Cytokine 110 (2018) 1–8

Contents lists available at ScienceDirect

Cytokine

journal homepage: www.elsevier.com/locate/cytokine

Sulfatase 1 mediates the attenuation of Ang II-induced hypertensive effects by CCL5 in vascular smooth muscle cells from spontaneously hypertensive rats

Hye Ju Cha, Hye Young Kim, Hee Sun Kim*

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

ARTICLE INFO

Keywords:
Sulfatases
CCL5
Ang II type 2 receptor
Hypertension

ABSTRACT

Extracellular sulfatases, sulfatase 1 (Sulf1) and sulfatase 2 (Sulf2), play a pivotal role in cell signaling and carcinogenesis. Chemokine CCL5 inhibits Ang II-induced hypertensive mediators via angiotensin II (Ang II) type 2 receptor (AT_2 R) pathway in vascular smooth muscle cells (VSMCs) from spontaneously hypertensive rats (SHR). In this study, we investigated the effect of Sulfs on anti-hypertensive effects of CCL5 in SHR VSMCs. CCL5 attenuated Ang II-induced inhibition of sulfatase activity in SHR VSMCs. Inhibition of Ang II-induced 12-lipoxygenase (12-LO) and endothelin-1 (ET-1) expression by CCL5 was reduced in Sulf1 small interfering RNA (siRNA)-transfected SHR VSMCs. In addition, attenuation of Ang II-induced dimethylarginine dimethylaminohydrolase-1 (DDAH-1) inhibition by CCL5 was reduced in Sulf1 siRNA-transfected SHR VSMCs. Downregulation of Sulf2 did not affect inhibitory effects of CCL5 on Ang II-induced 12-LO and ET-1 expression and Ang II-induced inhibition of DDAH-1 expression in SHR VSMCs. Downregulation of Sulf1 abrogated the expression of CCL5-induced AT_2 R messenger RNA (mRNA) and synergistic effect of CCL5 on Ang II-induced AT_2 R expression in SHR VSMCs. These findings suggest that Sulf1 is a potential up-regulatory factor in anti-hypertensive actions of CCL5 via AT_2 R pathway on Ang II-induced hypertensive effects in SHR VSMCs.

1. Introduction

Extracellular sulfatases (Sulfs) are well known as hydrolytic enzymes that control cell metabolism and signaling [1]. Sulfs regulate cell signaling by remodeling heparan sulfate proteoglycans (HSPGs) on the surface of cells. Sulf-mediated removal of 6-*O*-sulfate groups from HSPGs results in the release of bound growth factors, which initiate signaling pathways [1,2]. Therefore, the activity of Sulfs plays a pivotal role in both cell survival and proliferation. Aside from cell signaling, Sulfs modulate cellular processes such as cell development, muscle regeneration, neuromodulation, and tumor growth [1,3–5]. Sulfs are named as heparan sulfate 6-*O*-endosulfatases [2,6], and there are two types of heparan sulfate 6-*O*-endosulfatases: sulfatase 1 (Sulf1) or sulfatase FP1 and sulfatase 2 (Sulf2) or sulfatase FP2 [6]. Sulf1 and Sulf2 double knockout mice show significant developmental defects as well as reduced body weight [7,8]. Although Sulf1 and Sulf2 are structurally similar, they have reverse effects in tumor cells. Sulf1 inhibits angiogenesis and proliferation in cancer cells [9,10], whereas Sulf2 promotes angiogenesis and tumorigenic effects [11,12]. Thus, Sulfs have been suggested as therapeutic targets for cancer therapy.

Although the reduction in HSPG levels in the glomerular basement membrane has been related to hypertension [13], the role of Sulfs in hypertension development or maintenance is questionable. It has been reported that the maintenance of normal 6-*O*-sulfation levels by Sulf1 is important for the function of vascular smooth muscle cells (VSMCs) [14]. HSPGs are found in vascular walls, and overexpression or knockdown of the Sulf1 gene in normal VSMCs inhibits adhesion and increases of proliferation and apoptosis. Thus, Sulfs may play a functional role in hypertensive VSMCs.

The chemokines CC-chemokine ligand (CCL)-2 and CXC-chemokine ligand (CXCL)-8 play a major role in the development of hypertension [15–18]. Our previous studies show that CCL5 downregulates the

Abbreviations: CCL, CC-chemokine ligand; CXCL, CXC-chemokine ligand; Sulf1, sulfatase 1; Sulf2, sulfatase 2; VSMC, vascular smooth muscle cell; SHR, spontaneously hypertensive rat; Ang II, angiotensin II; AT_1 R, Ang II type 1 receptor; AT_2 R, Ang II type 2 receptor; 12-LO, 12-lipoxygenase; ET-1, endothelin-1; DDAH-1, dimethylarginine dimethylaminohydrolase-1; Sulfs, sulfatases; HSPGs, heparan sulfate proteoglycans; AMPK, AMP-activated protein kinase; ADMA, asymmetric (N^G,N^G) dimethylarginine; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; EDTA, ethylenediaminetetraacetic acid; PCR, polymerase chain reaction; cDNA, complementary DNA; siRNA, small interfering RNA; SEM, standard error of the means; eNOS, endothelial nitric oxide synthase

* 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 (H.S. Kim).

https://doi.org/10.1016/j.cyto.2017.12.027
Received 12 July 2017; Received in revised form 21 December 2017; Accepted 26 December 2017
Available online 21 April 2018
1043-4666/

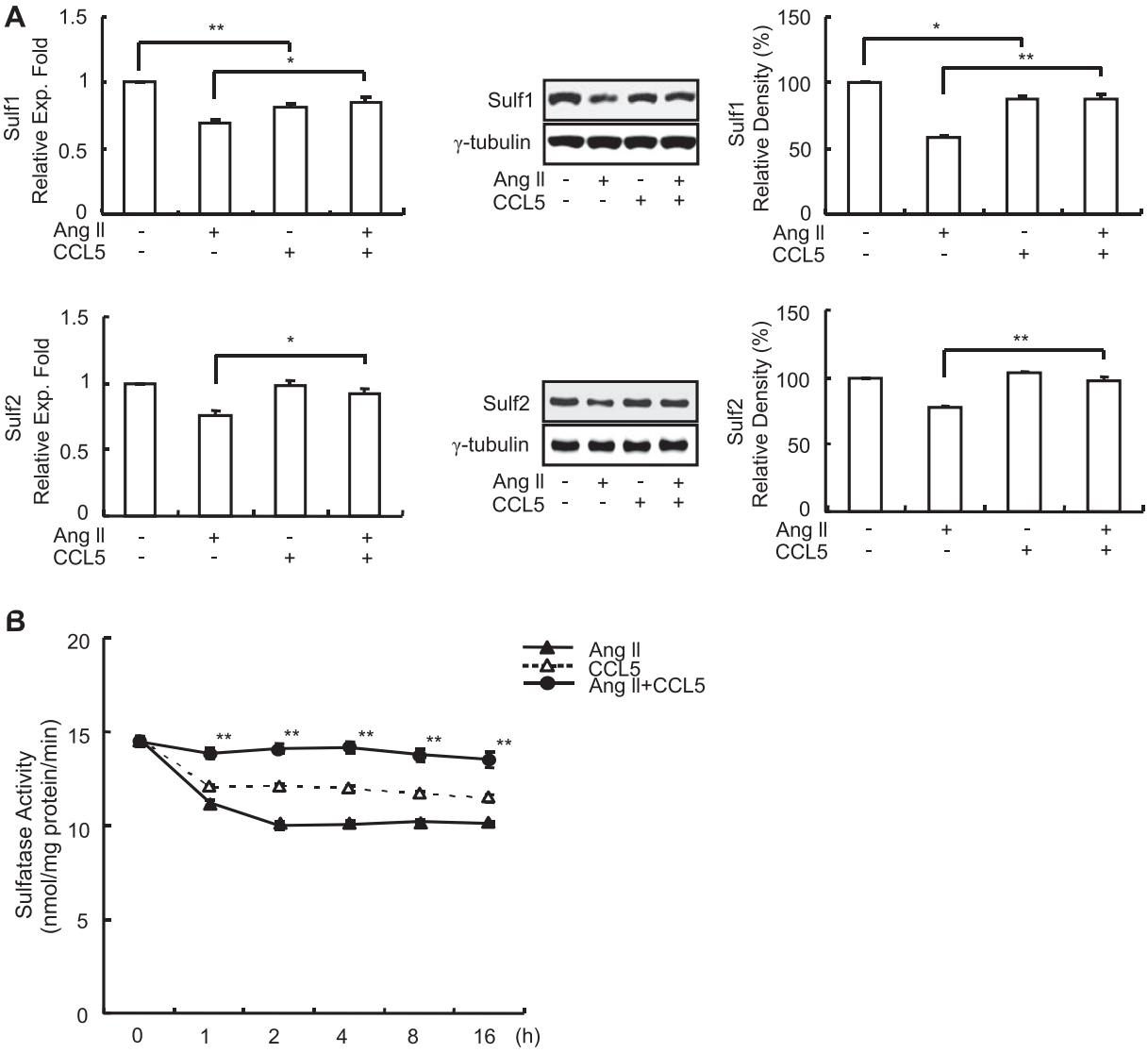

Fig. 1. CCL5 attenuates the inhibition of Ang II-induced sulfatase expressions and activities in VSMCs from SHR. (A) VSMCs were untreated or treated with Ang II (0.1 μmol/L) and/or CCL5 (100 ng/mL) for 2 h. Total RNAs and cell lysates were isolated and Sulf mRNAs and protein expressions determined by real-time PCR and immunoblotting, respectively. Data shown are representatives of three independent experiments. Bars represent the means ± SEM of three independent experiments. Statistical significance was tested by ANOVA or Kruskal-Wallis test, followed by the Bonferroni test. $^{*}p < 0.05$, $^{**}p < 0.01$. (B) VSMCs were untreated or treated with Ang II (0.1 μmol/L) and/or CCL5 (100 ng/mL) for the indicated time. Cell lysates were isolated and sulfatase activity assay was performed. Bars represent the means ± SEM of three independent experiments. $^{**}p < 0.01$ versus VSMCs treated with Ang II.

expression of angiotensin II (Ang II)-induced hypertensive mediators, 12-lipoxygenase (LO) and endothelin-1 (ET-1), in VSMCs obtained from spontaneously hypertensive rats (SHR) [19,20]. In addition, CCL5 up-regulates AMP-activated protein kinase (AMPK) activity and interleukin-10 (IL-10) and dimethylarginine dimethylaminohydrolase-1 (DDAH-1) expression [20–22]. Furthermore, CCL5 injection inhibits the elevation in blood pressure of the developing hypertension-state SHR [23]. Therefore, CCL5 is most likely to play a down-regulatory role in Ang II-induced vascular hypertension, which is contrary to the up-regulatory roles of chemokines CCL2 and CXCL8 in pathophysiologic features of hypertension [15–18].

Based on our previous studies, which demonstrated CCL5-mediated downregulation of Ang II-induced 12-LO and ET-1 production and up-regulation of DDAH-1 expression, we examined the relationship between CCL5-induced anti-hypertensive effects and Sulfs in VSMCs from SHR.

2. Materials and methods

2.1. Reagents

Total RNA extraction kit was purchased from iNtRON (Biotechnology, Seoul, Korea). Ang II was supplied by Calbiochem (San Diego, CA, USA). PD123319 was obtained from Sigma-Aldrich Co. (St. Louis, MO, USA) and CCL5, from R&D systems (Minneapolis, MN). LightCycler FastStart DNA SYBR Green I Mix was purchased from Roche (Mannheim, Germany) and Lipofectamine 2000, from Invitrogen (Carlsbad, CA, USA). Primer sequences for Ang II type 1 receptor (AT_1 R), Ang II subtype 2 receptor (AT_2 R), Sulf1, Sulf2, 12-LO, ET-1, DDAH-1, and β-actin were synthesized at Bionics (Daejeon, Korea). Sulf1, Sulf2, 12-LO, ET-1, and DDAH-1 antibodies were supplied by Santa Cruz Biotechnology (California, USA). AT_2 R antibody was purchased from Abcam (Cambridge, UK) and monoclonal anti-γ-tubulin antibody, from Sigma-Aldrich (St. Louis, MO, USA). Rat Sulf1 small interfering RNA (siRNA) Sulf2 siRNA sequences were purchased from Bioneer technology (Daejeon, Korea) and Santa Cruz Biotechnology (California,

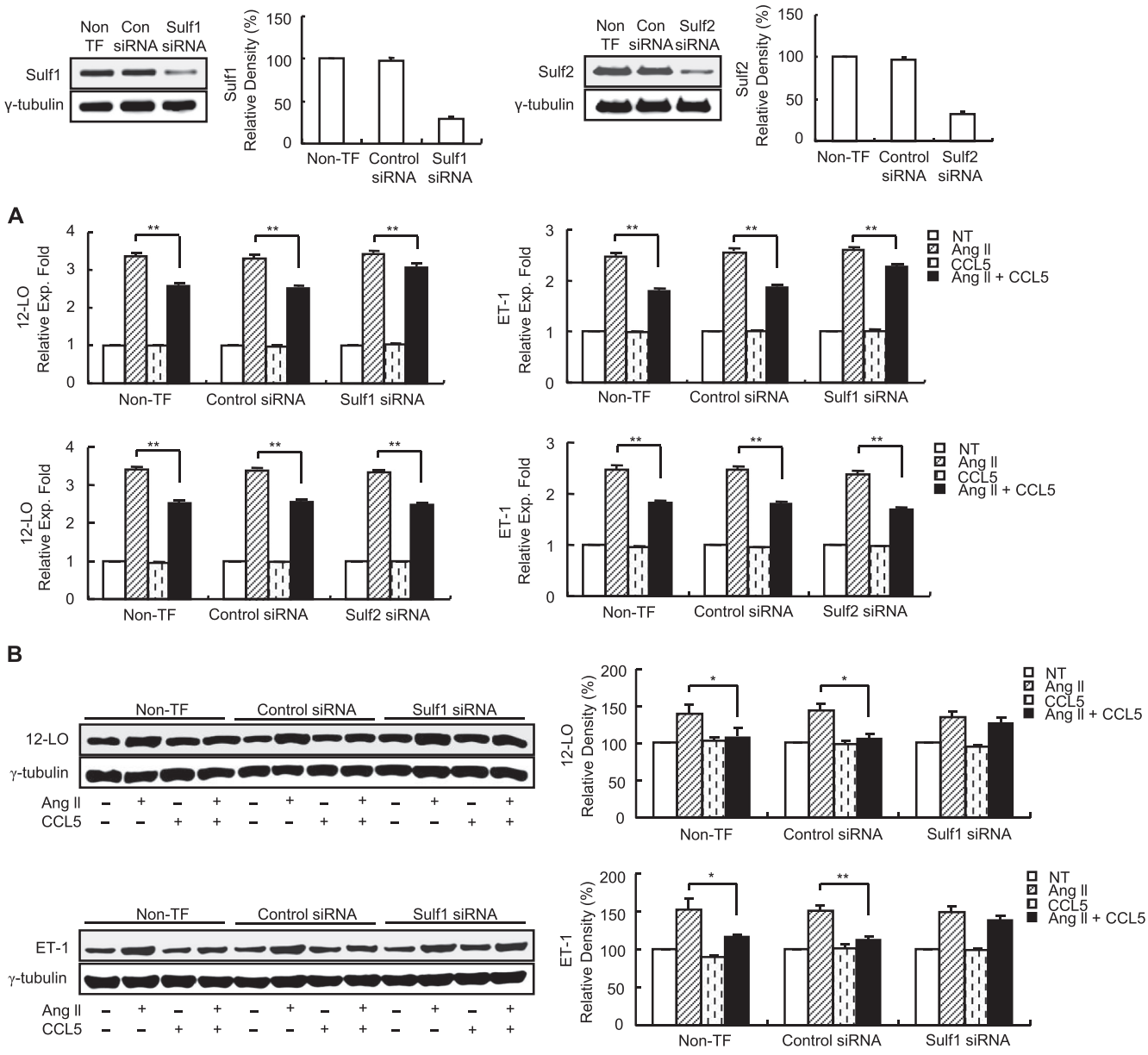

Fig. 2. The inhibitory effect of CCL5 on Ang II-induced 12-LO and ET-1 expression is mediated by Sulf1 in VSMCs from SHR. (A, B) VSMCs were plated in six-well plates, grown to 90% confluence, and transfected with Sulf1, Sulf2, or control siRNA oligomers (50 nmol/L). Successful transfection of Sulf1, Sulf2, or control siRNA oligomers in VSMCs was confirmed by immunoblotting analysis. Following transfection, VSMCs were untreated or treated with Ang II (0.1 μmol/L) and/or CCL5 (100 ng/mL) for 2 h. Total RNAs and cell lysates were isolated and mRNA expression (A) and protein production (B) of 12-LO and ET-1 were measured by real-time PCR and immunoblotting as well as densitometric analyses, respectively. Non-TF: non-transfected VSMCs. Data shown are representatives of three independent experiments. Bars represent means ± SEM of three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$.

USA), respectively. Negative control siRNA was obtained from Invitrogen (Carlsbad, CA, USA). pcDNA3.1 vector for transfection was purchased from Invitrogen Life technologies (Carlsbad, CA, USA). pcDNA3.1/Myc-His(-)-MSulf1 (Plasmid 13007) donated by professor Dr. Steven D. Rosen [6] was purchased from Addgene Inc. (Cambridge, MA, USA). All other reagents were pure-grade commercial preparations.

2.2. Animals

Specific pathogen-free male inbred SHR, all aged 22 weeks, were purchased from Japan SLC Inc. (Shizuka, Japan). All experimental animals were fed autoclaved food and received bedding in order to minimize the exposure to microbial pathogens. Rats were maintained in accordance with the Guide for the Care and Use of Experimental Animals of Yeungnam Medical Center.

2.3. Preparation of VSMCs

Vascular smooth muscle cells were isolated from thoracic aortas of 22-week-old male SHR following the explant method [24]. VSMCs were cultured in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. Cells were detached with 0.25% trypsin/ethylenediaminetetraacetic acid (EDTA) and seeded into 75 cm^2 tissue culture flasks at a density of 10^5 cells/mL. All experiments were conducted during cell passages 3–7. Prior to stimulation, 95% confluent VSMCs were serum-starved overnight in DMEM containing 0.1% FBS. Cell were incubated in a humidified incubator at 37 °C and 5% CO_2 in the presence or absence of stimuli for the indicated time.

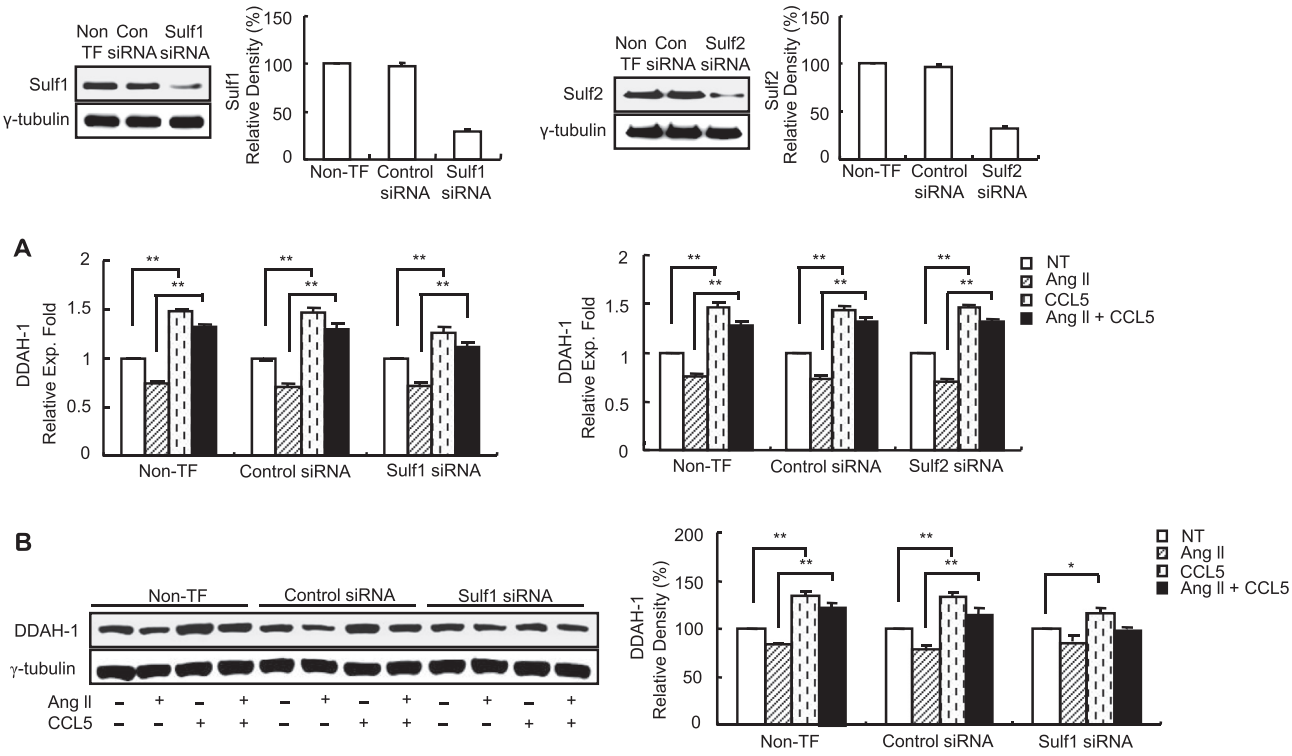

Fig. 3. Attenuation of Ang II-induced DDAH-1 inhibition by CCL5 is mediated through Sulf1 in VSMCs from SHR. (A, B) VSMCs were plated in six-well plates, grown to 90% confluence, and transfected with Sulf1, Sulf2, or control siRNA oligomers (50 nmol/L). Successful transfection of Sulf1, Sulf2, or control siRNA oligomers in VSMCs was confirmed by immunoblotting analysis. VSMCs were untreated or treated with Ang II (0.1 μmol/L) and/or CCL5 (100 ng/mL) for 2 h. Total RNAs and cell lysates were isolated and mRNA expression (A) and protein production (B) of DDAH-1 were measured by real-time PCR and immunoblotting as well as densitometric analyses, respectively. Non-TF: non-transfected VSMCs. Data shown are representatives of three independent experiments. Bars represent means ± SEM of three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$.

2.4. Preparation of total RNA and real-time polymerase chain reaction (PCR)

Total RNA was isolated using an easy-BLUE total RNA extraction kit (iNtRON Biotechnology, Seoul, Korea) according to the manufacturer's instructions. The quantity of total RNA obtained was determined by measuring the optical density (OD) at 260 and 280 nm.

One microgram of total RNA per sample was reverse-transcribed using a Maxime RT premix kit (iNtRON Biotechnology, Daejeon, Korea) according to the manufacturer's instructions. Complementary DNA (cDNA) synthesis was performed at 45 °C for 60 min, followed by RT inactivation at 95 °C for 5 min. AT_1 R, AT_2 R, Sulf1, Sulf2, 12-LO, ET-1, and DDAH-1 was amplified by real-time PCR using LightCycler (Roche, Germany). Total RNA was reverse-transcribed to cDNA from 1 μg of total RNA and then subjected to real-time PCR. The total PCR volume was 20 μL, and each PCR reaction consisted of LightCycler FastStart DNA SYBR Green I mix (Roche, Germany), primer, and 2 μL of cDNA. Prior to PCR amplification, the mixture was incubated at 95 °C for 10 min. The amplification step consisted of 45 cycles of denaturation (10 s at 95 °C), annealing (5 s at the primer-appropriate temperature), and extension (10 s at 72 °C) with fluorescence detection at 72 °C after each cycle. After the final cycle, melting point analyses of all samples were performed over a temperature range of 65–95 °C with continuous fluorescence detection. The primers used for PCR were as follows: AT_1 R (445 bp) sense, 5′-cacctatgtaagatcgcttc-3′ and antisense, 5′-gcacaatcgccataattatcc-3′; AT_2 R (65 bp) sense, 5′-ccgtgaccaagtcttgaagatg-3′ and antisense, 5′-agggaagccagcaaatgatg-3′; Sulf1 (136 bp) sense, 5′-aaacagtgcaacccaagacc-3′ and antisense, 5′-ttgccagttggtgtctgaag-3′; Sulf2 (73 bp) sense, 5′-ggcttagagacggaggaag-3′ and antisense, 5′-ggtctcttcatttctggcca-3′; 12-LO (312 bp) sense, 5′-tggggcaactggaagg-3′ and antisense, 5′-agagcgcttcagcaccat-3′; ET-1 (370 bp) sense, 5′-ctcctccttgatggacaagg-3′ and antisense, 5′-cttgatgctgttgctcatgg-3′; DDAH-1 (181 bp) sense, 5′-cgcaatagggtccagtgaat-3′ and antisense, 5′-ttgcgctttctgggtactct-3′; and β-actin (101 bp) sense, 5′-tactgccctggctcctagca-3′ and antisense, 5′-tggacagtgaggccaggatag-3′. The mRNA level of each sample was normalized to the mRNA level of β-actin, a housekeeping gene.

2.5. Measurement of sulfatase activity

Activity levels of Sulfs in cell lysate (30 μg) were measured using a Sulf activity assay kit obtained from Abcam (Cambridge, UK). All procedures were performed in accordance with the manufacturer's instructions.

2.6. Western blotting

Western blots were performed on cytoplasmic protein extracts of VSMCs from SHR using polyclonal antibody against AT_2 R (dilution 1/800), Sulf1 (dilution 1/200), Sulf2 (dilution 1/200), 12-LO (dilution 1/400), ET-1 (dilution 1/400), DDAH-1 (dilution 1/2000), and monoclonal antibody against γ-tubulin (dilution 1/2000), as described by Kim et al. [24].

2.7. Small interfering RNA (siRNA)

Vascular smooth muscle cells were plated in six-well plates and grown to 90% confluence. VSMCs were transfected with Sulf1 and Sulf2 siRNA oligomers (50 nmol/L) using Lipofectamine 2000, as per the manufacturer's instructions. After 24 h of incubation, VSMCs were placed in growth medium for 24 h before the experiments. Following incubation, cells were cultured in the presence or absence of stimuli for 2 h. Sense and antisense oligonucleotides used in these experiments were as follows: Sulf1 siRNA sense, 5′-gugacuucaggaaugagau-3′;

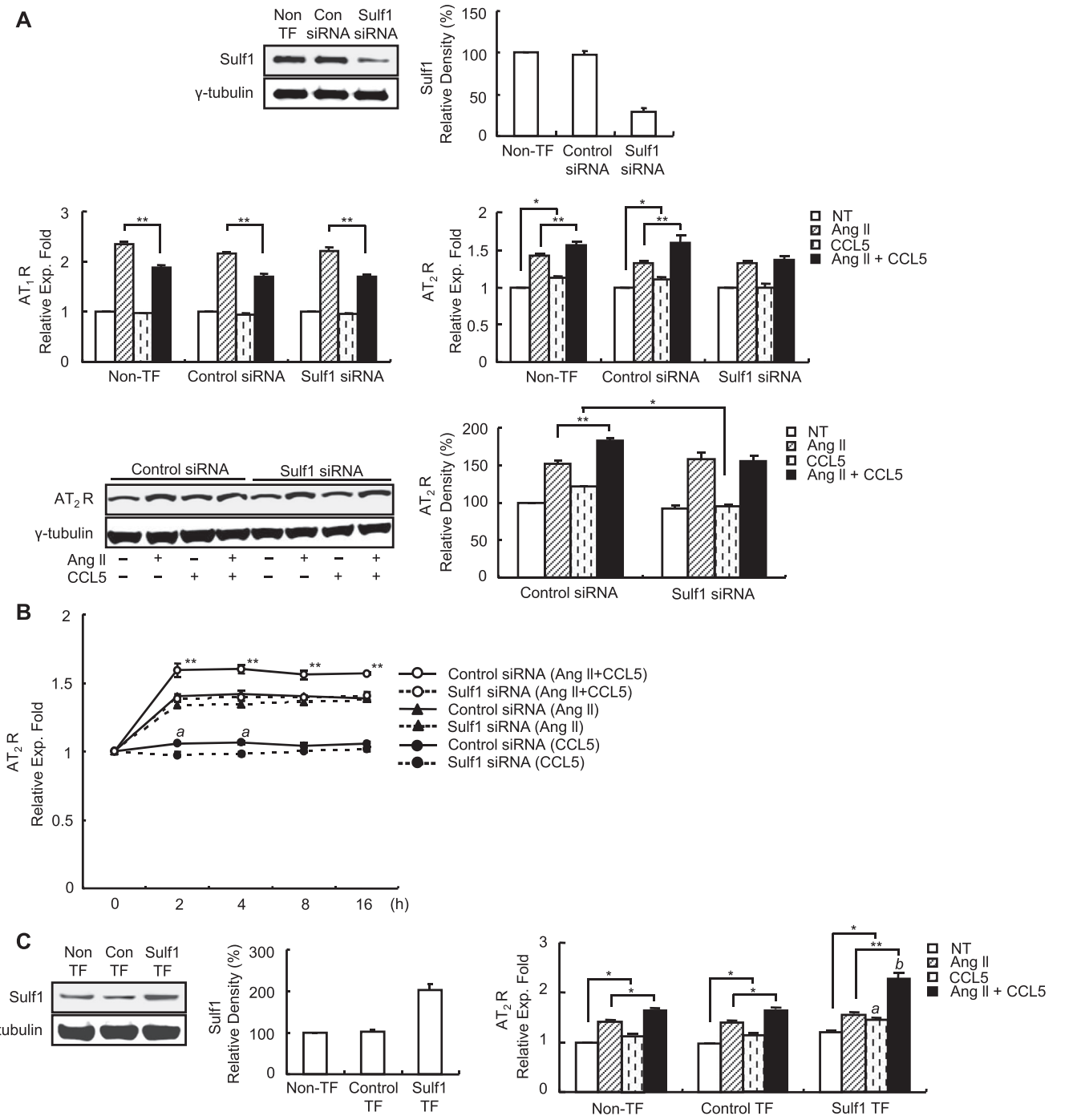

Fig. 4. Downregulation of Sulf1 results in the inhibition of CCL5-induced AT_2 R expression and synergistic effect of CCL5 on Ang II-induced AT_2 R expression in VSMCs from SHR. (A) VSMCs were plated in six-well plates, grown to 90% confluence, and transfected with Sulf1 or control siRNA oligomers (50 nmol/L). Successful transfection of Sulf1 or control siRNA oligomers in VSMCs was confirmed by immunoblotting analysis. Following transfection, VSMCs were untreated or treated with Ang II (0.1 μmol/L) and/or CCL5 (100 ng/mL) for 2 h. Total RNAs and cell lysates were isolated and AT_1 R and AT_2 R mRNA levels were determined by real-time PCR. The protein production of AT_2 R was determined by immunoblotting and densitometric analyses. Non-TF: non-transfected VSMCs. Data shown are representatives of three independent experiments. Bars represent means ± SEM of three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$. (B) VSMCs were transfected with Sulf1 or control siRNA oligomers (50 nmol/L) and untreated or treated with Ang II (0.1 μmol/L) and/or CCL5 (100 ng/mL) for the indicated time. Total RNAs were isolated and AT_2 R expression was determined by real-time PCR. Bars represent means ± SEM of three independent experiments. $^{**}p < 0.01$ versus Sulf1 siRNA-transfected VSMCs treated with Ang II/CCL5. $^{a}p < 0.05$ versus Sulf1 siRNA-transfected VSMCs treated with CCL5. (C) Overexpression of Sulf1 in VSMCs was performed with pcDNA3.1/Myc-His(-)-MSulf1. Successful transfection of pcDNA3.1/Myc-His(-)-MSulf1 in VSMCs was confirmed by immunoblotting analysis. Following transfection, VSMCs were untreated or treated with Ang II (0.1 μmol/L) and/or CCL5 (100 ng/mL) for 2 h. Total RNAs were isolated and AT_2 R mRNA level was determined by real-time PCR. Non-TF: non-transfected VSMCs, Control-TF: control (pcDNA3.1 empty vector)-transfected VSMCs. Bars represent means ± SEM of three independent experiments. $^{a}p < 0.01$ versus control-transfected VSMCs treated with CCL5. $^{b}p < 0.01$ versus control-transfected VSMCs treated with Ang II/CCL5. $^{*}p < 0.05$, $^{**}p < 0.01$.

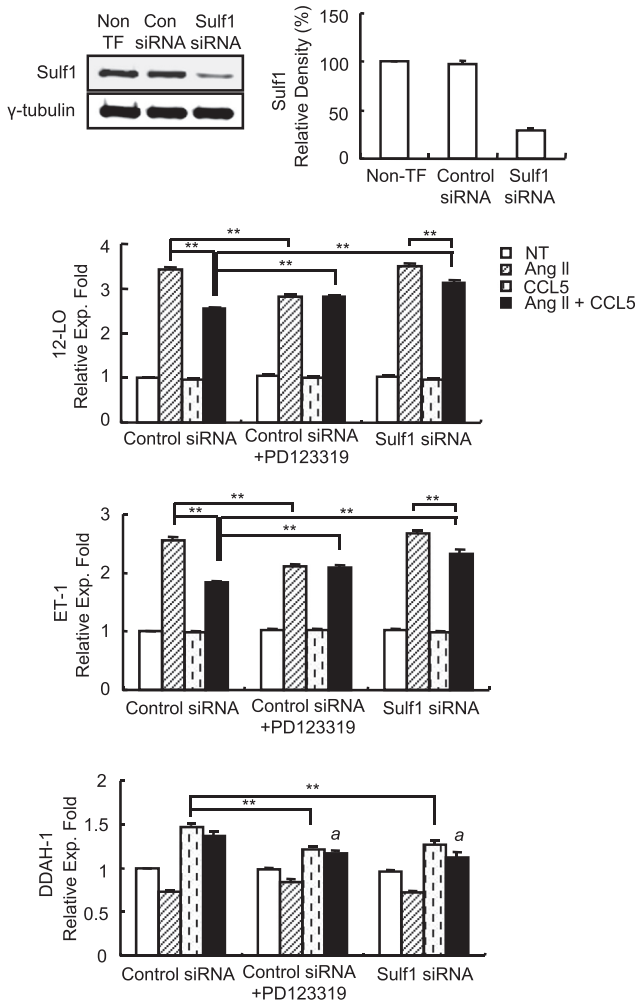

Fig. 5. Down-regulation of Sulf1 fails to completely abrogate the inhibition of Ang II-induced 12-LO and ET-1 expression by CCL5 via AT_2 R pathway. VSMCs from SHR were plated in six-well plates, grown to 90% confluence, and transfected with Sulf1 or control siRNA oligomers (50 nmol/L). Successful transfection of Sulf1 or control siRNA oligomers in VSMCs was confirmed by immunoblotting analysis. Following transfection, control siRNA- and Sulf1 siRNA-transfected VSMCs were untreated or treated with Ang II (0.1 μmol/L) and/or CCL5 (100 ng/mL) for 2 h. Another control siRNA-transfected VSMCs were untreated or treated with Ang II (0.1 μmol/L) and/or CCL5 (100 ng/mL) in the presence of PD123319 (AT_2 R inhibitor, 10 μmol/L) for 2 h. Total RNAs were isolated and 12-LO, ET-1, and DDAH-1 mRNA levels were measured by real-time PCR. Non-TF: non-transfected VSMCs. Bars represent means ± SEM from three independent experiments. $^{a}p < 0.01$ versus control siRNA-transfected VSMCs treated with Ang II/CCL5. $^{**}p < 0.01$.

antisense, 5′-aucucauuccugaagucac-3′ and Sulf2 siRNA sense, 5′-cacaucacaccgaguuaca-3′; antisense, 5′-uguaacucggugugaugug-3′.

2.8. Overexpression of Sulf1 gene

The recombinant plasmid expressing full-length Sulf1 cDNA, pcDNA3.1/Myc-His(-)-MSulf1, was amplified in liquid Luria Broth (LB) media with 100 μg/mL of ampicillin. The amplified plasmids were purified using a QIAGEN Plasmid Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. VSMCs were transfected with either Sulf1-expressing plasmid or pcDNA3.1 empty vector using Lipofectamine 2000, as per the manufacturer's instructions.

2.9. Statistical analysis

Results were expressed as means ± standard error of the means (SEM) of at least three or four independent experiments. Data were analyzed by one-way analysis of variance (ANOVA) or Kruskal-Wallis test (for comparison of four groups), followed by the Bonferroni's *post hoc* test. A value of $P < 0.05$ was considered as statistically significant. Statistical analysis was performed using IBM SPSS version 23.0 (IBM Co., Armonk, NY, USA).

3. Results

3.1. CCL5 reduces Ang II-induced inhibition of Sulf expressions and activities in VSMCs from SHR

It is known that Ang II reduces Sulf expressions and activities in VSMCs from SHR [unpublished data]. Therefore, we first examined the effect of CCL5 on the expressions of Sulfs and Ang II-induced inhibition of Sulfs in VSMCs. CCL5 inhibited Sulf1 mRNA expression, but showed no effect on Sulf2 mRNA expression. However, attenuation of Ang II-induced inhibition of Sulf1 and Sulf2 mRNA expressions was detected in VSMCs subjected to simultaneous treatment with Ang II and CCL5 (Ang II/CCL5) (Fig. 1A). The protein levels of Sulfs following CCL5 or Ang II/CCL5 treatment correlated with the mRNA levels (Fig. 1A). Next, we observed Sulf activity following CCL5 or Ang II/CCL5 treatment in VSMCs from SHR. Although CCL5 itself inhibited Sulf activity, it attenuated Ang II-induced inhibition of Sulf activity in VSMCs (Fig. 1B). The time courses of Sulf activity in response to Ang II, CCL5, or Ang II/CCL5 treatment were determined over a 16 h time period. Significant attenuation in Ang II-induced inhibition of Sulf activity by CCL5 was detected 1 h after Ang II/CCL5 treatment, which sustained for up to 16 h. Ang II-induced inhibition of Sulf activity remained almost constant from 2 to 16 h in VSMCs (Fig. 1B).

3.2. The inhibitory effect of CCL5 on Ang II-induced 12-LO and ET-1 expression is mediated via Sulf1 in VSMCs from SHR

Development of hypertension is associated with an increase in 12-LO and ET-1 activity [25,26]. CCL5 exerts an inhibitory effect on Ang II-induced 12-LO and ET-1 expression in VSMCs from SHR [19,20]. Therefore, we examined the effect of Sulfs on this inhibitory effect of CCL5 in VSMCs. Downregulation of Sulf1 failed to affect Ang II-induced 12-LO and ET-1 expression, but attenuated the inhibitory effect of CCL5 on Ang II-induced 12-LO and ET-1 expression. However, downregulation of Sulf2 failed to affect both the Ang II-induced 12-LO and ET-1 expression and the inhibitory effect of CCL5 on Ang II-induced 12-LO and ET-1 expression (Fig. 2A). The rate of inhibition of Ang II-induced 12-LO expression by CCL5 in VSMCs transfected with Sulf1 siRNA was reduced to 9.9 ± 1.7% as compared to 23.2 ± 1.4% in those transfected with the control siRNA. The rate of inhibition of Ang II-induced ET-1 expression by CCL5 in VSMCs transfected with Sulf1 siRNA was reduced to 12.8 ± 1.6% as compared to 27.0 ± 1.0% in those transfected with the control siRNA. The inhibition of Ang II-induced 12-LO and ET-1 protein production by CCL5 was also reduced in VSMCs transfected with Sulf1 siRNA (Fig. 2B).

3.3. The attenuation of Ang II-induced inhibition of DDAH-1 expression by CCL5 is mediated via Sulf1 in VSMCs from SHR

The protein DDAH-1 is a key enzyme that metabolizes ADMA, an inhibitor of endothelial nitric oxide synthase (eNOS) [27,28]. CCL5 increases DDAH-1 expression and attenuates the inhibitory effect of Ang II on DDAH-1 expression in VSMCs from SHR [21]. Therefore, we examined the effect of Sulfs on the attenuation of Ang II-induced inhibition of DDAH-1 by CCL5 in VSMCs. Ang II-induced inhibition of DDAH-1 was unaffected by both Sulf1 and Sulf2. In addition, down-

regulation of Sulf2 failed to affect the CCL5-meidated attenuation of Ang II-induced inhibition of DDAH-1 in VSMCs (Fig. 3A). However, downregulation of Sulf1 affected the expression of CCL5-induced DDAH-1 and attenuation of Ang II-induced DDAH-1 inhibition by CCL5. Both the expression of DDAH-1 and attenuation of Ang II-induced DDAH-1 inhibition by CCL5 were reduced in VSMCs transfected with Sulf1 siRNA (Fig. 3A). The rate of elevation of CCL5-induced DDAH-1 expression in Sulf1 siRNA-transfected VSMCs was reduced to 26.3 ± 5.5% as compared to 47.1 ± 3.8% in control siRNA-transfected VSMCs, and the rate of elevation of Ang II/CCL5-induced DDAH-1 expression in Sulf1 siRNA-transfected VSMCs was reduced to 11.7 ± 4.4% as compared to 29.3 ± 6.0% in control siRNA-transfected VSMCs. The protein levels of DDAH-1 and attenuation of Ang II-induced inhibition of DDAH-1 by CCL5 in VSMCs transfected with Sulf1 siRNA correlated with the mRNA levels noted in VSMCs transfected with Sulf1 siRNA (Fig. 3B).

3.4. Sulf1 mediates CCL5-induced AT_2 R expression and synergistic effect of CCL5 on Ang II-induced AT_2 R expression in VSMCs from SHR

The effects of CCL5 on the Ang II-induced expression of hypertensive mediators and inhibition of DDAH-1 are mediated via AT_2 R pathway in VSMCs from SHR [19–21]. Therefore, we examined whether the effects of Sulf1 on the anti-hypertensive action of CCL5 in VSMCs are related to Ang II receptors. In this study, Sulf1 downregulation had no effect on AT_1 R expression in VSMCs treated with CCL5 or Ang II/CCL5. In addition, Sulf1 downregulation failed to affect the expression of Ang II-induced AT_1 R and AT_2 R mRNA. However, Sulf1 downregulation abrogated CCL5-induced AT_2 R expression as well as the synergistic effect of CCL5 on Ang II-induced AT_2 R expression (Fig. 4A). The protein levels of CCL5- or Ang II/CCL5-induced AT_2 R correlated with the mRNA levels noted in the Sulf1 siRNA-transfected VSMCs (Fig. 4A). The time course of CCL5- or Ang II/CCL5-induced AT_2 R expression in Sulf1 siRNA-transfected VSMCs was determined over a 16 h time period. The synergistic effect of CCL5 on Ang II-induced AT_2 R expression was highest after 2 h Ang II/CCL5 treatment in control siRNA-transfected VSMCs and remained almost constant from 2 to 16 h. In comparison to the VSMCs transfected with the control siRNA, those transfected with the Sulf1 siRNA showed reduced expression of AT_2 R, which was almost constant from 2 to 16 h after Ang II/CCL5 treatment. The increase in AT_2 R expression in VSMCs by CCL5 remained almost constant from 2 to 4 h after CCL5 treatment (Fig. 4B). Next, to confirm the effect of Sulf1 on CCL5- or Ang II/CCL5-induced AT_2 R expression, we examined AT_2 R expression in VSMCs transfected with pcDNA3.1/Myc-His(-)-Sulf1. Although the increase in CCL5-induced AT_2 R expression was insignificant in Sulf1-overexpressing VSMCs, their AT_2 R expression was higher than that reported for VSMCs transfected with pcDNA3.1 empty vector. Overexpression of Sulf1 elevated the synergistic effect of CCL5 on Ang II-induced AT_2 R mRNA expression (Fig. 4C). The rate of increase of Ang II/CCL5-induced AT_2 R expression in Sulf1-overexpressing VSMCs was elevated to 36.2 ± 3.7% as compared to 16.1 ± 2.3% in VSMCs transfected with the pcDNA3.1 empty vector.

Next, we compared the effect of Sulf1 downregulation on CCL5-induced anti-hypertensive effects with that of AT_2 R blockade. We failed to observe any inhibitory effect of CCL5 on Ang II-induced 12-LO and ET-1 expression upon treatment of control siRNA-transfected VSMCs with AT_2 R blocker. However, Sulf1 downregulation failed to completely abrogate the inhibitory effect of CCL5 on Ang II-induced 12-LO and ET-1 expression. Ang II-induced 12-LO and ET-1 expression was also reduced by AT_2 R blocker. Therefore, the decrease in the inhibitory effect of CCL5 on Ang II-induced 12-LO and ET-1 expression following treatment of the control siRNA-transfected VSMCs with AT_2 R blocker was not significant as compared with the inhibitory effect of CCL5 on Ang II-induced 12-LO and ET-1 expression in control siRNA-transfected VSMCs without AT_2 R blocker treatment ($p < 0.01$; Fig. 5). Inhibition of DDAH-1 by Ang II was unaffected by AT_2 R blockade, but the expression of DDAH-1 by CCL5 or Ang II/CCL5 was reduced by AT_2 R blockade. In addition, no difference was observed in the expression level of DDAH-1 between control siRNA-transfected VSMCs treated with CCL5 (or Ang II/CCL5) plus PD123319 and Sulf1 siRNA-transfected VSMCs treated with CCL5 (or Ang II/CCL5) (Fig. 5).

4. Discussion

In the present study, we demonstrated that the downregulation of Ang II-induced 12-LO and ET-1 expression and attenuation of Ang II-induced DDAH-1 inhibition via AT_2 R mediated by CCL5 is associated with Sulf1 in VSMCs from SHR. The relationship between anti-hypertensive effects of CCL5 and Sulfs is yet unexplored. Sulfs have been studied mainly as modulators of cell signaling or regulators of carcinogenesis. However, hypertensive VSMCs may exhibit abnormal Sulf activity. Expressions and activities of Sulfs are elevated in VSMCs from SHR as compared with the VSMCs of normotensive Wistar-Kyoto rats (date not shown). In this study, CCL5 treatment resulted in a small decrease in Sulf activity, but abrogated Ang II-induced inhibition of Sulf activity in VSMCs from SHR. Therefore, we hypothesized that CCL5-mediated downregulation of Ang II-induced hypertensive mediators may be related to Sulf activity.

Both 12-LO and ET-1 are associated with the development of hypertension, while Ang II is a potent inducer of 12-LO and ET-1 expression in VSMCs [19,25,26,29]. DDAH-1 along with DDAH-2 is a major regulator of plasma ADMA [27,30]. Reduction in DDAH activity results in an increase in ADMA level, which leads to the contraction of blood vessels [28]. Ang II inhibits DDAH activity in VSMCs from SHR [21]. CCL5 has no direct effect on the expression of 12-LO and ET-1, but increases DDAH-1 expression directly in VSMCs [20,21]. In this study, both Sulf1 and Sulf2 failed to directly affect Ang II-induced 12-LO and ET-1 expression and Ang II-induced DDAH-1 inhibition in VSMCs from SHR. However, downregulation of Sulf1 resulted in the decrease in the inhibition of Ang II-induced 12-LO and ET-1 expression and attenuation of Ang II-induced DDAH-1 inhibition by CCL5 in VSMCs. Thus, the effect of CCL5 on the downregulation of Ang II-induced hypertensive mediators was mediated via Sulf1 in VSMCs. On the other hand, Sulf2 failed to influence the inhibitory effect of CCL5 on the expression of Ang II-induced hypertensive mediators and Ang II-induced DDAH-1 inhibition in VSMCs from SHR. Sulf1 and Sulf2 display similar structures and their *N*-terminal domains are highly conserved; however, their *C*-terminal regions display different structures. The *C*-terminal domains bind to heparan sulfate chains. Therefore, the differences in the heparan-sulfate binding sites at the *C*-terminal domains of Sulf1 and Sulf2 may result in functional differences. For instance, Sulf1 exerts tumor-suppressive effect through the inhibition of receptor tyrosine kinase signaling by HSPG desulfation, but Sulf2 displays an oncogenic activity via activation of receptor tyrosine kinases [31,32].

Angiotensin II has two receptor subtypes, AT_1 R and AT_2 R; the density of AT_1 R is higher than that of AT_2 R in VSMCs [33]. The stimulatory effect of Ang II in hypertension is dependent on the AT_1 R pathway [34]. In contrast, AT_2 R is known to antagonize the actions of AT_1 R [35,36]. However, studies have reported proinflammatory actions of AT_2 R in VSMCs [33,37]. In addition, a positive role of AT_2 R in anti-hypertensive effects of CCL5 has been demonstrated in VSMCs from SHR [19–22]. Stimulation with Ang II induces the expression of both AT_1 R and AT_2 R, and Ang II-induced expression of 12-LO and ET-1 mRNA is dependent on both AT_1 R and AT_2 R pathways [19,20]. On the other hand, Ang II-induced inhibition of DDAH-1 is dependent on the AT_1 R pathway in VSMCs of SHR [21]. In this study, we demonstrated that CCL5 has no effect on AT_1 R expression; however, it inhibits Ang II-induced AT_1 R expression in VSMCs from SHR. Moreover, CCL5 treatment resulted in a small increase in the expression of AT_2 R, but showed synergistic effect on Ang II-induced AT_2 R expression in VSMCs. The downregulation in the expression of Ang II-induced hypertensive

mediators, inhibition of VSMC proliferation, and upregulation of AMPK as well as DDAH-1 activity and IL-10 expression by CCL5 were dependent on the AT_2 R pathway [19–22,38]. We observed that Sulf1 downregulation resulted in the inhibition of CCL5-induced expression of AT_2 R mRNA as well as the synergistic effect of CCL5 on Ang II-induced AT_2 R expression. These results suggest that the anti-hypertensive effects of CCL5 through the AT_2 R pathway in VSMCs from SHR are related to Sulf1 expression. However, complete abrogation of the inhibitory effects of CCL5 on Ang II-induced 12-LO and ET-1 expression was not detected in Sulf1 siRNA-transfected VSMCs. This result indicates that the inhibitory effects of CCL5 on Ang II-induced 12-LO and ET-1 expression via AT_2 R pathway are mediated by Sulf1; however, Sulf1 mediates these anti-hypertensive effects partially in VSMCs. It is interesting that the reduction in CCL5-induced DDAH-1 expression and attenuation of the effect of CCL5 on Ang II-induced inhibition of DDAH-1 expression in Sulf1 siRNA-transfected VSMCs were comparable to those observed in control siRNA-transfected VSMCs stimulated with AT_2 R blocker. Thus, the CCL5-induced expression of DDAH-1 via AT_2 R pathway may be completely dependent on Sulf1 in VSMCs.

This is the first study to demonstrate the effects of Sulfs on anti-hypertensive actions of CCL5 on Ang II-induced hypertensive mediators in VSMCs from SHR. However, the vague mechanism between Sulf1 dependency of antihypertensive effects of CCL5 and the inhibitory action of CCL5 on Sulf1 expression and sulfatase activity must further be elucidated. This study provides the first evidence that Sulf1, not Sulf2, mediates the expression of CCL5-induced AT_2 R as well as the synergistic effect of CCL5 on Ang II-induced AT_2 R expression in VSMCs from SHR. Furthermore, the complete anti-hypertensive effects of CCL5 via AT_2 R pathway demand Sulf1 activity in VSMCs. Together with our previous results, these findings suggest that Sulf1 is a potential up-regulatory factor in anti-hypertensive effects of CCL5 on Ang II-induced hypertensive mediators in VSMCs from SHR.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (2015R1D1A1A01057212).

References

[1] M. Buono, M.P. Cosma, Sulfatase activities towards the regulation of cell metabolism and signaling in mammals, Cell Mol. Life Sci. 67 (2010) 769–780.
[2] S. Nagamine, S. Koike, K. Keino-Masu, M. Masu, Expression of a heparan sulfate remodeling enzyme, heparan sulfate 6-O-endosulfatase sulfatase FP2, in the rat nervous system, Brain Res. Dev. Brain Res. 159 (2005) 135–143.
[3] K. Honke, N. Taniguchi, Sulfotransferases and sulfated oligosaccharides, Med. Res. Rev. 22 (2002) 637–654.
[4] A. Langsdorf, A.T. Do, M. Kusche-Gullberg, C.P. Emerson Jr., X. Ai, Sulfs are regulators of growth factor signaling for satellite cell differentiation and muscle regeneration, Dev. Biol. 311 (2007) 464–477.
[5] Y. Dai, Y. Yang, V. MacLeod, X. Yue, A.C. Rapraeger, Z. Shriver, et al., HSulf-1 and HSulf-2 are potent inhibitors of myeloma tumor growth in vivo, J. Biol. Chem. 280 (2005) 40066–40073.
[6] M. Morimoto-Tomita, K. Uchimura, Z. Werb, S. Hemmerich, S.D. Rosen, Cloning and characterization of two extracellular heparin-degrading endosulfatases in mice and humans, J. Biol. Chem. 277 (2002) 49175–49185.
[7] C.R. Holst, H. Bou-Reslan, B.B. Gore, K. Wong, D. Grant, S. Chalasani, et al., Secreted sulfatases Sulf1 and Sulf2 have overlapping yet essential roles in mouse neonatal survival, PLoS One 2 (2007) e575.
[8] A. Ratzka, I. Kalus, M. Moser, T. Dierks, S. Mundlos, A. Vortkamp, Redundant function of the heparan sulfate 6-O-endosulfatases Sulf1 and Sulf2 during skeletal development, Dev. Dyn. 237 (2008) 339–353.
[9] K. Narita, J. Staub, J. Chien, K. Meyer, M. Bauer, A. Friedl, et al., HSulf-1 inhibits angiogenesis and tumorigenesis in vivo, Cancer Res. 66 (2006) 6025–6032.
[10] H. Zhang, D.R. Newman, P.L. Sannes, HSULF-1 inhibits ERK and AKT signaling and decreases cell viability in vitro in human lung epithelial cells, Respir. Res. 13 (2012) 69.
[11] M. Morimoto-Tomita, K. Uchimura, A. Bistrup, D.H. Lum, M. Egeblad, N. Boudreau, et al., Sulf-2, a proangiogenic heparan sulfate endosulfatase, is upregulated in breast cancer, Neoplasia 7 (2005) 1001–1010.
[12] K. Uchimura, M. Morimoto-Tomita, A. Bistrup, J. Li, M. Lyon, J. Gallagher, Z. Werb, et al., HSulf-2, an extracellular endoglucosamine-6-sulfatase, selectively mobilizes heparin-bound growth factors and chemokines: effects on VEGF, FGF-1, and SDF-1, BMC Biochem. 7 (2006) 2.
[13] B. Heintz, G. Stöcker, C. Mrowka, U. Rentz, H. Melzer, E. Stickeler, et al., Decreased glomerular basement membrane heparan sulfate proteoglycan in essential hypertension, Hypertension 25 (1995) 399–407.
[14] G.B. Sala-Newby, S.J. George, M. Bond, G.K. Dhoot, A.C. Newby, Regulation of vascular smooth muscle cell proliferation, migration and death by heparan sulfate 6-O-endosulfatase1, FEBS Lett. 579 (2005) 6493–6498.
[15] X.L. Chen, P.E. Tummala, M.T. Olbrych, R.W. Alexander, R.M. Medford, Angiotensin II induces monocyte chemoattractant protein-1 gene expression in rat vascular smooth muscle cells, Circ. Res. 83 (1998) 952–959.
[16] M. Buemi, D. Marino, R. Floccari, A. Ruello, L. Nostro, C. Aloisi, et al., Effect of interleukin 8 and ICAM-1 on calcium-dependent outflow of K+ in erythrocytes from subjects with essential hypertension, Curr. Med. Res. Opin. 20 (2004) 19–24.
[17] M. Ishibashi, K. Hiasa, Q. Zhao, S. Inoue, K. Ohtani, S. Kitamoto, et al., Critical role of monocyte chemoattractant protein-1 receptor CCR2 on monocytes in hypertension-induced vascular inflammation and remodeling, Circ. Res. 94 (2004) 1203–1210.
[18] J.H. Kim, Y.J. Kang, H.S. Kim, IL-8/CXCL8 upregulates 12-lipoxygenase expression in vascular smooth muscle cells from spontaneously hypertensive rats, Immune Netw. 9 (2009) 106–113.
[19] J.H. Kim, H.S. Kim, Downregulation of angiotensin II-induced 12-lipoxygenase expression and cell proliferation in vascular smooth muscle cells from spontaneously hypertensive rats by CCL5, Kor. J. Physiol. Pharmacol. 13 (2009) 385–392.
[20] H.Y. Kim, H.J. Cha, H.S. Kim, CCL5 upregulates activation of AMP-activated protein kinases in vascular smooth muscle cells of spontaneously hypertensive rats, Cytokine 67 (2014) 77–84.
[21] H.Y. Kim, J.H. Kim, H.S. Kim, Effect of CCL5 on dimethylarginine dimethylaminohydrolase-1 production in vascular smooth muscle cells from spontaneously hypertensive rats, Cytokine 64 (2013) 227–233.
[22] H.Y. Kim, H.J. Cha, H.S. Kim, CCL5 upregulates IL-10 expression and partially mediates the antihypertensive effects of IL-10 in the vascular smooth muscle cells of spontaneously hypertensive rats, Hypertens. Res. 38 (2015) 666–674.
[23] H.Y. Kim, H.J. Cha, J.H. Choi, Y.J. Kang, S.Y. Park, H.S. Kim, CCL5 inhibits elevation of blood pressure and expression of hypertensive mediators in developing hypertension state spontaneously hypertensive rats, J. Bacteriol. Virol. 45 (2015) 138–150.
[24] H.Y. Kim, Y.J. Kang, I.H. Song, H.C. Choi, H.S. Kim, Upregulation of interleukin-8/CXCL8 in vascular smooth muscle cells from spontaneously hypertensive rats, Hypertens. Res. 31 (2008) 515–523.
[25] M. Sasaki, M.T. Hori, T. Hino, M.S. Golub, M.L. Tuck, Elevated 12-lipoxygenase activity in the spontaneously hypertensive rat, Am. J. Hypertens. 10 (1997) 371–378.
[26] R.M. Touyz, E.L. Schiffrin, Role of endothelin in human hypertension, Can. J. Physio. Pharmacol. 81 (2003) 533–541.
[27] F. Palm, M.L. Onozato, Z. Luo, C.S. Wilcox, Dimethylarginine dimethylaminohydrolase (DDAH): expression, regulation, and function in the cardiovascular and renal systems, Am. J. Physiol. Heart. Circ. Physiol. 293 (2007) 3227–3245.
[28] V. Achan, M. Broadhead, M. Malaki, G. Whitley, J. Leiper, R. MacAllister, et al., Asymmetric dimethylarginine causes hypertension and cardiac dysfunction in humans and is actively metabolized by dimethylarginine dimethylaminohydrolase, Arterioscler. Thromb. Vasc. Biol. 23 (2003) 1455–1459.
[29] K. Nozawa, M.L. Tuck, M. Golub, P. Eggena, J.L. Nadler, N. Stern, Inhibition of lipoxygenase pathway reduces blood pressure in renovascular hypertensive rats, Am. J. Physiol. 259 (1990) 1774–1780.
[30] J.M. Leiper, J. Santa Maria, A. Chubb, R.J. MacAllister, I.G. Charles, G.S. Whitley, et al., Identification of two human dimethylarginine dimethylaminohydrolases with distinct tissue distributions and homology with microbial arginine deiminases, Biochem. J. 343 (1999) 209–214.
[31] J.D. Yang, Z. Sun, C. Hu, J. Lai, R. Dove, I. Nakamura, et al., Sulfatase 1 and sulfatase 2 in hepatocellular carcinoma: associated signaling pathways, tumor phenotypes, and survival, Genes Chromosomes Cancer 50 (2011) 122–135.
[32] J.P. Lai, D.S. Sandhu, C. Yu, T. Han, C.D. Moser, K.K. Jackson, et al., Sulfatase 2 up-regulates glypican 3, promotes fibroblast growth factor signaling, and decreases survival in hepatocellular carcinoma, Hepatology 47 (2008) 1211–1222.
[33] M.E. Mabrouk, R.M. Touyz, E.L. Schiffrin, Differential ANG II-induced growth activation pathways in mesenteric artery smooth muscle cells from SHR, Am. J. Physiol. Heart. Circ. Physiol. 281 (2001) 30–39.
[34] G. Wolf, E.G. Neilson, From converting enzyme inhibition to angiotensin II receptor blockade: new insight on angiotensin II receptor subtypes in the kidney, Exp. Nephrol. 4 (1996) 8–19.
[35] M. Horiuchi, J.Y. Lehtonen, L. Daviet, Signaling mechanism of the AT2 angiotensin II receptor: crosstalk between AT1 and AT2 receptors in cell growth, Trends Endocrinol. Metab. 10 (1999) 391–396.
[36] S. Gallinat, S. Busche, M.K. Raizada, C. Sumners, The angiotensin II type 2 receptor: an enigma with multiple variations, Am. J. Physiol. Endocrinol. Metab. 278 (2000) 357–374.
[37] G. Wolf, U. Wenzel, K.D. Burns, R.C. Harris, R.A. Stahl, F. Thaiss, Angiotensin II activates nuclear transcription factor-kappaB through AT1 and AT2 receptors, Kidney Int. 61 (2002) 1986–1995.
[38] H.Y. Kim, H.S. Kim, Dimethylarginine dimethylaminohydrolase-1 mediates inhibitory effect of interleukin-10 on angiotensin II-induced hypertensive effects in vascular smooth muscle cells of spontaneously hypertensive rats, Cytokine 77 (2016) 203–210.