

# Sulfatase 1 mediates IL-10-induced dimethylarginine dimethylaminohydrolase-1 expression and antiproliferative effects in vascular smooth muscle cells of spontaneously hypertensive rats

Hye Young Kim, Hee Sun Kim\*

Department of Microbiology, College of Medicine, Yeungnam University, Daegu, Republic of Korea

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## ABSTRACT

The extracellular sulfatases (exSulfs) sulfatase 1 (Sulf1) and sulfatase 2 (Sulf2) are well-known regulators of cell signaling and metabolism. In addition, exSulfs mediate the up- or downregulatory effects of cytokines on angiotensin II (Ang II)-induced expression of hypertensive mediators in vascular smooth muscle cells (VSMC) from spontaneously hypertensive rats (SHRs). Previously, we demonstrated that interleukin-10 (IL-10)-induced dimethylarginine dimethylaminohydrolase-1 (DDAH-1) expression was mediated by Ang II subtype 2 receptor (AT<sub>2</sub> R) and AMP-activated protein kinase (AMPK) activation, and that IL-10-mediated inhibition of Ang II-induced proliferation of SHRs VSMC was partially associated with DDAH-1. In this study, we examined the effects of exSulfs on IL-10-induced DDAH-1 expression, abrogation of Ang II-induced DDAH-1 downregulation, and inhibition of Ang II-induced proliferation of SHRs VSMC. IL-10-induced DDAH-1 expression and abrogation of Ang II-induced DDAH-1 downregulation were attenuated in Sulf1 siRNA-transfected SHRs VSMC. However, Sulf2 did not affect IL-10-induced DDAH-1 expression and abrogation of Ang II-induced DDAH-1 downregulation. Downregulation of Sulf1 inhibited IL-10-induced AT<sub>2</sub> R expression and the synergistic effects of IL-10 on Ang II-induced AT<sub>2</sub> R expression. Additionally, Sulf1 downregulation inhibited IL-10-induced AMPK activity and abrogation of Ang II-induced decrease in AMPK activity. Moreover, the IL-10-mediated inhibition of Ang II-induced proliferation was not detected in Sulf1 siRNA-transfected SHRs VSMC; IL-10-mediated inhibition of Ang II-induced VSMC proliferation was mediated via the AT<sub>2</sub> R pathway and AMPK activation. Specifically, IL-10-induced DDAH-1 expression, abrogation of Ang II-induced DDAH-1 downregulation, and inhibition of Ang II-induced proliferation, which is mediated by the AT<sub>2</sub> R pathway and AMPK activation, are mainly mediated by Sulf1 activity in SHRs VSMC. These results suggest that Sulf1, and not Sulf2, mediates the IL-10-induced inhibition of Ang II-induced hypertensive effects in SHRs VSMC.

## 1. Introduction

Sulfatases are classified as extracellular, non-lysosomal, or lysosomal sulfatases based on their localization. Seventeen distinct sulfatases in humans and fourteen in rodents have been identified thus far [1]. Among these, extracellular sulfatases (exSulfs), also known as heparin sulfate 6-O-endosulfatases, play an important role in cell signaling [2]. Removal of 6-O-sulfate from heparin sulfate proteoglycans (HSPGs)

by exSulfs results in the release of bound growth factors, which initiate signaling pathways. There are two types of exSulfs: sulfatase 1 (Sulf1) and sulfatase 2 (Sulf2) [3]. In addition to cell signaling, exSulfs play an active role in cell development, tumorigenesis, muscle regeneration, and immunomodulation [4–8].

Sulf1 and Sulf2 have opposite effects; Sulf1 inhibits cell proliferation and angiogenesis, whereas Sulf2 promotes these processes [5,6,9]. We had previously observed that Sulf1 activity affects the

**Abbreviations:** IL-10, interleukin-10; CCL, CC-chemokine ligand; CXCL, CXC-chemokine ligand; Sulf1, sulfatase 1; Sulf2, sulfatase 2; exSulfs, extracellular Sulfatases; VSMC, vascular smooth muscle cells; SHRs, spontaneously hypertensive rats; WKYs, Wistar-Kyoto rats; Ang II, angiotensin II; DDAH-1, dimethylarginine dimethylaminohydrolase-1; AT<sub>1</sub> R, Ang II type 1 receptor; AT<sub>2</sub> R, Ang II type 2 receptor; AMPK, AMP-activated protein kinase; HSPGs, heparan sulfate proteoglycans; ADMA, asymmetric (*N*<sup>G</sup>,*N*<sup>G</sup>) dimethylarginine; 12-LO, 12-lipoxygenase; ET-1, endothelin-1; PCR, polymerase chain reaction; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; siRNA, small interfering RNA; cDNA, complementary DNA; SEM, standard error of the mean

\* Corresponding author at: Department of Microbiology, College of Medicine, Yeungnam University, 170 Hyeonchung-ro, Nam-gu, Daegu 42415, Republic of Korea.

E-mail address: [heesun@med.yu.ac.kr](mailto:heesun@med.yu.ac.kr) (H.S. Kim).

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downregulatory effects of CC-chemokine ligand 5 (CCL5) on angiotensin II (Ang II)-induced expression of hypertensive mediators [10], while Sulf2 activity affects the upregulatory effects of CXC-chemokine ligand 8 (CXCL8) on the Ang II-induced expression of hypertensive mediators in vascular smooth muscle cells (VSMC) from spontaneously hypertensive rats (SHRs) [11]. However, the precise roles of exSulfs in hypertension have not been fully investigated at the cellular level.

Interleukin-10 (IL-10) is a well-known anti-inflammatory cytokine that plays a major role in immune homeostasis [12,13]. Studies have shown that IL-10 inhibits NADPH oxidase activity and limits Ang II-induced vascular contraction, while protecting against vascular inflammation during hypertension [12–14]. Systemic IL-10 expression in stroke-prone SHRs led to decreased vascular remodeling and systolic blood pressure, and intracerebroventricular infusion of IL-10 could reduce mean arterial blood pressure in SHRs [15,16]. Additionally, overexpression of IL-10 in the paraventricular nucleus has been shown to reduce Ang II-induced hypertension [17]. Downregulatory effects of IL-10 on Ang II-induced hypertensive mediator expression, anti-hypertensive mediator inhibition, and VSMC proliferation are similar to those of CCL5 in SHRs VSMC [18–22]. Both IL-10 and CCL5 have been shown to increase dimethylarginine dimethylaminohydrolase-1 (DDAH-1) expression, and attenuate Ang II-induced downregulation of DDAH-1 in SHRs VSMC [18,19]. DDAH-1 inhibits vascular contraction and downregulates the plasma level of asymmetric ( $N^G, N^G$ ) dimethylarginine (an inhibitor of nitric oxide synthase) [23]. Our previous studies demonstrated that Sulf1 mediates the inhibitory effects of CCL5 on Ang II-induced 12-lipoxygenase (12-LO) and endothelin-1 (ET-1) expression in SHRs VSMC [10], whereas Sulf2 mediates the upregulatory effects of CXCL8 on Ang II-induced ET-1 expression in SHRs VSMC [11]. Therefore, we hypothesized that IL-10-mediated abrogation of Ang II-induced DDAH-1 downregulation might be related to the activity of exSulfs.

Based on these previous studies, it can be suggested that exSulfs have a functional role in the up- or downregulatory effects of cytokines on the expression of hypertensive mediators in SHRs VSMC. Therefore, we investigated the effect of exSulfs on the IL-10-mediated inhibition of Ang II-induced hypertensive effects, focusing on DDAH-1 expression and the inhibition of the proliferation of SHRs-derived VSMC.

## 2. Materials and methods

### 2.1. Reagents

IL-10 was purchased from R&D Systems (Minneapolis, MN, USA). Losartan, PD123319, and Compound C were obtained from Sigma-Aldrich (St. Louis, MO, USA). Total RNA extraction kit was purchased from iNtRON Biotechnology (Seoul, Korea). Ang II was obtained from Calbiochem (San Diego, CA, USA). LightCycler FastStart DNA SYBR Green I Mix was supplied by Roche (Mannheim, Germany). The primers for DDAH-1, Ang II type 1 receptor (AT<sub>1</sub> R), Ang II type 2 receptor (AT<sub>2</sub> R), and  $\beta$ -actin were supplied by Bionics (Daejeon, Korea). Rat Sulf1 siRNA was purchased from Bioneer (Daejeon, Korea). Negative control small interfering RNA (siRNA), pcDNA3.1 vector, and Lipofectamine 2000 were obtained from Invitrogen Life Technologies (Carlsbad, CA, USA). The pcDNA3.1/Myc-His (-)-MSulf1 (Plasmid 13007) donated by Dr. Steven D. Rosen [3] was supplied by Addgene (Cambridge, MA, USA). Rat Sulf2 siRNA and primary antibodies against Sulf1 (dilution 1:200; cat. no. sc-98325), Sulf2 (dilution 1:200; cat. no. sc-271772), and DDAH-1 (dilution 1:200; cat. no. sc-26068) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Primary antibodies against AT<sub>2</sub> R (dilution 1:800; cat. no. ab19134) were purchased from Abcam (Cambridge, UK). Primary antibodies against AMPK (dilution 1:1000; cat. no. 2532) and p-AMPK (dilution 1:2000; cat. no. 4188) were purchased from Cell Signaling (Cambridge, UK). The anti- $\gamma$ -tubulin antibody (dilution 1:2000; cat. no. T6557) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Secondary antibodies against goat

anti-mouse IgG-HRP (dilution 1:2000; cat. no. sc-2005), goat anti-rabbit IgG-HRP (dilution 1:2000; cat. no. sc-2004), and donkey anti-goat IgG-HRP (dilution 1:2000; cat. no. sc-2020) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

### 2.2. VSMC preparation

Twenty-two-week-old, normotensive Wistar-Kyoto rats (WKYs) and specific pathogen-free male inbred SHRs were obtained from Japan SLC (Shizuoka, Japan). The rats were handled according to the principles of the Guide to the Care and Use of Experimental Animals of the Yeungnam Medical Center.

The VSMC were isolated from the thoracic aortas of male WKYs and SHRs and cultured as described by Kim et al. [24]. All experiments were conducted between cell passages 3 to 7. Prior to stimulation, VSMC were cultured overnight in Dulbecco's modified Eagle's medium (DMEM) under conditions of serum starvation.

### 2.3. Real-time polymerase chain reaction

Total RNA from VSMC was isolated using an easy-BLUE total RNA extraction kit (iNtRON Biotechnology, Seoul, Korea) according to the manufacturer's instructions. One microgram of total RNA was used in a reaction mixture (reaction volume, 20  $\mu$ L) prepared using the Maxime RT PreMix kit (iNtRON Biotechnology, Daejeon, Korea) according to the manufacturer's instructions. DDAH-1, AT<sub>1</sub> R, or AT<sub>2</sub> R were amplified by real-time PCR using the LightCycler system (Roche, Germany). PCR amplification was performed as previously described by Kim et al. [11]. The primer sets used were as follows: DDAH-1, forward, 5'-cgcaatagggtccagtgaat-3' and reverse, 5'-ttgcgcttctgggtactct-3' (181 bp); AT<sub>1</sub> R, forward, 5'-cacctatgtaagatcgcttc-3' and reverse, 5'-gcacaatgcacataattatcc-3' (445 bp); AT<sub>2</sub> R, forward, 5'-ccgtgaccaagtcttgaagatg-3' and reverse, 5'-agggaagccagcaaatgatg-3' (65 bp); and  $\beta$ -actin, forward, 5'-tactgcctggctcctagca-3' and reverse, 5'-tgga-cagtggagccagtag-3' (101 bp). The mRNA level of each sample was normalized to the mRNA level of  $\beta$ -actin, which served as the house-keeping gene.

### 2.4. DDAH activity

DDAH activity was determined using the procedure described by Ueda et al. [23]. Twenty micrograms of protein was incubated with 4 mmol/L ADMA-0.1 mol/L sodium phosphate buffer (pH 6.5) in a total volume of 0.5 mL for 3 h at 37 °C. After the addition of an equal volume of 4% sulfosalicylic acid, the supernatants (100  $\mu$ L) were treated with diacetyl monoxime (0.8% wt/vol in 5% acetic acid) and antipyrine (0.5% wt/vol in 50% sulfuric acid). The amount of L-citrulline formed was measured using a spectrophotometer at 466 nm (UV-Visible spectrophotometer, Shimadzu UV-160, Kyoto, Japan).

### 2.5. Western blot analysis

Western blot analysis was performed as described by Kim et al. [24]. Protein concentrations in the extracts were measured using the Bradford assay (Bio-Rad, Hercules, CA, USA). Equal amounts (20  $\mu$ g) of each of the protein samples were separated on 10% SDS-polyacrylamide gels and then transferred onto nitrocellulose membranes. The membranes were soaked in 5% non-fat dried milk in TBST (10 mmol/L Tris/HCl pH 7.5, 150 mmol NaCl, and 0.05% Tween-20) for 1 h and then incubated for 16–18 h with primary antibodies against Sulf1, Sulf2, AT<sub>2</sub> R, AMPK, p-AMPK, DDAH-1, and  $\gamma$ -tubulin 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 antigen–antibody complexes were detected using an enhanced chemiluminescence detection system (LAS-3000, Fujifilm, Tokyo, Japan).

## 2.6. Small interfering RNAs (siRNAs)

VSMC were seeded on 6-well plates and transfected with exSulf-specific siRNA or non-targeting control siRNA using Lipofectamine 2000, according to the manufacturer's instructions. The sequences used were as follows: Sulf1 siRNA sense sequence, 5'-gugacuucaggaaugagau-3' and antisense sequence, 5'-aucucauuccugaagucac-3'; Sulf2 siRNA – sense sequence, 5'-cacacacacaggaguuaca-3' and antisense sequence, 5'-uguuacucggugaugug-3'. The siRNAs were used at a concentration of 50 nmol/L. The transfected VSMC were cultured in growth medium for 24 h before the experiments. After culture, the cells were treated with stimulants for 2 h.

## 2.7. Sulf1 gene overexpression

A recombinant plasmid, pcDNA3.1/Myc-His(-)-MSulf1, was amplified in Luria-Bertani Broth media with 100 µg/mL ampicillin. The amplified plasmids were purified using a QIAGEN Plasmid Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The VSMC were transfected with either pcDNA3.1/Myc-His(-)-MSulf1 or the pcDNA3.1 empty vector using Lipofectamine 2000, as per the manufacturer's instructions.

## 2.8. VSMC proliferation

SHRs VSMC were plated on 24-well plates in serum-free medium for 24 h and then exposed to the stimulants. [<sup>3</sup>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, [<sup>3</sup>H]-thymidine-labeled cells were collected with 0.1% SDS, and radioactivity was measured using a Packard scintillation counter (Packard Instrument Company, Meriden, CT, USA).

## 2.9. Statistical analysis

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

## 3. Results

### 3.1. Sulf1 mediates IL-10-induced DDAH-1 expression and abrogation of Ang II-induced DDAH-1 downregulation in SHRs VSMC

Ang II inhibits DDAH-1 expression, whereas IL-10 increases it, and Ang II-induced DDAH-1 downregulation is prevented by IL-10 in SHRs VSMC [18]. Initially, we compared the effects of exSulfs on the IL-10-induced expression of DDAH-1 and abrogation of Ang II-induced DDAH-1 downregulation in SHRs VSMC with those in WKYs VSMC. Ang II or IL-10 did not affect the expression of DDAH-1 in WKYs VSMC, and both Sulf1 and Sulf2 did not affect the unresponsiveness of DDAH-1 expression to Ang II and/or IL-10 in WKYs VSMC. However, downregulation of Sulf1 inhibited IL-10-induced DDAH-1 expression and abrogation of Ang II-induced DDAH-1 downregulation in SHRs VSMC. Contrastingly, Sulf2 did not affect IL-10-induced DDAH-1 expression and abrogation of Ang II-induced DDAH-1 downregulation in SHRs VSMC (Fig. 1A). The disappearance of DDAH-1 protein production and abrogation of Ang II-induced downregulation of DDAH-1 protein production by IL-10 was also detected in Sulf1 siRNA-transfected SHRs VSMC (Fig. 1B). DDAH-1 activity levels following IL-10 and/or Ang II treatment in Sulf1 siRNA-transfected SHRs VSMC were correlated with the mRNA levels of DDAH-1 (Fig. 1C). Additionally, overexpression of Sulf1 elevated the expression of DDAH-1 following treatment with IL-

10 alone or simultaneous treatment with IL-10 and Ang II (IL-10/Ang II) in SHRs VSMC (Fig. 1D).

### 3.2. Sulf1 mediates IL-10-induced DDAH-1 expression and abrogation of Ang II-induced DDAH-1 downregulation via the AT<sub>2</sub> R pathway and AMPK activation in SHRs VSMC

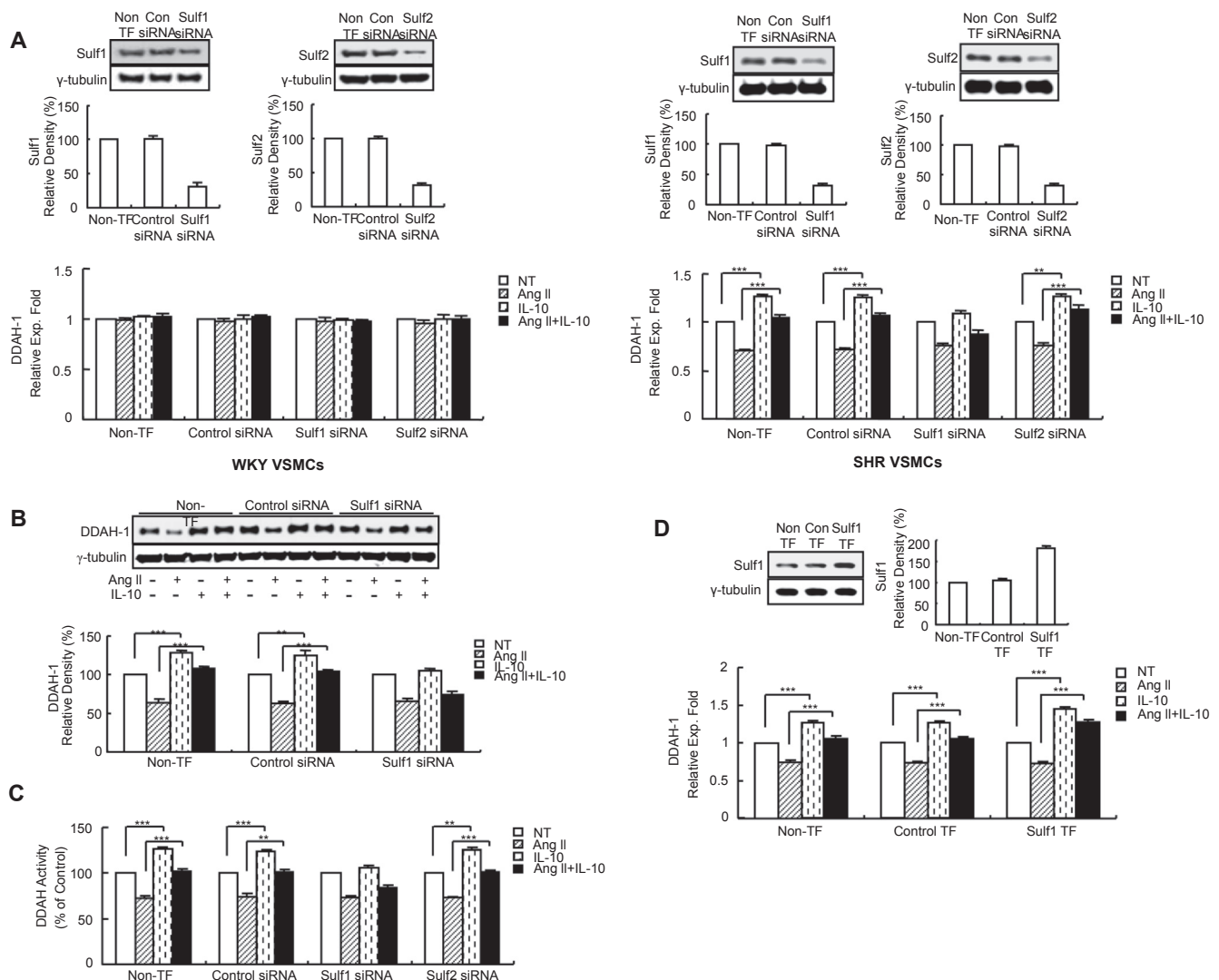
Ang II induces the expression of both AT<sub>1</sub> R and AT<sub>2</sub> R, whereas IL-10 has no effect on AT<sub>1</sub> R expression, though it induces AT<sub>2</sub> R expression very weakly in SHRs VSMC [20]. Moreover, IL-10-induced DDAH-1 expression is dependent on the AT<sub>2</sub> R pathway in SHRs VSMC [18,20]. Therefore, we examined whether the effects of Sulf1 on IL-10-induced DDAH-1 expression and abrogation of Ang II-induced DDAH-1 downregulation is linked to Ang II receptors. Further, IL-10-induced AT<sub>2</sub> R expression and the IL-10-induced synergistic increase in Ang II-induced AT<sub>2</sub> R expression were abrogated in Sulf1 siRNA-transfected SHRs VSMC; however, the IL-10-mediated inhibition of Ang II-induced AT<sub>1</sub> R expression remained unaffected (Fig. 2A). The protein levels of AT<sub>2</sub> R in Sulf1 siRNA-transfected SHRs VSMC treated with IL-10 or IL-10/Ang II were correlated with the mRNA levels shown in Fig. 2A (Fig. 2B). Additionally, the overexpression of Sulf1 elevated IL-10-induced AT<sub>2</sub> R expression and the IL-10-induced synergistic increase in Ang II-induced AT<sub>2</sub> R expression (Fig. 2C).

Cardiovascular diseases, including hypertension, are associated with the dysfunction of AMPK (phosphorylation of the AMPK activation site Thr172) [25]. IL-10 increases AMPK activation in SHRs VSMC [20], and IL-10-induced DDAH-1 expression is partially mediated by AMPK activity in SHRs VSMC [18]. Therefore, we examined whether the involvement of AMPK activity in IL-10-induced DDAH-1 expression and abrogation of Ang II-induced DDAH-1 downregulation is associated with Sulf1 expression in SHRs VSMC. IL-10-induced AMPK activation and suppression of Ang II-induced downregulation of AMPK activation were abrogated in Sulf1 siRNA-transfected SHRs VSMC (Fig. 3A). Overexpression of Sulf1 elevated the activation of AMPK by IL-10 or IL-10/Ang II in SHRs VSMC (Fig. 3B). Next, we examined whether the effects of AMPK activation on IL-10-induced DDAH-1 expression and abrogation of Ang II-induced DDAH-1 downregulation are associated with the AT<sub>2</sub> R pathway in SHRs VSMC. IL-10-induced AMPK phosphorylation and suppression of Ang II-induced downregulation of AMPK phosphorylation were sustained in SHRs VSMC treated with losartan, an AT<sub>1</sub> R inhibitor. However, PD123319, an AT<sub>2</sub> R inhibitor, abrogated IL-10-induced AMPK phosphorylation and suppressed the IL-10-mediated inhibition of Ang II-induced downregulation of AMPK phosphorylation (Fig. 4).

Next, we compared the effects of Sulf1 downregulation on IL-10-induced DDAH-1 expression and abrogation of Ang II-induced DDAH-1 downregulation with those of PD123319 or Compound C, an AMPK activation inhibitor. No significant differences were observed in the expression of DDAH-1 among the three groups: the control siRNA-transfected SHRs VSMC treated with PD123319, the control siRNA-transfected SHRs VSMC treated with Compound C, and the Sulf1 siRNA-transfected SHRs VSMC (Fig. 5). There were no statistically significant differences between the basal expression levels of DDAH-1 and those after IL-10 exposure in the three groups. Additionally, no statistically significant differences were observed in DDAH-1 expression following treatment with Ang II alone and with IL-10/Ang II in all three groups.

### 3.3. Sulf1 mediates the IL-10-induced inhibition of Ang II-induced SHRs VSMC proliferation via the AT<sub>2</sub> R pathway and AMPK activation

IL-10 does not affect VSMC proliferation but rather inhibits Ang II-induced SHRs VSMC proliferation. In addition, DDAH-1 partially mediates the inhibitory effects of IL-10 on Ang II-induced SHRs VSMC proliferation [18]. Thus, we examined whether exSulfs mediate the IL-10-induced inhibition of Ang II-induced VSMC proliferation. Sulf2 did not affect the IL-10-mediated inhibition of Ang II-induced VSMC



**Fig. 1.** IL-10-induced DDAH-1 expression and abrogation of Ang II-induced DDAH-1 downregulation is mediated by Sulf1 in SHRs VSMC. (A–C) SHRs or WKYs VSMC were plated in 6-well plates, grown to 90% confluence, and transfected with Sulf1, Sulf2, or control siRNA oligomers (50 nmol/L). Successful transfection of Sulf1, Sulf2, or control siRNA oligomers in VSMC was confirmed by immunoblotting analysis. Following transfection, (A) SHRs or WKYs VSMC were untreated or treated with Ang II (0.1  $\mu\text{mol/L}$ ) and/or IL-10 (25 ng/mL) for 2 h. After the total RNAs were isolated, real-time PCR assays were performed. (B, C) SHRs VSMC were left untreated or treated with Ang II (0.1  $\mu\text{mol/L}$ ) and/or IL-10 (25 ng/mL) for 2 h. After the cell lysates were isolated, immunoblotting analysis (B), and measurement of DDAH activity (C) were performed. The activity of DDAH was measured by converting ADMA to L-citrulline. (D) Overexpression of Sulf1 in SHRs VSMC was examined following transfection with pcDNA3.1/Myc-His(-)-MSulf1. Successful transfection of pcDNA3.1/Myc-His(-)-MSulf1 in SHRs VSMC was confirmed by immunoblotting analysis. Following transfection, SHRs VSMC were left untreated or treated with Ang II (0.1  $\mu\text{mol/L}$ ) and/or IL-10 (25 ng/mL) for 2 h. Total RNAs were isolated and the DDAH-1 mRNA levels were determined by real-time PCR. Non-TF: non-transfected VSMC, Control-TF: control (pcDNA3.1 empty vector)-transfected VSMC. The data shown are representative of three independent experiments. The bars represent the means  $\pm$  SEMs of three independent experiments. \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

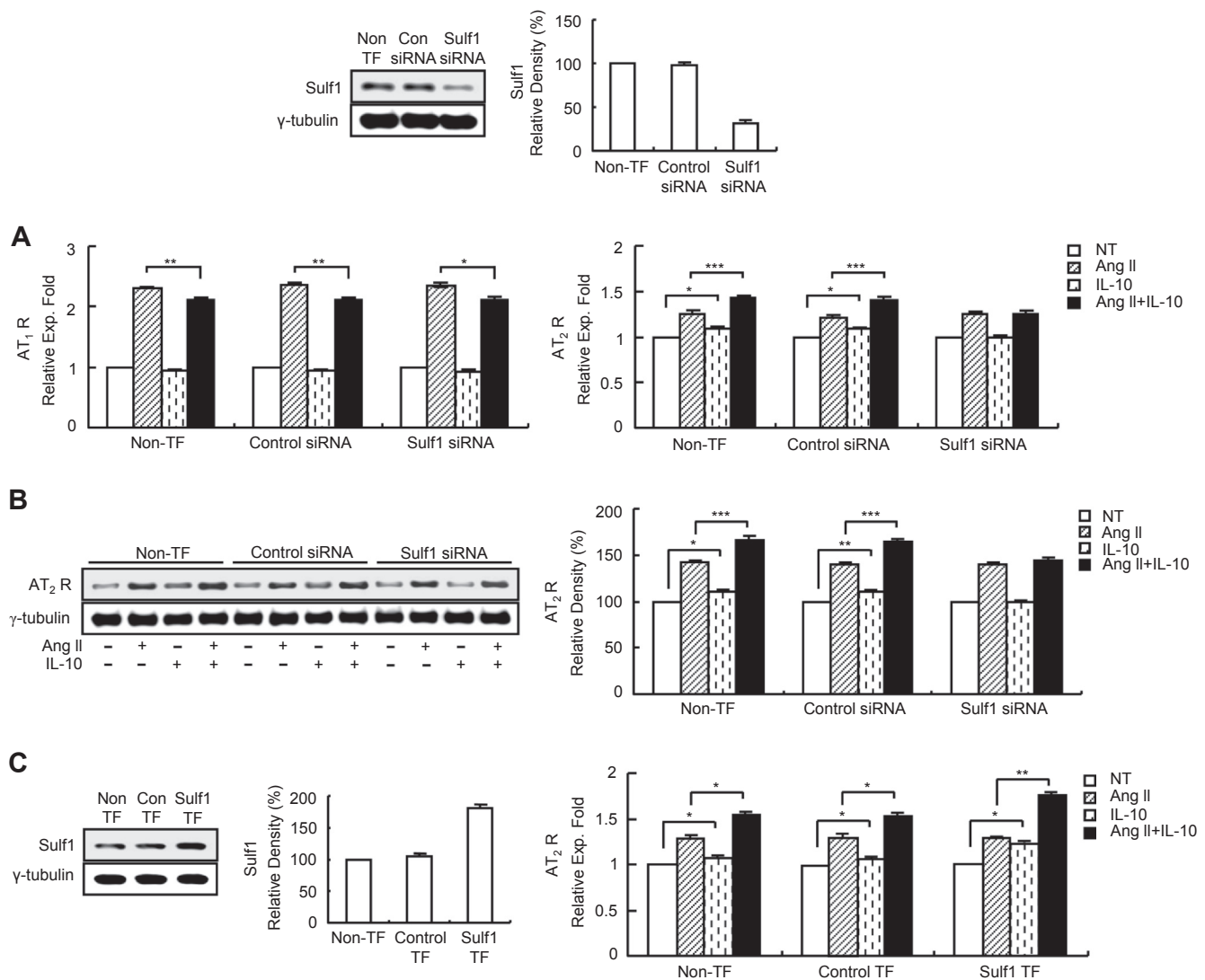
proliferation. Conversely, downregulation of Sulf1 attenuated the IL-10-induced inhibition of Ang II-induced SHRs VSMC proliferation (Fig. 6A). In addition, we observed that the IL-10-induced inhibition of Ang II-induced SHRs VSMC proliferation was mediated via the  $\text{AT}_2$  R pathway, and not the  $\text{AT}_1$  R pathway (Fig. 6B). Next, we compared the effects of Sulf1 and treatment with PD123319 or Compound C on the IL-10-induced inhibition of Ang II-induced VSMC proliferation. The control siRNA-transfected SHRs VSMC treated with PD123319 showed lower levels of Ang II (or Ang II/IL-10)-induced proliferation than the control siRNA-transfected SHRs VSMC treated with Compound C or Sulf1 siRNA-transfected SHRs VSMC. However, no statistically significant difference was observed between the proliferation levels of the control siRNA-transfected SHRs VSMC treated with Compound C and Sulf1 siRNA-transfected SHRs VSMC. Further, the proliferation levels of VSMC treated with Ang II alone did not differ significantly compared to

those of VSMC treated with IL-10/Ang II in all three groups (Fig. 6C).

#### 4. Discussion

In this study, we observed the effects of Sulf1 on IL-10-induced DDAH-1 expression, abrogation of Ang II-induced DDAH-1 downregulation, and inhibition of Ang II-induced proliferation in SHRs VSMC. Sulf1 mediated IL-10-induced DDAH-1 expression and abrogation of Ang II-induced DDAH-1 downregulation via the  $\text{AT}_2$  R pathway and AMPK activity in SHRs VSMC. Additionally, the IL-10-induced inhibition of Ang II-induced SHRs VSMC proliferation was mediated by Sulf1 via the  $\text{AT}_2$  R pathway and AMPK activity. Unlike Sulf1, Sulf2 showed no effect on IL-10-induced DDAH-1 expression and inhibition of Ang II-induced DDAH-1 downregulation and proliferation in SHRs VSMC.



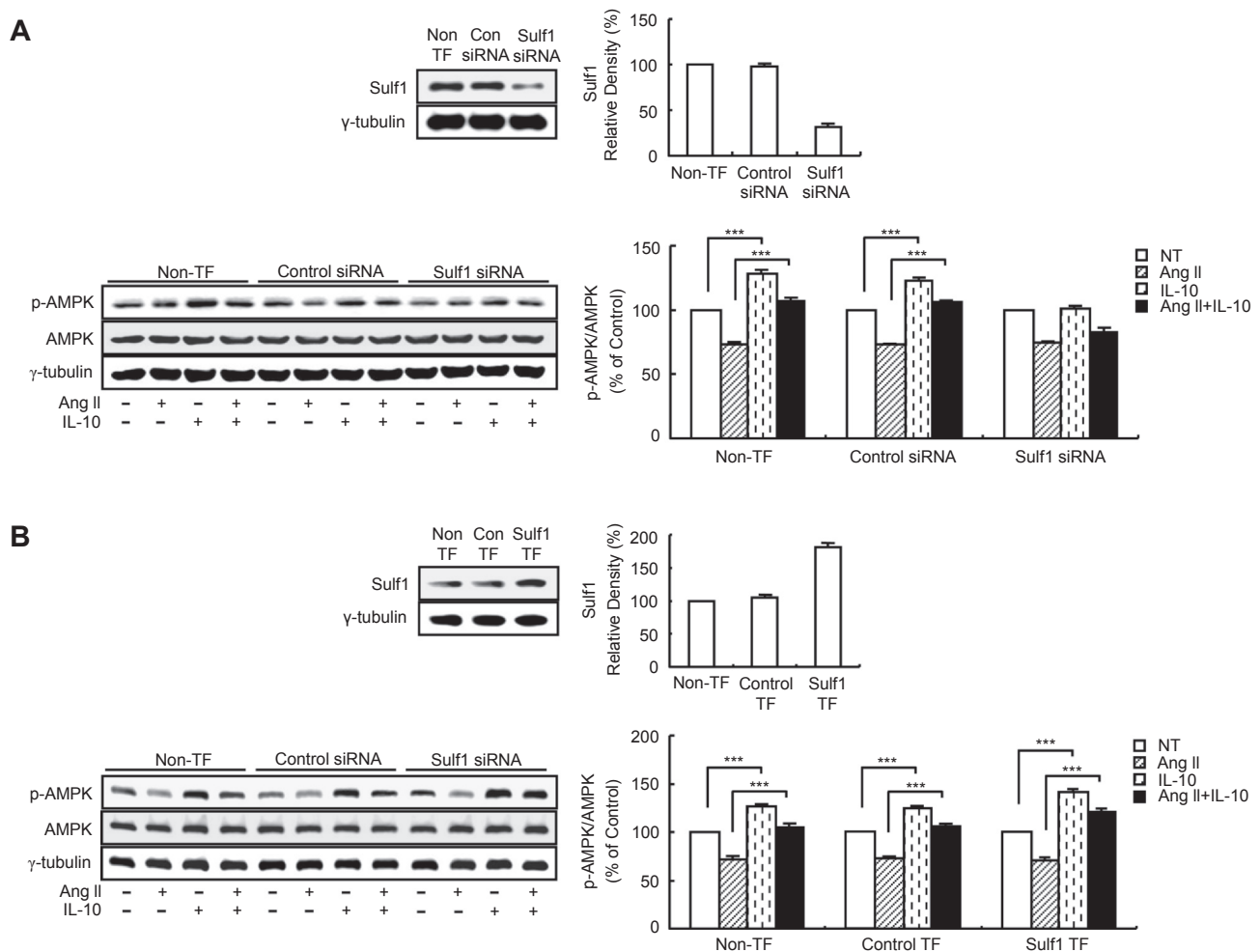


**Fig. 2.** Downregulation of Sulf1 inhibits IL-10-induced AT<sub>2</sub> R expression and IL-10-induced synergistic increase in Ang II-induced AT<sub>2</sub> R expression in SHR VSMC. (A, B) SHRs VSMC were plated in 6-well plates, grown to 90% confluence, and transfected with Sulf1 or control siRNA oligomers (50 nmol/L). Successful transfection of Sulf1 or control siRNA oligomers in VSMC was confirmed by immunoblotting analysis. Following transfection, SHRs VSMC were left untreated or treated with Ang II (0.1  $\mu$ mol/L) and/or IL-10 (25 ng/mL) for 2 h. Total RNAs and cell lysates were isolated. AT<sub>1</sub> R and AT<sub>2</sub> R mRNA levels were determined by real-time PCR (A). The protein production of AT<sub>2</sub> R was determined by immunoblotting and densitometric analyses (B). (C) Overexpression of Sulf1 in SHRs VSMC was examined following transfection with pcDNA3.1/Myc-His(-)-MSulf1. Successful transfection of pcDNA3.1/Myc-His(-)-MSulf1 in SHRs VSMC was confirmed by immunoblotting analysis. Following transfection, SHRs VSMC were left untreated or treated with Ang II (0.1  $\mu$ mol/L) and/or IL-10 (25 ng/mL) for 2 h. Total RNAs were isolated and the AT<sub>2</sub> R mRNA level was determined by real-time PCR. Non-TF: non-transfected VSMC, Control-TF: control (pcDNA3.1 empty vector)-transfected VSMC. The data shown are representative of three independent experiments. The bars represent the means  $\pm$  SEMs of three independent experiments. \* $p$  < 0.05, \*\* $p$  < 0.01, \*\*\* $p$  < 0.001.

It was observed that exSulfs did not affect the basal expression of DDAH-1 in WKYs VSMC, although it was reduced in Sulf1 siRNA-transfected SHRs VSMC (data not shown). Our previous studies demonstrated that exSulfs did not affect the basal expression of ET-1 in WKYs VSMC; however, in Sulf2 siRNA-transfected SHRs VSMC, the basal expression of ET-1 was reduced [11]. In addition, the basal expression levels of Sulf1 and Sulf2 were elevated in SHRs VSMC, compared to those in WKYs VSMC, and downregulation of Sulf1 abrogated the inhibition of Ang II-induced expression of hypertensive mediators via an AT<sub>2</sub> R blockade in SHRs VSMC [26]. These results suggested a potential role of exSulfs in the pathogenic process of SHRs VSMC.

Sulf1 mediated IL-10-induced DDAH-1 expression and abrogation of Ang II-induced DDAH-1 downregulation in SHRs VSMC. In one of our previous studies that focused on CCL5, the downregulation of Sulf1 attenuated the inhibitory effects of CCL5 on Ang II-induced 12-LO and ET-1 expression and Ang II-induced DDAH-1 downregulation in SHRs VSMC [10]. On the contrary, Sulf2 did not affect the inhibitory effects

of CCL5 on Ang II-induced 12-LO and ET-1 expression and Ang II-induced DDAH-1 downregulation in SHRs VSMC [10]. These results correspond to those of IL-10 observed in this study (data for 12-LO and ET-1 not shown). IL-10 also inhibits Ang II-induced 12-LO and ET-1 expression in SHRs VSMC [18,20]. However, unlike the effects of IL-10 or CCL5, the hypertensive effects of CXCL8 were partially dependent on Sulf2 activity, but not on Sulf1 activity, in SHRs VSMC [11]. Along with CCL2, CXCL8 plays a major role in vascular inflammation in hypertension [27–30]. CXCL8-induced ET-1 expression and CXCL8-mediated synergistic increase in Ang II-induced ET-1 expression were associated with Sulf2 activity in SHRs VSMC [11]. Sulf1 and Sulf2 exert opposite effects on tumor cell proliferation [5,6]. Consistently, Sulf1 and Sulf2 showed opposite effects on the expression of hypertensive mediators in SHRs VSMC. Although no other studies are available for comparison, these results suggest that downregulation of hypertensive mediators or upregulation of anti-hypertensive mediators are associated with Sulf1 activity, while the upregulation of hypertensive mediators is



**Fig. 3.** IL-10-induced AMPK activation and abrogation of Ang II-induced inhibition of AMPK activity is mediated by Sulf1 in SHR VSMC. (A) SHR VSMC were plated in 6-well plates, grown to 90% confluence, and transfected with Sulf1 or control siRNA oligomers (50 nmol/L). Successful transfection of Sulf1 or control siRNA oligomers in SHR VSMC was confirmed by immunoblotting analysis. Following transfection, SHR VSMC were left untreated or treated with Ang II (0.1  $\mu$ mol/L) and/or IL-10 (25 ng/mL) for 2 h. The p-AMPK expression was determined by immunoblotting and densitometric analyses. (B) Overexpression of Sulf1 in SHR VSMC was examined following transfection with pcDNA3.1/Myc-His(-)-MSulf1. Successful transfection of pcDNA3.1/Myc-His(-)-MSulf1 in SHR VSMC was confirmed by immunoblotting analysis. Following transfection, SHR VSMC were left untreated or treated with Ang II (0.1  $\mu$ mol/L) and/or IL-10 (25 ng/mL) for 2 h. The p-AMPK expression was determined by immunoblotting and densitometric analyses. Non-TF: non-transfected VSMC. Control-TF: control (pcDNA3.1 empty vector)-transfected VSMC. The data shown are representative of three independent experiments. The bars represent the means  $\pm$  SEMs of three independent experiments. \*\*\* $p$  < 0.001.

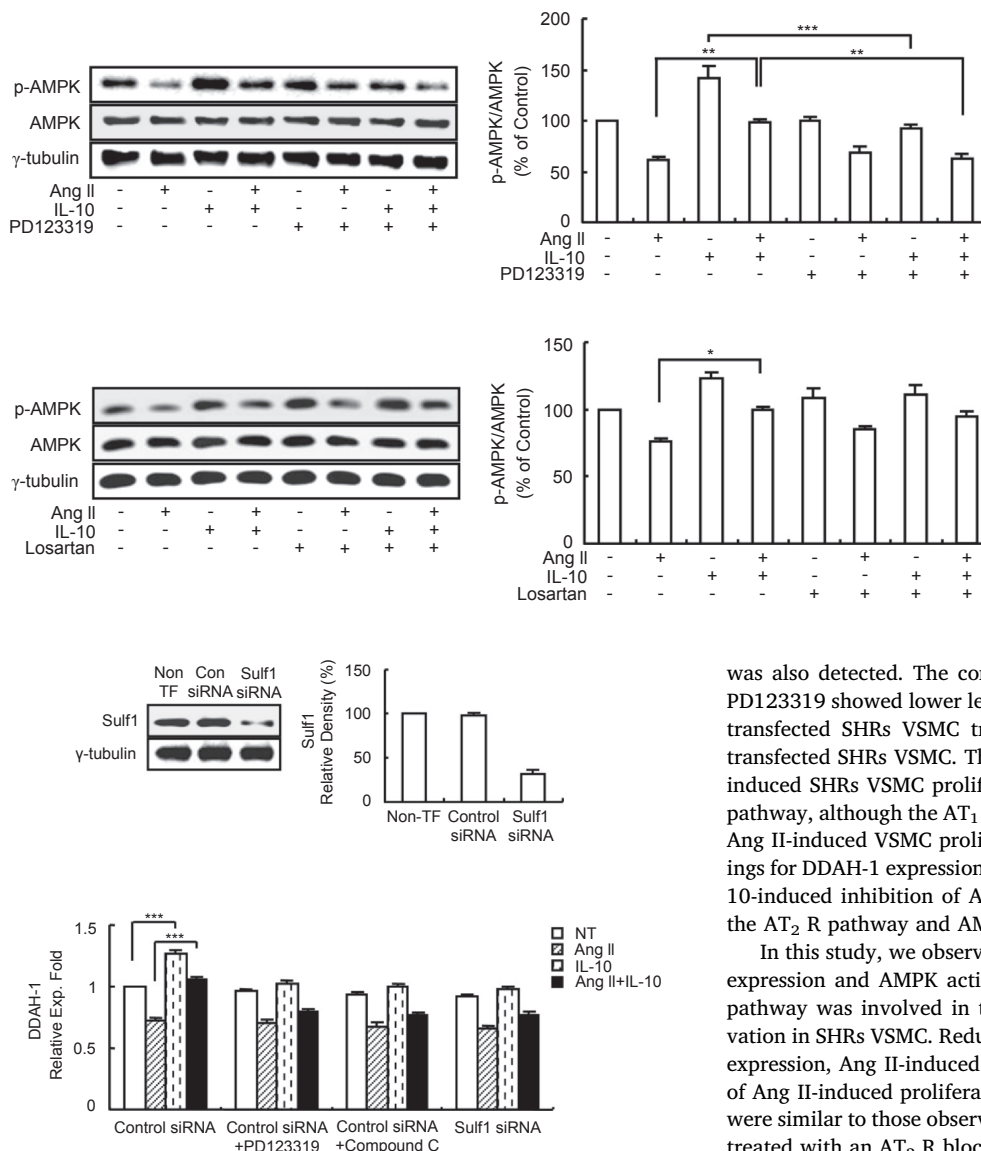
associated with Sulf2 activity in SHR VSMC. Structural differences in the C-terminal domain of exSulfs that bind to heparin sulfate may result in functional differences [3].

Sulf1 mediated IL-10-induced  $AT_2$  R expression and the IL-10-mediated synergistic increase of Ang II-induced  $AT_2$  R in SHR VSMC. Downregulation of Sulf1 abrogated the expression of IL-10-induced  $AT_2$  R and the IL-10-mediated synergistic increase of Ang II-induced  $AT_2$  R expression. IL-10-induced DDAH-1 expression and suppression of Ang II-induced downregulation of DDAH-1 by IL-10 are mediated by the  $AT_2$  R pathway [18]. Downregulatory effects of IL-10 on ET-1 and 12-LO expression in SHR VSMC have also been associated with the  $AT_2$  R pathway [18,20]. Moreover, Sulf1 mediates the CCL5-induced downregulation of Ang II-induced hypertensive mediator expression via the  $AT_2$  R pathway in SHR VSMC [10], whereas Sulf2 mediates the expression of ET-1 and the CXCL8-mediated synergistic increase in Ang II-induced ET-1 expression via the  $AT_1$  R pathway in SHR VSMC [11]. Many stimulatory actions of Ang II, such as vascular contraction, proinflammatory cytokine production, and cell proliferation, are mediated by the  $AT_1$  R pathway [31,32]. In contrast,  $AT_2$  R has opposing vascular actions compared to those of  $AT_1$  R [33]. Thus, we

suggest that Sulf1 mediates IL-10-induced DDAH-1 expression and inhibition of Ang II-induced DDAH-1 downregulation through the  $AT_2$  R pathway in SHR VSMC.

AMPK activation is important in the maintenance of cellular metabolic homeostasis [34]. AMPK activation has protective roles in hypertension, including the inhibition of VSMC proliferation, improvement of vascular endothelial function, and regulation of blood pressure [25,35]. In this study, the downregulation of Sulf1 inhibited IL-10-induced AMPK activation and abrogation of the Ang II-induced downregulation of AMPK activation in SHR VSMC. We also observed that the increase in IL-10-induced AMPK activity was mediated by  $AT_2$  R. AMPK activation mediates IL-10-induced DDAH-1 expression in SHR VSMC [18]. In addition, CCL5 increases AMPK activation and attenuates Ang II-induced inhibition of AMPK activation. The increase in AMPK activity by CCL5 is also mediated by  $AT_2$  R in SHR VSMC [21]. Taken together, we suggest that the AMPK activity-mediated downregulatory effects of IL-10 on Ang II-induced DDAH-1 inhibition is dependent on the  $AT_2$  R pathway and Sulf1 activity.

Although IL-10 alone had no effect on SHR VSMC proliferation, IL-10 inhibited Ang II-induced SHR VSMC proliferation. Moreover,



**Fig. 5.** Sulf1 mediates IL-10-induced DDAH-1 expression and abrogation of Ang II-induced DDAH-1 downregulation via the AT<sub>2</sub> R pathway and AMPK activation in SHR VSMCs. SHR VSMCs were plated in 6-well plates, grown to 90% confluence, and transfected with Sulf1 or control siRNA oligomers (50 nmol/L). Successful transfection of Sulf1 or control siRNA oligomers in SHR VSMCs was confirmed by immunoblotting analysis. Following transfection, control siRNA- and Sulf1 siRNA-transfected VSMCs were left untreated or treated with Ang II (0.1  $\mu$ mol/L) and/or IL-10 (25 ng/mL) for 2 h. Other control siRNA-transfected SHR VSMCs were untreated or treated with Ang II (0.1  $\mu$ mol/L) and/or IL-10 (25 ng/mL) in the presence of PD123319 (AT<sub>2</sub> R inhibitor, 10  $\mu$ mol/L) or Compound C (inhibitor of AMPK activity, 10  $\mu$ mol/L) for 2 h. Total RNAs were isolated and DDAH-1 mRNA levels were measured by performing real-time PCR. Non-TF: non-transfected VSMC. The bars represent the means  $\pm$  SEMs from three independent experiments. \*\*\* $p$  < 0.001.

DDAH-1 mediates the IL-10-induced inhibition of Ang II-induced SHR VSMC proliferation [18]. Changes in 6-O-sulfation levels by Sulf1 mediate the proliferation of VSMC [36]. Therefore, we examined whether Sulf1 mediates the IL-10-induced inhibition of Ang II-induced SHR VSMC proliferation. The IL-10-induced inhibition of Ang II-induced proliferation was suppressed in SHR VSMC transfected with Sulf1 siRNA. Additionally, the IL-10-induced inhibition of Ang II-induced VSMC proliferation was mediated via the AT<sub>2</sub> R pathway. Suppression of the IL-10-induced inhibition of the Ang II-induced proliferation of control siRNA-transfected VSMC treated with Compound C

**Fig. 4.** IL-10-induced AMPK activation in SHR VSMC is mediated by AT<sub>2</sub> R. SHR VSMCs were left untreated or treated with Ang II (0.1  $\mu$ mol/L) and/or IL-10 (25 ng/mL) in the presence or absence of losartan (AT<sub>1</sub> R inhibitor, 10  $\mu$ mol/L) or PD123319 (AT<sub>2</sub> R inhibitor, 10  $\mu$ mol/L) for 2 h. The p-AMPK expression was determined by immunoblotting and densitometric analyses. The data shown are representative of three independent experiments. The bars represent the means  $\pm$  SEMs of three independent experiments. \* $p$  < 0.05, \*\* $p$  < 0.01, \*\*\* $p$  < 0.001.

was also detected. The control siRNA-transfected VSMC treated with PD123319 showed lower levels of proliferation than the control siRNA-transfected SHR VSMC treated with Compound C or Sulf1 siRNA-transfected SHR VSMC. This can be attributed to the fact that Ang II-induced SHR VSMC proliferation is partially mediated via the AT<sub>2</sub> R pathway, although the AT<sub>1</sub> R pathway is the major pathway involved in Ang II-induced VSMC proliferation (Fig. 6B). Consistent with the findings for DDAH-1 expression, the results show that Sulf1 mediates the IL-10-induced inhibition of Ang II-induced SHR VSMC proliferation via the AT<sub>2</sub> R pathway and AMPK activation.

In this study, we observed that Sulf1 mediated IL-10-induced AT<sub>2</sub> R expression and AMPK activation in SHR VSMC, and that the AT<sub>2</sub> R pathway was involved in the mediation of IL-10-induced AMPK activation in SHR VSMC. Reduction in the levels of IL-10-induced DDAH-1 expression, Ang II-induced downregulation of DDAH-1, and inhibition of Ang II-induced proliferation in Sulf1 siRNA-transfected SHR VSMC were similar to those observed in control siRNA-transfected SHR VSMC treated with an AT<sub>2</sub> R blocker or an inhibitor of AMPK activity. Taken together, we suggest that IL-10-induced DDAH-1 expression, abrogation of Ang II-induced DDAH-1 downregulation, and inhibition of Ang II-induced proliferation, which is mediated via the AT<sub>2</sub> R pathway and AMPK activation, are mainly dependent on Sulf1 activity in SHR VSMC (Fig. 7).

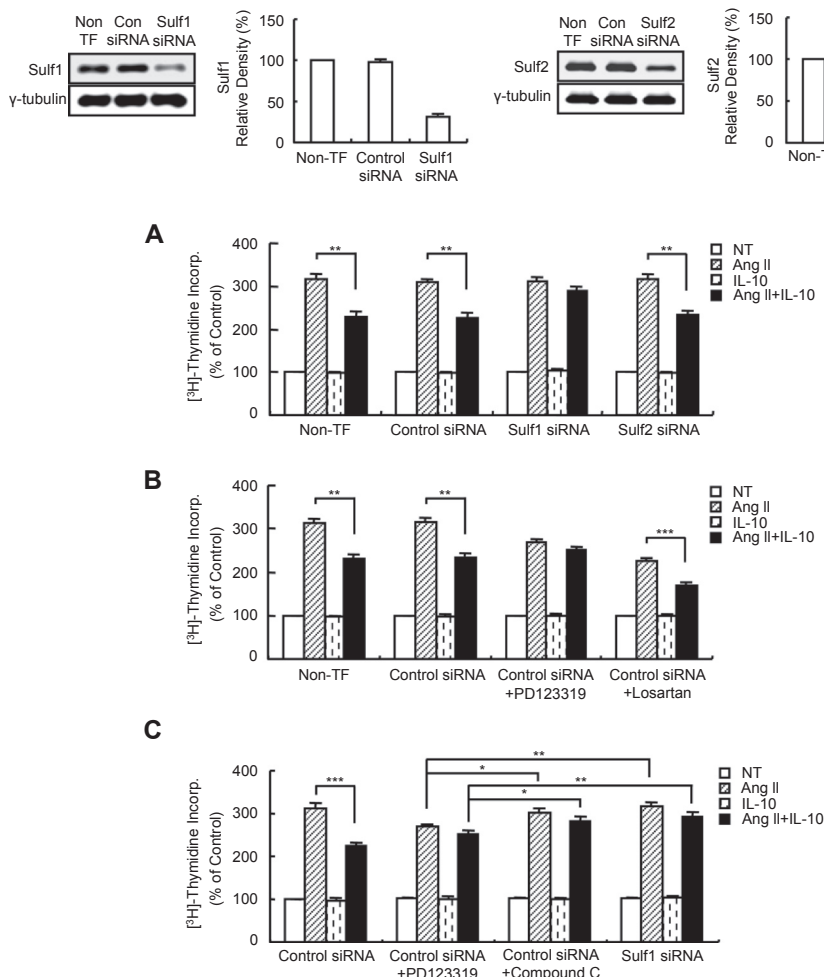
In conclusion, we provided the first evidence that Sulf1, and not Sulf2, mediates IL-10-induced DDAH-1 expression, abrogation of Ang II-induced DDAH-1 downregulation, and inhibition of Ang II-induced proliferation, which is mediated by the AT<sub>2</sub> R pathway and AMPK activity, in SHR VSMC. These findings suggest that Sulf1 plays an important role in promoting the regulatory actions of IL-10 on Ang II-induced hypertensive effects in SHR VSMC.

#### CRedit authorship contribution statement

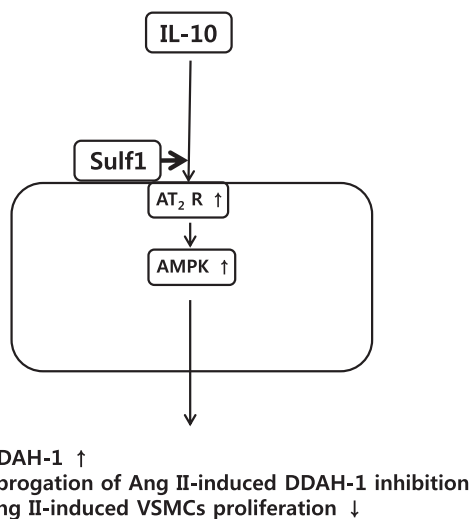
**Hye Young Kim:** Investigation, Data curation, Resources. **Hee Sun Kim:** Conceptualization, Validation, Formal analysis, Data curation, Writing - original draft, Writing - review & editing, Supervision, Funding acquisition.

#### Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to



**Fig. 6.** Sulf1 mediates the IL-10-induced inhibition of Ang II-induced SHR VSMC proliferation. (A–C) SHR VSMC were plated in 24-well plates, grown to 90% confluence, and transfected with Sulf1, Sulf2, or control siRNA oligomers (50 nmol/L). Successful transfection of Sulf1, Sulf2, or control siRNA oligomers in SHR VSMC was confirmed by immunoblotting analysis. Following transfection, (A) Non-transfected, control siRNA-, Sulf1 siRNA-, and Sulf2 siRNA-transfected SHR VSMC were left untreated or treated with Ang II (0.1  $\mu$ M/L) and/or IL-10 (25 ng/mL) for 24 h in medium containing [ $^3$ H]-thymidine (1  $\mu$ Ci/mL). (B) Non-transfected and control siRNA-transfected SHR VSMC were left untreated or treated with Ang II (0.1  $\mu$ M/L) and/or IL-10 (25 ng/mL) for 24 h in medium containing [ $^3$ H]-thymidine (1  $\mu$ Ci/mL). Other control siRNA-transfected VSMC were left untreated or treated with Ang II (0.1  $\mu$ M/L) and/or IL-10 (25 ng/mL) in the presence of losartan ( $AT_1$  R inhibitor, 10  $\mu$ M/L) or PD123319 ( $AT_2$  R inhibitor, 10  $\mu$ M/L) for 24 h in medium containing [ $^3$ H]-thymidine (1  $\mu$ Ci/mL). (C) Control siRNA- or Sulf1 siRNA-transfected SHR VSMC were then left untreated or treated with Ang II (0.1  $\mu$ M/L) and/or IL-10 (25 ng/mL) for 24 h in medium containing [ $^3$ H]-thymidine (1  $\mu$ Ci/mL). Other control siRNA-transfected SHR VSMC were left untreated or treated with Ang II (0.1  $\mu$ M/L) and/or IL-10 (25 ng/mL) in the presence of PD123319 ( $AT_2$  R inhibitor, 10  $\mu$ M/L) or Compound C (an inhibitor of AMPK activity, 10  $\mu$ M/L) for 24 h in medium containing [ $^3$ H]-thymidine (1  $\mu$ Ci/mL). The levels of [ $^3$ H]-thymidine incorporation are shown on the Y-axis. The bars represent the means  $\pm$  SEMs of four independent experiments. \* $p$  < 0.05, \*\* $p$  < 0.01, \*\*\* $p$  < 0.001.



**Fig. 7.** Flow diagram of the effect of Sulf1 on the antihypertensive actions of IL-10 in SHR VSMC.

influence the work reported in this paper.

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