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Sulfatase 2 mediates, partially, the expression of endothelin-1 and the additive effect of Ang II-induced endothelin-1 expression by CXCL8 in vascular smooth muscle cells from spontaneously hypertensive rats

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ABSTRACT

The extracellular sulfatases (Sulfs), sulfatase 1 (Sulf1) and sulfatase 2 (Sulf2), have an important role in cell signaling by modulating the 6-*O*-sulfation of heparan sulfate proteoglycans (HSPGs) on the cell surface. Gene expression and enzyme activity of Sulfs are elevated in hypertensive vascular smooth muscle cells (VSMCs) compared to those in normotensive VSMCs. CXC-chemokine ligand (CXCL) 8 has a pathogenic role in the development and progression of hypertension. In this study, we investigated the effect of Sulfs on the expression of CXCL8-induced endothelin (ET)-1, a hypertensive mediator, in VSMCs from spontaneously hypertensive rats (SHR). Expression of ET-1 and elevation of angiotensin (Ang) II-induced ET-1 expression by CXCL8 were reduced in Sulf2 small interfering RNA (siRNA)-transfected SHR VSMCs. But, downregulation of Sulf1 did not affect the expression of CXCL8-induced ET-1 and additive effect of CXCL8 on Ang II-induced ET-1 expression in SHR VSMCs. CXCL8-induced ET-1 expression and the additive effect of CXCL8 on Ang II-induced ET-1 expression were dependent on the Ang II type 1 receptor (AT_1 R) pathway, not the Ang II type 2 receptor (AT_2 R) pathway. In addition, downregulation of Sulf2 reduced the expression of CXCL8-induced AT_1 R and abrogated the additive effect of CXCL8 on Ang II-induced AT_1 R expression in SHR VSMCs. Sulf2 mediated, partially, the expression of ET-1 and the additive expression of Ang II-induced ET-1 mRNA by CXCL8 via the AT_1 R pathway in SHR VSMCs. These findings suggest that Sulf2 is an up-regulatory factor in the additive action of CXCL8 via the AT_1 R pathway on Ang II-induced ET-1 expression in VSMCs under hypertension environment.

1. Introduction

Sulfatases (Sulfs) are hydrolytic enzymes that cut sulfate groups from sulfamates and sulfate esters. To date, 17 distinct Sulfs in humans and 14 in rodents have been identified and classified on the basis of their subcellular localization and pH-dependent activity [1,2]. Extracellular Sulfs, including sulfatase 1 (Sulf1) and sulfatase 2 (Sulf2), are neutral-basic pH-dependent sulfatases, referred to as heparan sulfate 6-*O*-endosulfatases [1,3]. Although Sulf1 and Sulf2 are structurally similar, they have opposite effects in tumorigenesis. Sulf1 inhibits angiogenesis and tumorigenesis [4,5], whereas Sulf2 has pro-angiogenic and oncogenic effects [6,7]. In addition, it is well known that Sulfs control cell signaling by remodeling heparan sulfate proteoglycans (HSPGs) on the surface of cells. Removal of 6-*O*-sulfate groups from HSPGs by Sulfs results in the release of bound growth factors, which initiates signaling pathways [1,7]. Aside from their involvement in cell signaling, Sulfs are associated with diverse cellular processes such as tumor growth, neuroanatomical development, and muscle regeneration [1,8–10].

Inflammatory cell infiltration by chemokines in vascular walls contributes to the pathogenesis of vascular hypertension [11–15]. Along with CC-chemokine ligand (CCL) 2, CXC-chemokine ligand (CXCL) 8 has an important role in the development and maintenance of vascular hypertension and atherosclerosis [12–15]. CXCL8 is increased in arterial walls of hypertensive animals and elevates hypertensive mediators, including 12-lipoxygensae (LO) and endothelin (ET)-1, in hypertensive vascular smooth muscle cells (VSMCs) [15–17]. Additionally, it stimulates proliferation and migration of VSMCs and

Abbreviations: ET-1, Endothelin-1; CXCL, CXC-chemokine ligand; Sulf1, sulfatase 1; Sulf2, sulfatase 2; VSMCs, vascular smooth muscle cells; SHR, spontaneously hypertensive rat; WKY, Wistar-Kyoto rats; Ang II, angiotensin II; AT_1 R, Ang II type 1 receptor; AT_2 R, Ang II type 2 receptor; HSPGs, heparan sulfate proteoglycans; Sulfs, sulfatases; PCR, polymerase chain reaction; FBS, fetal bovine serum; EDTA, ethylenediaminetetraacetic acid; DMEM, Dulbecco's modified Eagle's medium; siRNA, small interfering RNA; cDNA, complementary DNA; SEM, standard errors of the mean

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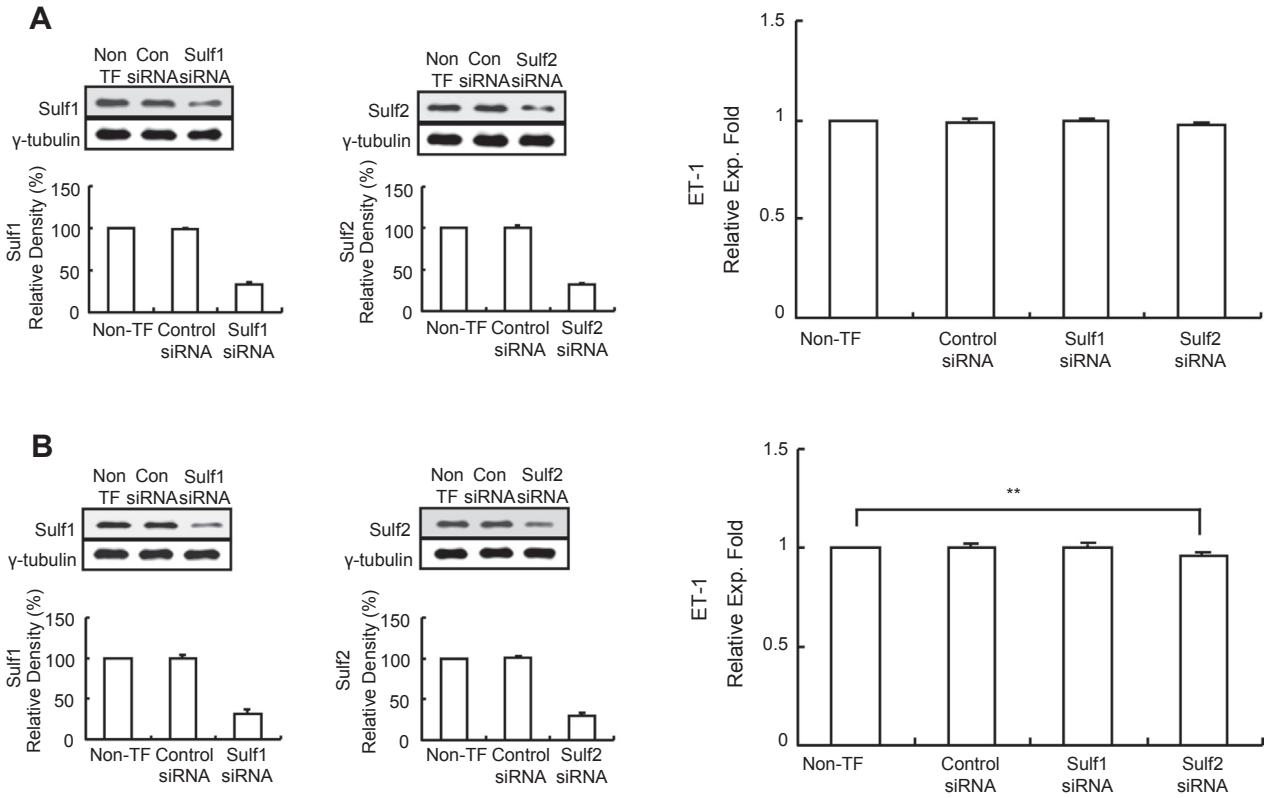

Fig. 1. Basal expression of ET-1 is reduced in Sulf2 siRNA-transfected SHR VSMCs. WKY (A) and SHR (B) VSMCs were plated in six-well plates, grown to 90% confluence, and transfected with Sulf1, Sulf2, or control siRNA oligomers (50 nmol/L). Successful transfection of Sulf1, Sulf2, or control siRNA oligomers in VSMCs was confirmed by immunoblot analysis. Following transfection, Total RNAs were isolated and mRNA expression of ET-1 was measured by real-time PCR Non-TF: non-transfected VSMCs. Bars represent means ± SEM of three independent experiments. $^{**}p < 0.01$.

arterial neointimal formation [12].

Sulf1 is associated with the mediation of several functions of VSMCs [18]. Knockdown or overexpression of Sulf1 in normal VSMCs inhibits adhesion but induces apoptosis and proliferation [18]. Therefore, the maintenance of normal 6–*O*–sulfation levels by Sulf1 is important in the functioning of VSMCs. In our previous research, Sulf1 was shown to mediate the inhibitory effect of PD123319, an angiotensin (Ang) II type 2 receptor (AT_2 R) inhibitor, on Ang II-induced 12-LO and ET-1 expressions and on the proliferation of hypertensive VSMCs [19]. Moreover, Sulf1, not Sulf2, had a modulatory role in the AT_2 R pathway of Ang II-induced hypertensive effects in VSMCs from spontaneously hypertensive rats (SHR) [19]. Furthermore, Sulf1 mediates the attenuation of Ang II-induced hypertensive effects by CCL5 in SHR VSMCs [20].

Previous research into extracellular Sulfs has focused mainly on cell signaling or carcinogenesis [1–5,10]. The relationship between the activity of Sulfs and the hypertensive effects of chemokines has not been described, except in our previous studies [20]. Inhibitory actions of CCL5 on Ang II-induced 12-LO and ET-1 expressions are mediated by Sulf1 in hypertensive VSMCs [20]. Chemokine CCL5 has down-regulatory effects on hypertensive mediators [21–23], but CXCL8 has up-regulatory effects on hypertensive mediators [15–17]. Therefore, we doubted whether CXCL8-mediated upregulation of hypertensive mediator expressions was related to Sulfs activity in hypertensive VSMCs. To our knowledge, there have been few studies evaluating the functional role of Sulfs in hypertension. Therefore, as a fundamental study to evaluate the relationship between Sulfs activity and hypertensive mediators in hypertensive cells, we examined the effect of Sulfs on the expression of CXCL8-induced ET-1, a powerful vasoconstrictor, in SHR VSMCs.

2. Materials and methods

2.1. Reagents

The total RNA extraction kit was obtained from iNtRON Biotechnology (Seoul, Korea). Ang II was obtained from Calbiochem (San Diego, CA, USA). CXCL8 was purchased from R&D systems (Minneapolis, MN). Losartan, PD123319, and monoclonal anti-γ-tubulin antibody were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). Lipofectamine 2000 and negative control small interfering RNA (siRNA) were obtained from Invitrogen (Carlsbad, CA, USA). LightCycler FastStart DNA SYBR Green I Mix was obtained from Roche (Mannheim, Germany). The forward and reverse primers for ET-1, Ang II type 1 receptor (AT_1 R), AT_2 R, and β-actin were supplied by Bionics (Daejeon, Korea). ET-1, Sulf1, Sulf2 antibodies, and rat Sulf2 siRNA sequences were purchased from Santa Cruz Biotechnology (Santa Cruz, California, USA). AT_1 R antibody was obtained from Abcam (Cambridge, UK). Rat Sulf1 siRNA sequences was purchased from Bioneer (Daejeon, Korea). The pcDNA3.1 vector was obtained from Invitrogen Life Technologies (Carlsbad, CA, USA), and the pcDNA3.1/Myc-His(−)-MSulf2 (Plasmid 13008), donated by Dr. Steven D. Rosen [3], was obtained from Addgene (Cambridge, MA, USA). All other chemicals were obtained from pure-grade commercial sources.

2.2. Animals

Specific pathogen-free 22-week-old male inbred SHR and normotensive Wistar-Kyoto rats (WKY) were supplied by Japan SLC (Shizuka, Japan). All rats were fed autoclaved food and received bedding in order to minimize their exposure to microbial pathogens. Rats were cared for in accordance with the principles of the Guide to the Care and Use of

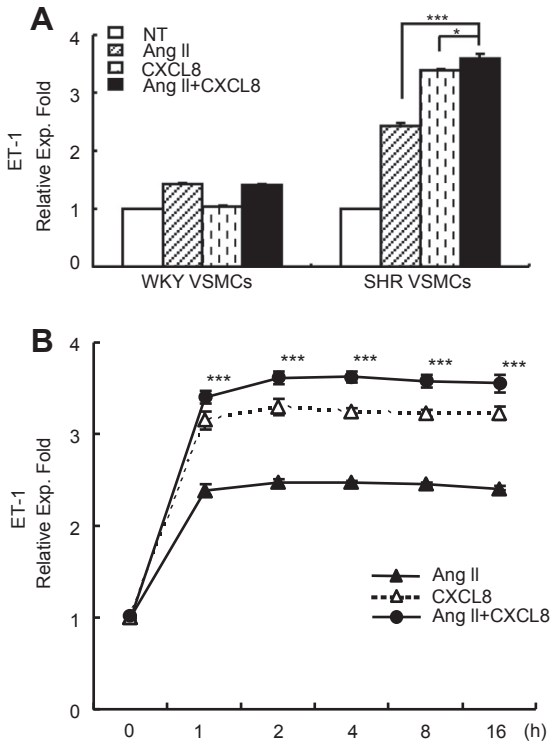

Fig. 2. CXCL8 increases expression of ET-1 mRNA and the expression of Ang II-induced ET-1 mRNA is elevated by CXCL8 in SHR VSMCs. (A) WKY and SHR VSMCs were untreated or treated with Ang II (0.1 μmol/L) and/or CXCL8 (100 ng/mL) for 2 h. Total RNAs were isolated and ET-1 mRNA expressions determined by real-time PCR. Bars represent the means ± SEM of three independent experiments. $^{*}p < 0.05$, $^{***}p < 0.001$. (B) SHR VSMCs were untreated or treated with Ang II (0.1 μmol/L) and/or CXCL8 (100 ng/mL) for the indicated time. Total RNAs were isolated and ET-1 mRNA expressions determined by real-time PCR. Bars represent the means ± SEM of three independent experiments. $^{***}p < 0.001$ *versus* VSMCs treated with Ang II.

Experimental Animals of the Yeungnam Medical Center.

2.3. Preparation of VSMCs

Vascular smooth muscle cells were from 22-week-old male SHR and WKY were isolated from thoracic aortas as described previously [16]. The VSMCs were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 1% penicillin-streptomycin and 10% heat-inactivated fetal bovine serum (FBS). All experiments were performed during 3 to 7 cell passages. VSMCs were cultured in serum-starved DMEM for overnight before stimulations. Cells were incubated in a humidified incubator at 37 °C and 5% CO_2 in the presence or absence of stimuli for the indicated time.

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

Total RNA was extracted from VSMCs using the easy-BLUE total RNA extraction kit (iNtRON Biotechnology, Seoul, Korea) following the manufacturer's instructions.

Isolated total RNA was reverse-transcribed by using a Maxime RT premix kit (iNtRON Biotechnology, Daejeon, Korea) according to the manufacturer's instructions. Synthesis of complementary DNA (cDNA) was performed at 45 °C for 60 min, followed by room temperature inactivation at 95 °C for 5 min. The AT_1 R, AT_2 R and ET-1 were amplified by real-time PCR using the LightCycler system (Roche, Germany). Twenty microliter of each PCR reaction included primer, 2 μL of cDNA and LightCycler FastStart DNA SYBR Green I mix (Roche, Germany). The mixture was incubated for 10 min at 95 °C. The amplification step consisted of 45 cycles of denaturation (for 10 s at 95 °C), annealing (primer-appropriate temperature at 5 s), and extension (for 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 °C to 95 °C with continuous fluorescence detection. The following primer sets were used: AT_1 R, forward, 5′-caccctatgtaagatcgcttc-3′ and reverse, 5′-gcacaatcgccataattatcc-3′ (445 bp); AT_2 R, forward, 5′-ccgtgaccaagtcttgaagatg-3′ and reverse, 5′-aggaagccagcaaatgatg-3′ (65 bp); ET-1, forward, 5′-ctcctccttgatggacaagg-3′ and reverse, 5′-cttgatgctgttgctcatgg-3′ (370 bp); and β-actin, forward, 5′-tactgccctggctcctagca-3′ and reverse, 5′-tggacagtgaggccaggatag-3′ (101 bp). The mRNA level of each sample was normalized to the mRNA level of β-actin. β-actin was used as housekeeping gene.

2.5. Western blotting

Cytoplasmic protein extracts were collected in VSMCs from SHR or WKY. Polyclonal antibodies against AT_1 R (2.5 mg/mL), Sulf1 (200 μg/mL), Sulf2 (100 μg/mL), ET-1 (200 μg/mL), or monoclonal antibody against γ-tubulin (1 mg/mL) were used as described by Kim et al. [16].

2.6. Small interfering RNA (siRNA)

Vascular smooth muscle cells were cultured until reaching 90% confluence in six-well plates. The VSMCs were transfected with sequence-specific siRNA or non-targeting control siRNA using Lipofectamine 2000, as per the manufacturer's instructions. The following sequences were used: 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′. The working concentration of siRNA in cell experiments was 50 nmol/L. After 24 h of incubation, the transfected VSMCs were cultured in growth medium for 24 h before undertaking the experiments. Following incubation, cells were cultured in the presence or absence of stimuli for 2 h.

2.7. Overexpression of Sulf2 gene

A recombinant plasmid expressing full-length Sulf2 cDNA, pcDNA3.1/Myc-His(−)-MSulf2 was amplified in liquid Luria-Bertani Broth media with 100 μg/mL of ampicillin. The amplified plasmids were purified by using a QIAGEN Plasmid Mini kit (Qiagen, Hilden, Germany) as described by the manufacturer's instructions. VSMCs were transfected with either pcDNA3.1/Myc-His(−)-MSulf2 or the pcDNA3.1 empty vector by using Lipofectamine 2000, according to the manufacturer's instructions.

2.8. Statistical analysis

All values are presented as means ± standard errors of the mean (SEM) of at least three or four independent experiments. Data were analyzed by using one-way analysis of variance followed by applying the Bonferroni or Dunnett's T3 *post-hoc* test by SPSS version 23.0 (IBM Co., Armonk, NY, USA). A value of $p < 0.05$ was considered to represent a statistically significant.

3. Results

3.1. CXCL8 has an additive effect on Ang II-induced ET-1 expression in SHR VSMCs

Initially, we observed the direct effect of extracellular Sulfs on basal expression of ET-1 mRNA in SHR and WKY VSMCs. Neither Sulf1 nor Sulf2 had a significant effect on the basal expression of ET-1 mRNA in WKY VSMCs (Fig. 1A). However, the basal expression of ET-1 was

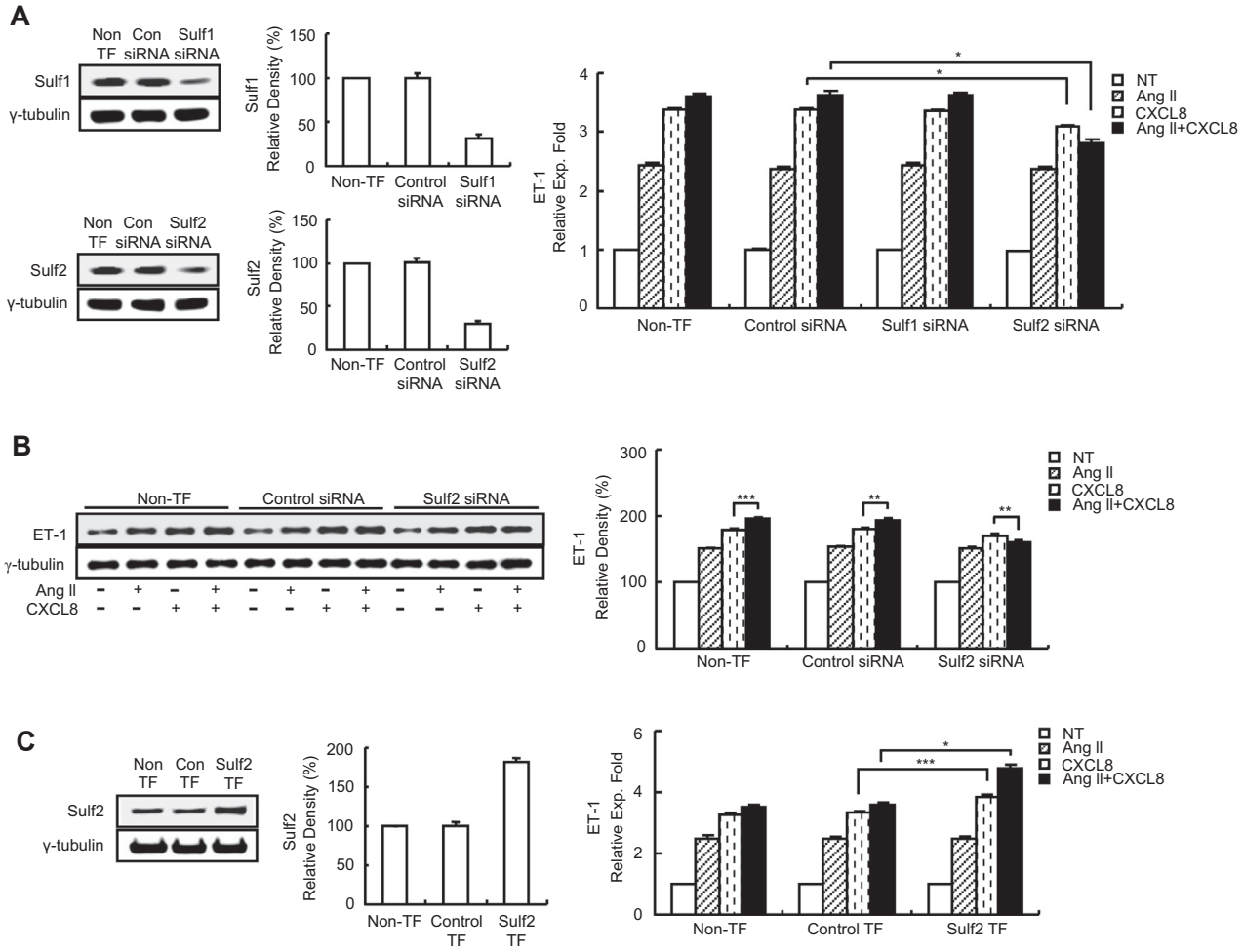

Fig. 3. Downregulation of Sulf2 reduces the expression of CXCL8-induced ET-1 and the additive effect of CXCL8 on Ang II-induced ET-1 expression in SHR VSMCs. (A, B) SHR VSMCs were plated in six-well plates, grown to 90% confluence, and transfected with Sulf1, Sulf2, or control siRNA oligomers (50 nmol/L). Successful transfection of Sulf1, Sulf2, or control siRNA oligomers in VSMCs was confirmed by immunoblot analysis. SHR VSMCs were untreated or treated with Ang II (0.1 μmol/L) and/or CXCL8 (100 ng/mL) for 2 h. Total RNAs and cell lysates were isolated, and mRNA expression (A) and protein production (B) of 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. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. (C) Overexpression of Sulf2 in SHR VSMCs was obtained by using pcDNA3.1/Myc-His(−)-MSulf2. Successful transfection of pcDNA3.1/Myc-His(−)-MSulf2 in SHR VSMCs was confirmed by immunoblot analysis. Following transfection, VSMCs were untreated or treated with Ang II (0.1 μmol/L) and/or CXCL8 (100 ng/mL) for 2 h. Total RNAs were isolated; ET-1 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. $^{*}p < 0.05$, $^{***}p < 0.001$.

reduced slightly in SHR VSMCs transfected with Sulf2 siRNA (Fig. 1B).

Basal expression of ET-1 in SHR VSMCs is reported to be higher than that in WKY VSMCs [24], and CXCL8 increases expression of ET-1 mRNA in SHR VSMCs [15,25]. Therefore, we examined the effect of CXCL8 on the expression of Ang II-induced ET-1 mRNA in SHR VSMCs and WKY VSMCs. CXCL8 had no effect on expression of ET-1 mRNA and Ang II-induced ET-1 mRNA expression in WKY VSMCs. However, CXCL8 elevated ET-1 mRNA expression higher than that induced by Ang II and the combined effect was larger than the single stimulation of Ang II or CXCL8 (Fig. 2A). In addition, the time courses of ET-1 expression and Ang II-induced ET-1 expression in response to CXCL8 stimulation in SHR VSMCs were observed over 16 h. The increased ET-1 expression level in SHR VSMCs treated with CXCL8 remained approximately constant from 1 h to 16 h upon CXCL8 treatment in SHR VSMCs. In addition, an additive effect on expression of Ang II-induced ET-1 by CXCL8 was detected 1 h after simultaneous treatment of Ang II and CXCL8 (Ang II/CXCL8) and was sustained for up to 16 h (Fig. 2B).

3.2. Expression of ET-1 and the additive effect on Ang II-induced ET-1 expression by CXCL8 are mediated via Sulf2 in VSMCs from SHR

Neither Sulf1 nor Sulf2 has a reported effect on Ang II-induced ET-1 expression in SHR VSMCs [19]. However, Sulf1 mediates the attenuation of Ang II-induced 12-LO and ET-1 expressions by CCL5 in SHR VSMCs [20]. In this study, we examined the effects of Sulf1 and Sulf2 on CXCL8-induced ET-1 expression and the additive effect of CXCL8 on Ang II-induced ET-1 expression in SHR VSMCs. Downregulation of Sulf1 did not affect the expression of CXCL8-induced ET-1 mRNA expression or the additive effect of CXCL8 on Ang II-induced ET-1 mRNA expression. However, downregulation of Sulf2 reduced the expression of CXCL8-induced ET-1 mRNA and the additive effect of CXCL8 on Ang II-induced ET-1 mRNA expression (Fig. 3A). The rate of additive expression of Ang II-induced ET-1 mRNA by CXCL8 in SHR VSMCs transfected with Sulf2 siRNA was reduced to 18.8% ± 2.4% from the 52.3% ± 1.9% in those transfected with the control siRNA. The protein levels of ET-1 and reduction of the additive effect of CXCL8 on Ang II-induced ET-1 expression in SHR VSMCs transfected with Sulf2 siRNA were correlated with the mRNA levels noted in SHR VSMCs transfected

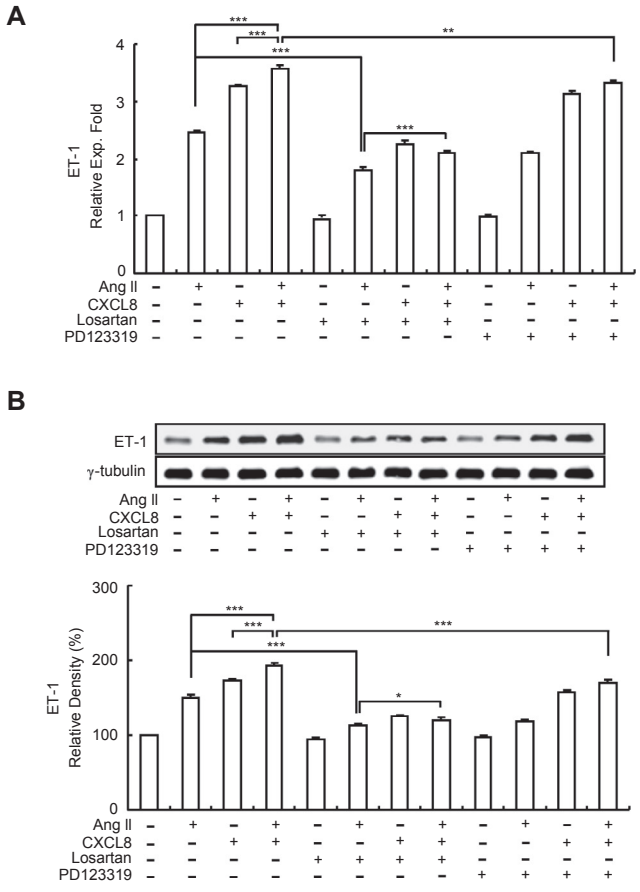

Fig. 4. Expression of CXCL8-induced ET-1 and the additive effect of CXCL8 on Ang II-induced ET-1 expression are mediated by the AT_1 R pathway in SHR VSMCs. SHR VSMCs were untreated or treated with Ang II (0.1 μmol/L) and/or CXCL8 (100 ng/mL) in the presence or absence of losartan (AT_1 R inhibitor, 10 μmol/L) or PD123319 (AT_2 R inhibitor, 10 μmol/L) for 2 h, after which total RNAs and cell lysates were isolated and ET-1 mRNA levels were determined by real-time PCR (A). The protein production of ET-1 (B) was determined by immunoblotting and densitometric analyses. Bars represent the means ± SEM of three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

with Sulf2 siRNA (Fig. 3B). To confirm the effect of Sulf2 on the expression of CXCL8-induced ET-1 and the additive effect of CXCL8 on Ang II-induced ET-1 expression, we examined ET-1 expressions in SHR VSMCs transfected with pcDNA3.1/Myc-His(−)-MSulf2. Overexpression of Sulf2 elevated the expression of CXCL8-induced ET-1 mRNA and the additive effect of CXCL8 on Ang II-induced ET-1 mRNA expression (Fig. 3C). The rate of additive expression of Ang II-induced ET-1 mRNA by CXCL8 in Sulf2-overexpressing SHR VSMCs was elevated to 94.2% ± 2.9% from the 43.5% ± 0.8% in SHR VSMCs transfected with the pcDNA3.1 empty vector.

3.3. Sulf2 mediates CXCL8-induced AT_1 R expression and the additive effect of CXCL8 on Ang II-induced AT_1 R expression in SHR VSMCs

Expression of Ang II-induced ET-1 mRNA is dependent on both the AT_1 R and AT_2 R pathways in SHR VSMCs, and dependence on the AT_1 R pathway is stronger than that on the AT_2 R pathway [19]. In addition, the AT_1 R pathway mediates the expression of CXCL8-induced ET-1 mRNA [15]. Therefore, we examined whether the effects of Sulf2 on the expression of CXCL8-induced ET-1 and the additive effect of CXCL8 on Ang II-induced ET-1 expression in SHR VSMCs are related to the Ang II receptor pathways. First, because dependency on the AT_2 R pathway in CXCL8-induced ET-1 expression has been not elucidated, we examined whether the expression of CXCL8-induced ET-1 and the additive effect of CXCL8 on Ang II-induced ET-1 expression are mediated by the AT_1 R and/or AT_2 R pathways in SHR VSMCs. The expression levels of ET-1 mRNA and Ang II-induced ET-1 mRNA by CXCL8 were reduced in SHR VSMC treated with losartan, an AT_1 R inhibitor. In contrast, PD123319, an AT_2 R inhibitor, did not affect the expression levels of ET-1 mRNA and Ang II-induced ET-1 mRNA by CXCL8 (Fig. 4A). Although the additive expression of AngII-induced ET-1 mRNA by CXCL8 in SHR VSMCs treated with PD123319 was significantly reduced ($^{**}p < 0.01$), this reduction was attributable to the reduction of Ang II-induced ET-1 mRNA expression by PD123319. The expression of CXCL8-induced ET-1 mRNA and the additive effect of CXCL8 on Ang II-induced ET-1 mRNA expression were not dependent on AT_2 R pathway in SHR VSMCs. The protein levels of ET-1 and a reduction of the additive effect of CXCL8 on Ang II-induced ET-1 expression in SHR VSMCs treated with losartan or PD123319 were correlated with the mRNA levels noted in SHR VSMCs treated with losartan or PD123319 (Fig. 4B).

Next, we examined the effect of Sulf2 on the expression of CXCL8-induced Ang II receptors in SHR VSMCs. CXCL8 increases AT_1 R mRNA expression but decreases AT_2 R mRNA expression in SHR VSMCs [17]. In the present study, the increased level of CXCL8-induced AT_1 R mRNA expression was lower than that of Ang II-induced AT_1 R mRNA expression. The reduction of CXCL8–induced AT_1 R expression was detected in Sulf2 siRNA-transfected SHR VSMCs, and Sulf2 downregulation abrogated the additive effect of CXCL8 on Ang II-induced AT_1 R expression. On the other hand, Sulf2 downregulation failed to affect AT_2 R expressions in SHR VSMCs treated with CXCL8 and/or Ang II (Fig. 5A). Sulf1 downregulation had no effect on either AT_1 R or AT_2 R expressions in SHR VSMCs treated with CXCL8 and/or Ang II. The protein levels of CXCL8- or Ang II/CXCL8-induced AT_1 R in the Sulf2 siRNA-transfected SHR VSMCs were correlated with the mRNA levels noted in the Sulf2 siRNA-transfected SHR VSMCs (Fig. 5B). Next, to confirm the effect of Sulf2 on CXCL8- or Ang II/CXCL8-induced AT_1 R expression, we examined AT_1 R expression in VSMCs transfected with pcDNA3.1/Myc-His(−)-MSulf2. Although CXCL8–induced AT_1 R expression was not elevated highly in Sulf2-overexpressing VSMCs, the AT_1 R expression was higher than that in SHR VSMCs transfected with the pcDNA3.1 empty vector ($^{**}p < 0.01$). Overexpression of Sulf2 elevated the additive effect of CXCL8 on Ang II-induced AT_1 R mRNA expression (Fig. 5C). The rate of additive expression of Ang II-induced AT_1 R mRNA by CXCL8 in Sulf2-overexpressing VSMCs was elevated to 35.5% ± 2.6% from the 13.9% ± 3.2% in SHR VSMCs transfected with the pcDNA3.1 empty vector.

3.4. Sulf2 mediates, partially, the expression of CXCL8-induced ET-1 and the additive effect of CXCL8 on Ang II-induced ET-1 expression via the AT_1 R pathway in SHR VSMCs

Next, we compared the effects of Sulf2 downregulation on the expression of CXCL8-induced ET-1 and the additive effect of CXCL8 on Ang II-induced ET-1 expression with those associated with the losartan, AT_1 R blockade. Although a reduction of the additive effect of CXCL8 on Ang II-induced ET-1 expression was detected with both AT_1 R blockade and Sulf2 downregulation, the reduction levels of CXCL8-induced ET-1 expression and the additive effect of CXCL8 on Ang II-induced ET-1 expression by Sulf2 downregulation were less than those resulting from AT_1 R blockade (Fig. 6).

4. Discussion

Neither Sulf1 nor Sulf2 has been reported to affect Ang II-induced hypertensive mediators expression in SHR VSMCs [19]. However, in the present study, we have demonstrated that the expression of ET-1 and the additive expression of Ang II-induced ET-1 gene by CXCL8 are mediated via Sulf2, not Sulf1, and that these effects of Sulf2 are associated with the AT_1 R pathway in SHR VSMCs.

Inflammation of the vascular wall has a pivotal role in the

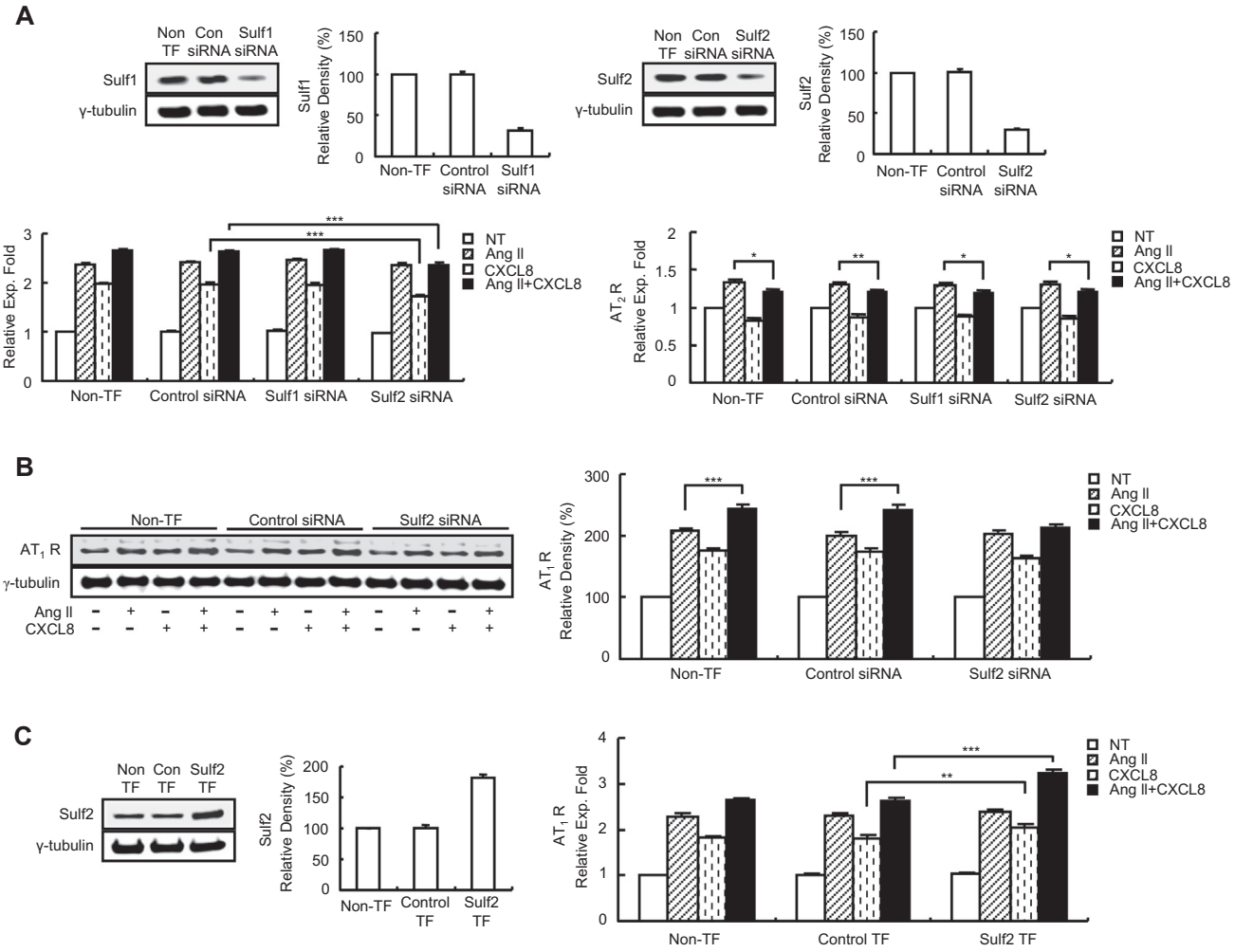

Fig. 5. Downregulation of Sulf2 reduces the expression of CXCL8-induced AT_1 R and abrogates the additive effect of CXCL8 on Ang II-induced AT_1 R expression in SHR VSMCs. (A, B) SHR VSMCs were plated in six-well plates, grown to 90% confluence, and transfected with Sulf1, Sulf2, or control siRNA oligomers (50 nmol/L). Successful transfection of Sulf1, Sulf2, or control siRNA oligomers in VSMCs was confirmed by immunoblot analysis. Following transfection, SHR VSMCs were untreated or treated with Ang II (0.1 μmol/L) and/or CXCL8 (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 (A). The protein production of AT_1 R (B) was determined by immunoblot and densitometric analyses. Non-TF: non-transfected VSMCs. Data shown are representatives of three independent experiments. Bars represent means ± SEM of three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. (C) Overexpression of Sulf2 in SHR VSMCs was obtained by using pcDNA3.1/Myc-His(−)-MSulf2. Successful transfection of pcDNA3.1/Myc-His (−)-MSulf2 in SHR VSMCs was confirmed by immunoblot analysis. Following transfection, SHR VSMCs were untreated or treated with Ang II (0.1 μmol/L) and/or CXCL8 (100 ng/mL) for 2 h. Total RNAs were isolated, and AT_1 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. $^{**}p < 0.01$, $^{***}p < 0.001$.

pathogenesis and progression of hypertension, and ET-1 is an important mediator of inflammation and elicits vascular remodeling in hypertensive vasculature [26]. Basal expression of ET-1 is increased in SHR VSMCs compared to that in WKY VSMCs [15,26]. Ang II induces ET-1 production [26,27], and CXCL8 is a potent inducer of ET-1 expression in SHR VSMCs [15]. In this study, CXCL8 showed an additive effect on Ang II-induced ET-1 expression in SHR VSMCs, but that additive effect was reduced by Sulf2 downregulation. Sulf2, not Sulf1, affected the expression of CXCL8-induced ET-1 and the additive effect of CXCL8 on Ang II-induced ET-1 expression in SHR VSMCs. In addition, we have observed that the expression of CXCL8-induced 12-LO and the additive effect of CXCL8 on Ang II-induced 12-LO expression were also reduced by Sulf2 downregulation in SHR VSMCs (data not shown). In our previous study with chemokine CCL5 [20], downregulation of Sulf1 reduced the inhibition of Ang II-induced 12-LO and ET-1 expressions and the attenuation of Ang II-induced dimethylarginine dimethylaminohydrolase-1 (DDAH-1, a regulator of nitric oxide bioavailability) inhibition by CCL5 in SHR VSMCs. On the other hand, Sulf2 did not affect the inhibitory effect of CCL5 on the expression of Ang II-induced hypertensive mediators and Ang II-induced DDAH-1 inhibition in SHR VSMCs. CXCL8 and CCL2 have been reported to act as up-regulatory mediators in the pathogenesis and progression of vascular hypertension [12,16,17]. In contrast, CCL5 downregulates the expressions of Ang II-induced hypertensive mediators and inhibits the elevation of blood pressure in hypertensive animals [23]. Taken together, our results suggest that Sulf1 has a down-regulatory role and Sulf2 has an up-regulatory role in the effects of chemokines on Ang II-induced hypertension. Namely, although further systematic studies are demanded, Sulf1 and Sulf2 may have opposite roles in the pathogenesis of hypertensive vasculature, like their opposite roles in tumorigenesis.

Although Sulf1 and Sulf2 have similar structures, their *C*-terminal regions, which bind to heparan sulfate chains, display structural differences [1,5]. Therefore, the differences in the heparan sulfate binding sites in the *C*-terminal domains of extracellular Sulfs may lead to their functional differences. For example, Sulf1 shows a tumor–suppressive effect through the inhibition of receptor tyrosine kinase signaling, whereas Sulf2 elicits a tumorigenesis effect via activation of receptor tyrosine kinase signaling [5].

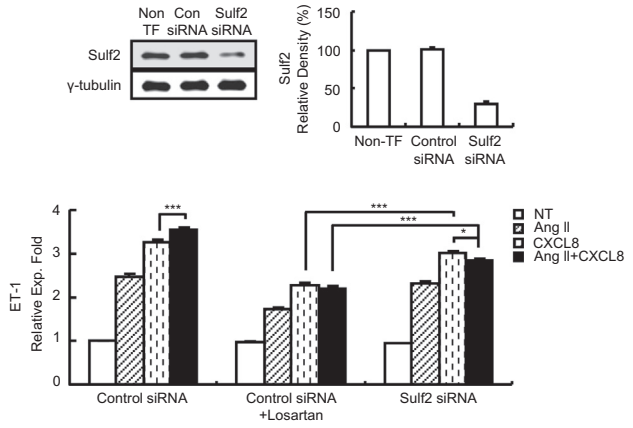

Fig. 6. Sulf2 mediates, partially, the expression of CXCL8-induced ET-1 mRNA and the additive effect of CXCL8 on Ang II-induced ET-1 mRNA expression in SHR VSMCs. SHR VSMCs were plated in six-well plates, grown to 90% confluence, and transfected with Sulf2 or control siRNA oligomers (50 nmol/L). Successful transfection of Sulf2 or control siRNA oligomers in VSMCs was confirmed by immunoblot analysis. Following transfection, control siRNA- and Sulf2 siRNA-transfected VSMCs were untreated or treated with Ang II (0.1 μmol/L) and/or CXCL8 (100 ng/mL) for 2 h. Other control siRNA-transfected VSMCs were untreated or treated with Ang II (0.1 μmol/L) and/or CXCL8 (100 ng/mL) in the presence of losartan (AT_1 R inhibitor, 10 μmol/L) for 2 h. Total RNAs were isolated, and ET-1 mRNA levels were measured by real-time PCR. Non-TF: non-transfected VSMCs. Bars represent means ± SEM from three independent experiments. $^{*}p < 0.05$, $^{***}p < 0.001$.

Ang II is one of the main mediators in the development and pathophysiology of hypertension [11]. Two subtypes of Ang II receptor are AT_1 R and AT_2 R. The density of AT_1 R is higher than that of AT_2 R in VSMCs [28]. The hypertensive effect of Ang II is related to the AT_1 R pathway [29]. In contrast, although proinflammatory effects of AT_2 R in VSMCs have been reported [28,30], AT_2 R has opposing effects on the actions of AT_1 R [25,31]. A positive role of AT_2 R in the anti-hypertensive effects of CCL5 has been demonstrated in SHR VSMCs [21,22,32]. Ang II increases the expression of both AT_1 R and AT_2 R, and expression of ET-1 mRNA by Ang II is dependent on both the AT_1 R and AT_2 R pathways [19]. In this study, expression of CXCL8-induced ET-1 was dependent on the AT_1 R pathway only, and a reduction of the additive effect of Ang II-induced ET-1 expression by CXCL8 was detected in SHR VSMCs treated with an AT_1 R inhibitor. In addition, Sulf2 downregulation resulted in the inhibition of CXCL8-induced expression of AT_1 R mRNA as well as the abrogation of the additive effect of CXCL8 on Ang II-induced AT_1 R expression. Consequently, Sulf2 activity affects the up-regulatory effect of CXCL8 on the expression of ET-1, as well as Ang II-induced ET-1 expression, via the AT_1 R pathway in SHR VSMCs. In a previous study, we evaluated the relationship between the activity of extracellular Sulfs and Ang II receptors in SHR VSMCs. Neither Sulf1 nor Sulf2 affected the basal expression of AT_1 R, but downregulation of Sulf1 reduced basal expression of AT_2 R in SHR VSMCs [19]. Additionally, downregulation of Sulf1 abrogated the expression of CCL5-induced AT_2 R mRNA and the additive effect of CCL5 on Ang II-induced AT_2 R expression [20]. Downregulation of Ang II-induced hypertensive effects by CCL5 is dependent on AT_2 R pathway in SHR VSMCs [21,22]. Considering these results together, we suggest that an action mechanism involving Sulf1 and the AT_2 R pathway results in a down-regulatory effect on hypertensive mediators, while that involving Sulf2 and the AT_1 R pathway results in an up-regulatory effect on hypertensive mediators in hypertensive VSMCs.

Although a significant reduction of the additive effect of CXCL8 on Ang II-induced ET-1 expression was detected in Sulf2 siRNA-transfected SHR VSMCs, overall expression levels of ET-1 in Sulf2 siRNA-transfected SHR VSMCs were higher than those in SHR VSMCs treated with an AT_1 R inhibitor. This result indicates that Sulf2 mediates partially, not totally, the expression of ET-1 and the additive expression of Ang II-induced ET-1 expression by CXCL8 via the AT_1 R pathway in SHR VSMCs.

This is the first fundamental study to examine the relationship between the activity of extracellular Sulfs and the hypertensive effect of CXCL8 in hypertensive VSMCs. Although the exact mechanisms of interaction between extracellular Sulfs activity and the hypertensive effects of chemokines must be elucidated further, the present results suggest that Sulf2 is a potential up-regulatory factor in the additive action of chemokines on Ang II-induced hypertensive mediators expression in hypertensive VSMCs.

Disclosures

The authors declare no conflicts of interest.

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