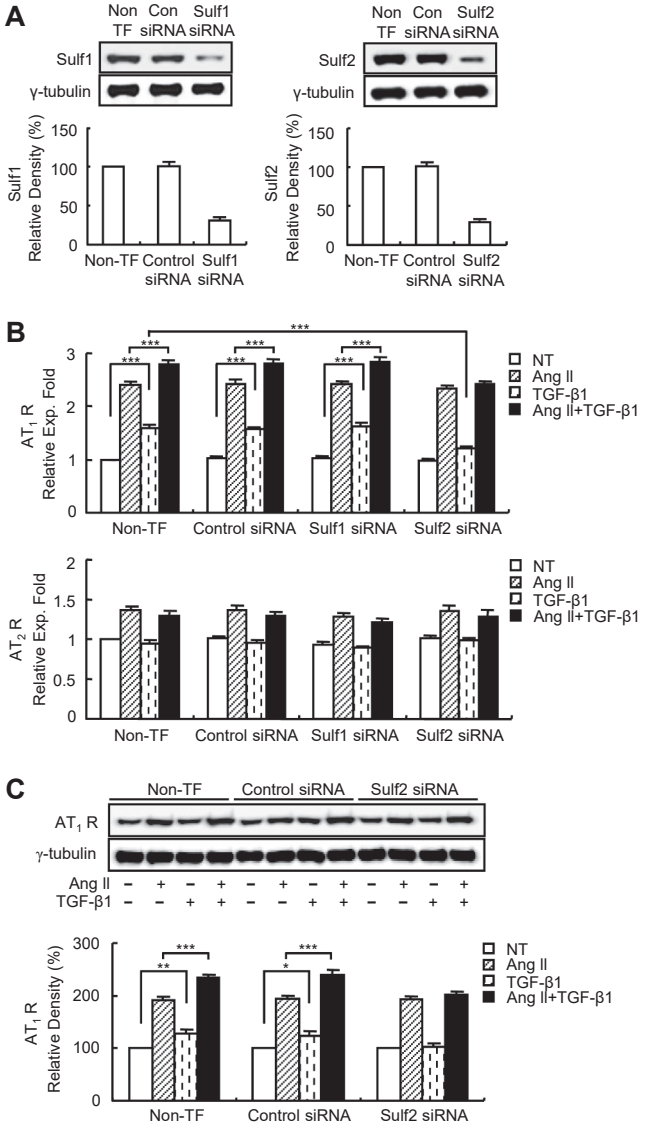

Fig. 4. TGF-β1 increases the expression of AT_1 R, not AT_2 R and down-regulation of Sulf2 abrogates TGF-β1-induced AT_1 R expression and the additive effect of TGF-β1 on Ang II-induced AT_1 R expression in SHR VSMCs. SHR VSMCs were plated in 6-well plates, grown to 90% confluency, and transfected with Sulf1, Sulf2 or control siRNA oligomers (50 nmol/L). Successful transfection of Sulf1, Sulf2, or control siRNA oligomers in VSMCs was confirmed by immunoblotting analysis (A). Following transfection, SHR VSMCs were untreated or treated with Ang II (0.1 μmol/L) and/or TGF-β1 (5 ng/mL) for 2 h. Total RNA was isolated and cell lysates were prepared. AT_1 R and AT_2 R mRNA levels were determined by real-time PCR (B). The protein levels of AT_1 R were determined by immunoblotting and densitometric analyses (C). Non-TF: non-transfected VSMCs. The data shown are representative of three independent experiments. The bars represent the mean ± SEM of three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

that of SHR VSMCs treated with TGF-β1 and Ang II simultaneously in both the groups (Fig. 6D).

4. Discussion

The findings of this study reveal that hypertensive effects such as increased 12-LO and ET-1 expression, inhibition of DDAH-1 activity and AMPK activation, and SHR VSMCs proliferation induced by TGF-β1 and the additive effects of TGF-β1 on Ang II-induced similar hypertensive actions are mediated by Sulf2 via the AT_1 R pathway, and not Sulf1 in SHR VSMCs. This result is consistent with our previous study on extracellular sulfatases which indicated that the upregulatory effect of CXCL8 on ET-1 expression is related to Sulf2 activity via the AT_1 R pathway [8].

The role of extracellular sulfatases in hypertension has not yet been elucidated. However, the up- or downregulation of the Sulf1 gene affects adhesion, proliferation, and apoptosis in normal VSMCs, and therefore, the maintenance of normal 6-*O*-sulfation levels by Sulf1 is important for the function of these cells [31]. In addition, our previous studies have demonstrated that the activity of extracellular sulfatases is involved in the expression of mediators related to hypertension in SHR VSMCs, but not in WKY rats VSMCs [7–9]. Moreover, the expression of extracellular sulfatases is increased in SHR VSMCs as compared to WKY rats VSMCs (data not shown). Therefore, hypertensive VSMCs might exhibit abnormal activity of extracellular sulfatases which might play a functional role in hypertensive cells.

Both, 12-LO and ET-1 are major hypertensive mediators, and Ang II is a potent stimulator of these molecules [32–34]. Induction of ET-1 expression by TGF-β1 has been demonstrated in vascular endothelial

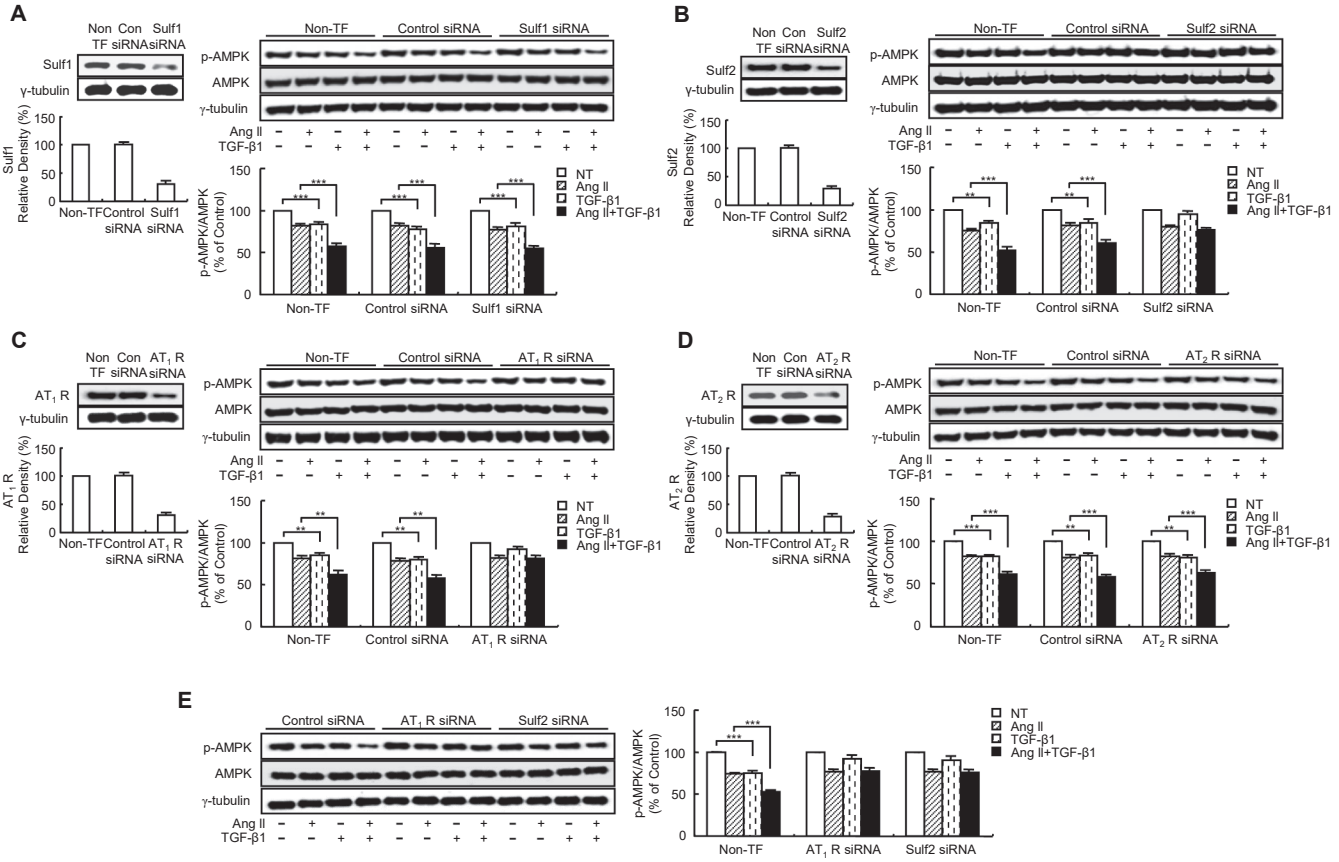

Fig. 5. TGF-β1-induced AMPK inhibition and the additive effect of TGF-β1 on Ang II-induced inhibition of AMPK activity are mediated by Sulf2 via the AT_1 R pathway in SHR VSMCs. SHR VSMCs were plated in 6-well plates, grown to 90% confluency, and transfected with Sulf1, Sulf2, AT_1 R, AT_2 R or control siRNA oligomers (50 nmol/L). Successful transfection of Sulf1, Sulf2, AT_1 R, AT_2 R or control siRNA oligomers in SHR VSMCs was confirmed by immunoblotting analysis. Following transfection, SHR VSMCs were untreated or treated with Ang II (0.1 μmol/L) and/or TGF-β1 (5 ng/mL) for 2 h. The expression levels of p-AMPK were determined by immunoblotting and densitometric analyses. Non-TF: non-transfected VSMCs. The data shown are representative of three independent experiments. The bars represent the mean ± SEM of three independent experiments. $^{**}p < 0.01$, $^{***}p < 0.001$.

cells [35]. DDAH-1 regulates the plasma levels of ADMA, which upon accumulation causes contraction of blood vessels [36]. In this study, TGF-β1 had no effect on the expression of 12-LO, ET-1, and DDAH-1 in WKY rats VSMCs. However, it enhanced 12-LO and ET-1 expression and inhibited DDAH-1 expression, as well as upregulated Ang II-induced 12-LO and ET-1 expression and inhibition of DDAH-1 in SHR VSMCs. The expression of TGF-β type II receptor is increased in SHR VSMCs compared to that in WKY rat VSMCs, and Ang II causes abnormal regulation of TGF-β receptors in SHR VSMCs [18]. Thus, these differences may contribute to the different results between SHR VSMCs and WKY rats VSMCs.

The upregulatory effects of TGF-β1 on 12-LO and ET-1 expression, inhibition of DDAH-1, Ang II-induced 12-LO and ET-1 expression, and Ang II-induced inhibition of DDAH-1 were mediated by Sulf2, and not Sulf1 via the AT_1 R pathway, and not via the AT_2 R pathway in SHR VSMCs. It is well-known that AT_1 R and AT_2 R exhibit opposite effects [37]. A positive role of AT_2 R in the anti-hypertensive effects of IL-10 or CCL5 and that of AT_1 R in the hypertensive effects of CXCL8 in SHR VSMCs have been demonstrated [7–9,29]. We observed that the effect of TGF-β1 on the expression of 12-LO, ET-1, and DDAH-1 was also dependent on the AT_1 R pathway, and Sulf2 downregulation resulted in the abrogation of TGF-β1-induced expression of AT_1 R mRNA as well as the additive effect of TGF-β1 on Ang II-induced AT_1 R expression. These results suggest that the hypertensive effects of TGF-β1 through the AT_1 R pathway in SHR VSMCs are related to Sulf2 activation. Ang II increases both AT_1 R and AT_2 R expression, and Ang II-induced expression of 12-LO and ET-1 mRNA is dependent on both the pathways and is independent of both Sulf1 and Sulf2 activity [7,34]. Furthermore, the inhibition of DDAH-1 by Ang II is solely dependent on the AT_1 R pathway and is independent of both Sulf1 and Sulf2 activity in SHR VSMCs [9,38]. Therefore, the overall expression levels of 12-LO and ET-1 in control siRNA-transfected SHR VSMCs stimulated with an AT_1 R blocker were lower than those in Sulf2 siRNA-transfected SHR VSMCs (Fig. 3). However, complete abrogation of the increased effect of TGF-β1 on 12-LO and ET-1 expression and the additive effect of TGF-β1 on Ang II-induced 12-LO and ET-1 expression was detected in both control siRNA-transfected SHR VSMCs treated with an AT_1 R blocker and Sulf2 siRNA-transfected SHR VSMCs. Thus, TGF-β1-induced expression of 12-LO and ET-1 via the AT_1 R pathway is completely dependent on Sulf2 in SHR VSMCs. On the other hand, complete abrogation of the inhibitory effect of TGF-β1 on DDAH-1 expression and the additive effect of TGF-β1 on Ang II-induced inhibition of DDAH-1 was not detected in control siRNA-transfected SHR VSMCs treated with an AT_1 R blocker. This result indicates that the inhibitory effect of TGF-β1 on DDAH-1 expression and the additive effect of TGF-β1 on Ang II-induced inhibition of DDAH-1 do not entirely depend on the AT_1 R pathway, however, these events are completely dependent on Sulf2 activation in SHR VSMCs. It is well known that Smad proteins mediate the intracellular signaling of TGF-β1, which mediates TGF-β1 signaling via TGF-β receptors (TGF-β R) in rat VSMCs [39,40]. Of the Smad proteins, Smad2 and Smad3 play a pivotal role in optimal TGF-β1 signal transduction [41]. Therefore, we also observed the effects of extracellular sulfatases on TGF-β1-induced Smad2 and Smad3 phosphorylation in SHR VSMCs. Downregulation of Sulf2 inhibited TGF-β1-induced Smad2 and Smad3 phosphorylation and

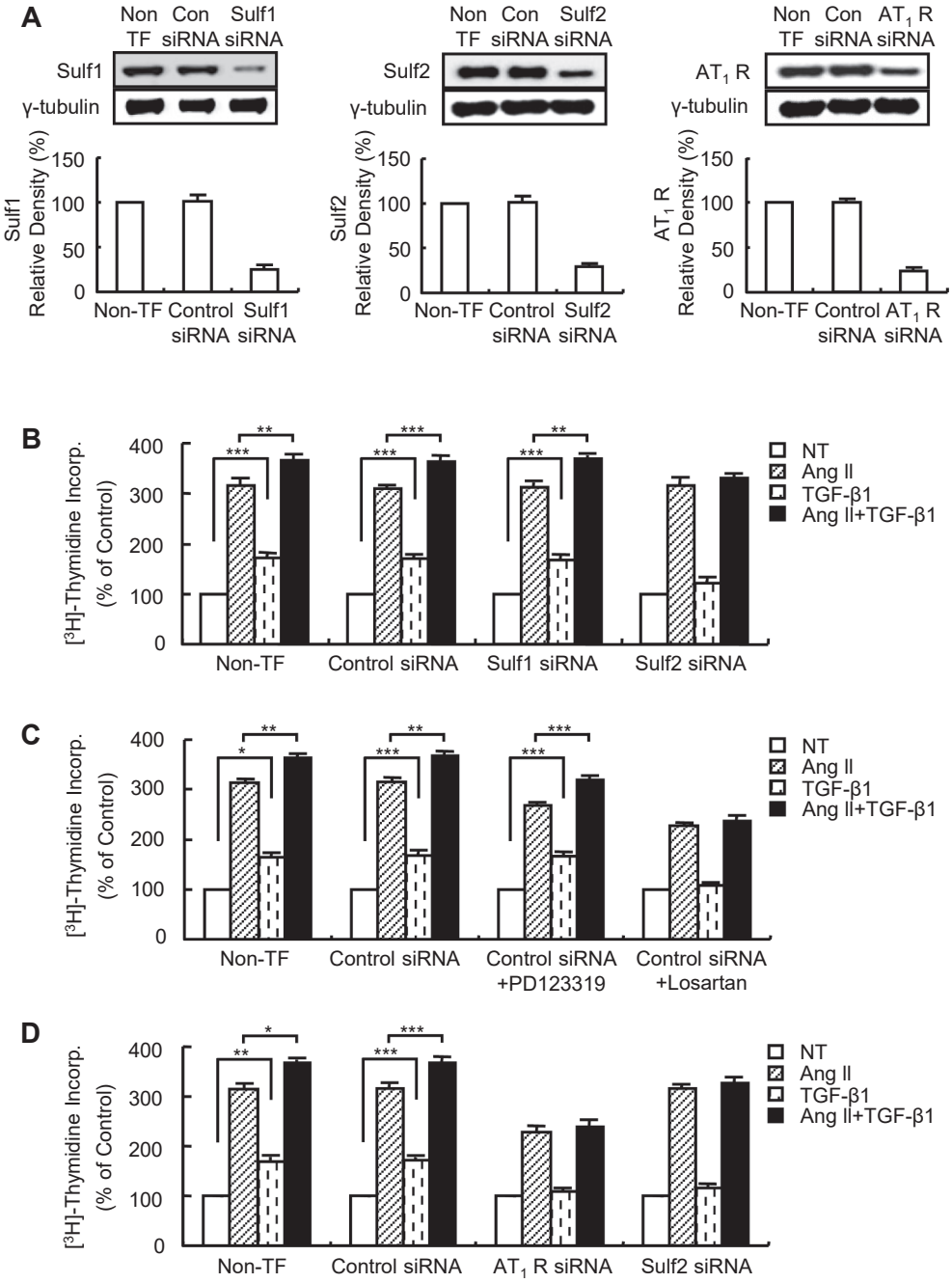

Fig. 6. Sulf2 mediates TGF-β1-induced SHR VSMCs proliferation and the additive effect of TGF-β1 on Ang II-induced SHR VSMCs proliferation. SHR VSMCs were plated in 24-well plates, grown to 90% confluency, and transfected with Sulf1, Sulf2, AT_1 R or control siRNA oligomers (50 nmol/L). Successful transfection of Sulf1, Sulf2, AT_1 R or control siRNA oligomers in SHR VSMCs was confirmed by immunoblotting analysis (A). Following transfection, non-transfected, control siRNA-, Sulf1 siRNA-, Sulf2 siRNA- and AT_1 R siRNA-transfected SHR VSMCs were untreated or treated with Ang II (0.1 μmol/L) and/or TGF-β1 (5 ng/mL) for 24 h in medium containing [^{3}H]-thymidine (1 μCi/mL) (B,D). In addition, control siRNA-transfected VSMCs were untreated or treated with Ang II (0.1 μmol/L) and/or TGF-β1 (5 ng/mL) in the presence of losartan (AT_1 R inhibitor, 10 μmol/L) or PD123319 (AT_2 R inhibitor, 10 μmol/L) for 24 h in medium containing [^{3}H]-thymidine (1 μCi/mL) (C). The levels of [^{3}H]-thymidine incorporation are shown on the Y-axis. The bars represent the mean ± SEM of four independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

abrogated the additive effect of TGF-β1 on Ang II-induced Smad2 and Smad3 phosphorylation (data not shown). In a hepatocellular carcinoma study, Sulf2-induced angiogenesis was mediated via periostin, and the expression of Sulf2-mediated periostin was dependent on the TGF-β1/Smad2 and Smad3 pathway [42]. Moreover, the Ang II/ AT_1 R pathway resulted in Smad2 and Smad3 phosphorylation in a tissue fibrosis study [43]. In the present study, we observed the TGF-β1/Sulf2/AT_1 R pathway in TGF-β1-induced hypertensive effects in SHR VSMCs. Further studies are required to clarify the relationship between the extracellular sulfatases/TGF-β R/Smad pathway and extracellular sulfatases/AT_1 R/Smad pathway in TGF-β-induced hypertensive effects in SHR VSMCs.

AMPK activity plays an important role in the regulation of energy homeostasis and cellular metabolism [44]. AMPK activation affects VSMCs proliferation, vascular endothelial function, and blood pressure. Moreover, it has a defensive role in hypertension [28]. In this study, downregulation of Sulf2 abrogated the TGF-β1-induced inhibition of AMPK activation and the additive effect of TGF-β1 on the Ang II-induced inhibition of AMPK activation in SHR VSMCs. We also observed that the TGF-β1-induced effects were mediated by the AT_1 R pathway. The AMPK activity-mediated downregulatory effect of IL-10 on the Ang II-induced inhibition of DDAH-1 is dependent on Sulf1 activity and the AT_2 R pathway [9]. In addition, CCL5 enhances AMPK activation and inhibits the Ang II-induced reduction of AMPK activation via the AT_2 R pathway [45]. Thus, we suggest that increased AMPK activity depends on Sulf1 via the AT_2 R pathway, while decreased AMPK activity depends on Sulf2 via the AT_1 R pathway.

TGF-β1 has a dual role in cell proliferation. TGF-β1 has a proliferative effect in SHR VSMCs but inhibits VSMCs proliferation in WKY rats [19,30]. TGF-β1 upregulated Ang II-induced SHR VSMCs proliferation, and Sulf2 mediated this process as well as the additive effect of TGF-β1

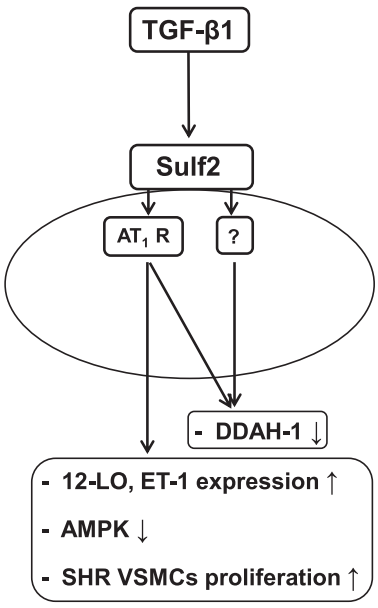

Fig. 7. Schematic representation of the action of Sulf2 on the hypertensive effects of TGF-β1 in SHR VSMCs.

on Ang II-induced SHR VSMCs proliferation. Additionally, the AT_1 R pathway mediated TGF-β1-induced SHR VSMCs proliferation. Complete abrogation of TGF-β1-induced SHR VSMCs proliferation and the additive effect of TGF-β1 on Ang II-induced SHR VSMCs proliferation was detected in both AT_1 R siRNA-transfected SHR VSMCs and Sulf2 siRNA-transfected SHR VSMCs. However, the AT_1 R siRNA-transfected SHR VSMCs showed lower levels of proliferation than the Sulf2 siRNA-transfected SHR VSMCs. This can be attributed to the fact that Ang II-induced SHR VSMCs proliferation is mediated via both the AT_1 R and AT_2 R pathways and is independent of extracellular sulfatases (Fig. 6B, C). Sulf1 regulates VSMCs proliferation through changes in 6-*O*-sulfation levels [31], and we have previously demonstrated that the IL-10-induced downregulation of Ang II-induced proliferation is mediated via the AT_2 R pathway and is inhibited in Sulf1 siRNA-transfected SHR VSMCs [9]. Consistent with the findings for 12-LO, ET-1, and DDAH-1 expression, this result shows that Sulf2 mediates TGF-β1-induced VSMCs proliferation and the additive effect of TGF-β1 on Ang II-induced VSMCs proliferation via the AT_1 R pathway in SHR VSMCs.

In this study, we demonstrated that Sulf2 mediated TGF-β1-induced AT_1 R expression, and that the AT_1 R pathway is involved in the mediation of TGF-β1-induced hypertensive effects in SHR VSMCs (Fig. 7). Considering the results of this study and our previous studies together, we suggest that extracellular sulfatases, Sulf1 and Sulf2, counteract the expression of cytokine-induced mediators related to hypertension in hypertensive VSMCs. Furthermore, these findings suggest that a signaling cascade involving Sulf2 and the AT_1 R pathway leads to an upregulation of hypertensive mediators, while that involving Sulf1 and the AT_2 R pathway results in an upregulation of anti-hypertensive mediators in hypertensive VSMCs.

CRediT authorship contribution statement

Hee Sun Kim: Conceptualization, Validation, Formal analysis, Writing – original draft, Writing – review & editing, Supervision, Funding acquisition. **Hye Young Kim:** Investigation, Data curation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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