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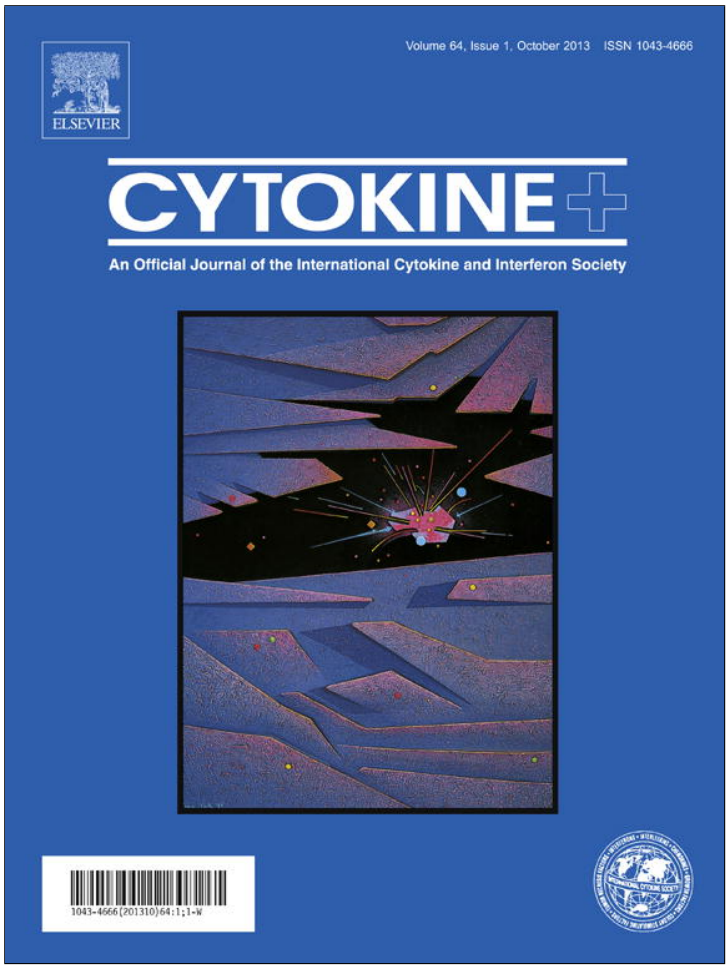

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Effect of CCL5 on dimethylarginine dimethylaminohydrolase-1 production in vascular smooth muscle cells from spontaneously hypertensive rats

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ABSTRACT

Chemokines promote vascular inflammation and play a pathogenic role in the development and maintenance of hypertension. However, in our previous study, chemokine CCL5 was shown to reduce Ang II-induced 12-lipoxygenase (12-LO) production as well as proliferation in vascular smooth muscle cells (VSMCs) obtained from spontaneously hypertensive rats (SHR). Dimethylarginine dimethylaminohydrolase (DDAH) acts as an important regulator of vascular function by metabolizing and regulating plasma asymmetric (N^G,N^G) dimethylarginine (ADMA), a major risk factor for cardiovascular disease. Therefore, in this study, we investigated the effect of CCL5 on DDAH-1 production in SHR VSMCs. Constitutive expression of DDAH-1 in VSMCs from SHR was higher than that in VSMCs from normotensive Wistar Kyoto rats (WKY), whereas expression of DDAH-2 was not significantly different between SHR and WKY VSMCs. CCL5 increased DDAH-1 production and attenuated Ang II-induced DDAH-1 inhibition in SHR VSMCs. In addition, although CCL5 did not affect the level of asymmetric (N^G,N^G) dimethylarginine (ADMA), it attenuated Ang II-induced ADMA production through DDAH-1 activity. DDAH-1 induction by CCL5 was mediated by the Ang II subtype 2 receptor (AT_2 R) pathway. Further, attenuation of Ang II-induced 12-LO and endothelin-1 (ET-1) expression by CCL5 could be attributed to DDAH-1 activity. These findings combined with our previous results suggest that CCL5 is a potential down-regulatory factor in Ang II-induced vascular hypertension.

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1. Introduction

Chemokines play important roles in Ang II-induced vascular hypertension [1,2]. These compounds mediate the migration and infiltration of inflammatory cells into vascular walls, which in combination with oxidative stress, contribute to hypertension-related pathogenesis. Therefore, inhibition of chemokine production is important in the regulation of inflammatory reactions in hypertensive vascular walls.

Dimethylarginine dimethylaminohydrolase (DDAH) is known as an important regulator of plasma asymmetric (N^G,N^G) dimethylarginine (ADMA), a major risk factor for cardiovascular disease and nitric oxide (NO) bioavailability [3,4]. Inhibition of DDAH production increases the level of ADMA and inhibits NO-mediated relaxation of blood vessels [5]. DDAH exists as two isoforms, DDAH-1 and DDAH-2 [6]. Plasma ADMA levels are regulated by DDAH-1, whereas DDAH-2 acts more importantly to preserve endothelial function in blood vessel resistance [3].

Chemokine CCL5 (regulated upon activation, normally T-cell expressed, and presumably secreted; RANTES) is a potent chemoattractant for memory T lymphocytes and monocytes/macrophages [7,8]. Although overproduction of CCL5 is associated with diverse disease progression [9,10], we previously demonstrated reduced expression of CCL5 in VSMCs from spontaneously hypertensive rats (SHR) compared to those from normotensive Wistar–Kyoto rats (WKY) as well as inhibition of angiotensin II (Ang II)-induced 12-lipoxygenase (LO) mRNA production due to CCL5 [11]. Moreover, Ang II was shown to suppress CCL5 expression in SHR VSMCs [12]. Therefore, we propose that although CCL5 acts as an inflammatory mediator in various diseases, it most likely plays a down-regulatory role in Ang II-induced vascular hypertension in contrast to the up-regulatory roles of chemokines CCL2 or CXCL8 [13–15].

Based on our previous study, which demonstrated the down-regulatory effects of CCL5 on Ang II-induced 12-LO production and VSMCs proliferation, we examined the up-regulatory effect of CCL5 on DDAH-1 expression as well as the relationship between CCL5-induced DDAH-1 activity and attenuation of Ang II-induced 12-LO and ET-1 production by CCL5 in SHR VSMCs.

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2. Materials and methods

2.1. Reagents

Trizol reagent for total RNA isolation was purchased from Invitrogen (Carlsbad, CA, USA). Dulbecco's phosphate-buffered saline (PBS), Dulbecco's modified Eagle's medium (DMEM), penicillin–streptomycin, and fetal bovine serum (FBS) were purchased from Gibco/BRL (Life Technologies, Gaithersburg, MD, USA). Ang II was obtained from Calbiochem (San Diego, CA, USA). ADMA, diacetyl monoxime, antipyrine, PD123319 and losartan were obtained from Sigma–Aldrich Co. (St. Louis, MO, USA). CCL5 was obtained from R&D systems (Minneapolis, MN). nor-NOHA was obtained from Cayman Chemical (Ann Arbor, Michigan, USA). LightCycler FastStart DNA SYBR Green I Mix was obtained from Roche (Mannheim, Germany). Goat anti-human DDAH-1, 12-LO, and ET-1 polyclonal antibodies were purchased from Santa Cruz Biotechnology (California, USA). Primer sequences for DDAH-1, DDAH-2, 12-LO, ET-1, Ang II subtype 2 receptor (AT_2 R), and β-actin were purchased from Bionics (Daejeon, South Korea). Rat AT_2 R and DDAH-1 siRNA sequences were purchased from Bioneer technology (Daejon, South Korea). All other reagents were pure-grade commercial preparations.

2.2. Animals and experimental protocols

Specific pathogen-free male inbred SHR or WKY, all aged 22–23 weeks, were purchased from Japan SLC Inc. (Shizuka, Japan). All experimental animals received autoclaved food and bedding in order to minimize exposure to viral and/or microbial pathogens. Rats were cared for in accordance with the Guide for the Care and Use of Experimental Animals of Yeungnam Medical Center.

2.3. Preparation of VSMCs

VSMCs were obtained from thoracic aortas of 22–23-week-old male SHR and WKY following the explant method as described by Kim et al. [15]. VSMCs were cultured in DMEM supplemented with 10% FBS and 1% penicillin–streptomycin. All experiments were conducted between cell passages three to six. Prior to stimulation, 95% confluent VSMCs were serum-starved overnight by incubation in DMEM with 0.1% FBS. Cell cultures were incubated

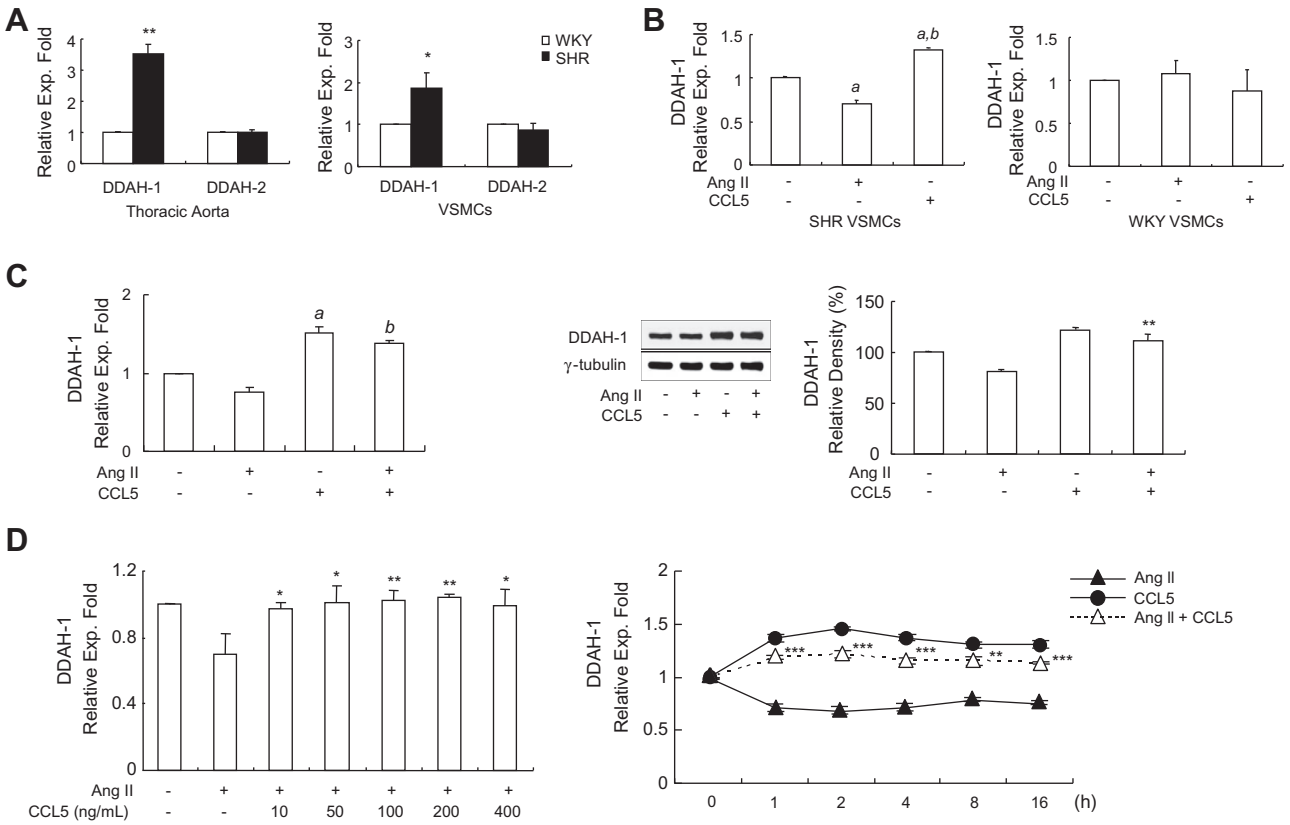

Fig. 1. CCL5 increases DDAH-1 expression and attenuates Ang II-induced DDAH-1 inhibition in SHR VSMCs. (A) After total RNAs were isolated from SHR or WKY thoracic aorta tissues and VSMCs, real-time PCR was performed. Relative transcription levels of DDAH-1 or DDAH-2 were normalized with the signal intensity of WKY thoracic aorta tissues or VSMCs. β-actin was used as an internal control. Bars represent the means ± SEM of three independent experiments. [a] $p < 0.01$ [**] $p < 0.01$ vs. WKY thoracic aorta tissues, and [*] $p < 0.05$ vs. WKY VSMCs. (B) SHR and WKY VSMCs were untreated or treated with Ang II (0.1 μmol/L) or CCL5 (100 ng/mL) for 2 h. After total RNAs were isolated from SHR or WKY thoracic aorta tissues and VSMCs, real-time PCR was performed. Relative transcription levels of DDAH-1 were normalized with the signal intensity of the untreated SHR or WKY VSMCs. β-actin was used as an internal control. Bars represent the means ± SEM of three independent experiments. [a] $p < 0.01$ vs. untreated SHR VSMCs, and [b] $p < 0.001$ vs. SHR VSMCs treated with Ang II. (C) SHR VSMCs were untreated or treated with Ang II (0.1 μmol/L) and/or CCL5 (100 ng/mL) for 2 h. Real-time PCR and immunoblotting were performed. Data shown are representative of three independent experiments. Bars represent the means ± SEM of three independent experiments. [a] $p < 0.01$ vs. untreated SHR VSMCs, and [b] $p < 0.01$ vs. SHR VSMCs treated with Ang II. In Western blotting analysis, each ratios were normalized by the ratio of untreated SHR VSMCs withγ-tubulin. γ-tubulin was used as an internal control. [**] $p < 0.01$ vs. SHR VSMCs treated with Ang II. (D) Dose response and time course of inhibitory effect of Ang II on DDAH-1 mRNA expression in response to CCL5 treatment in SHR VSMCs. SHR VSMCs were untreated or treated with Ang II (0.1 μmol/L) and 0, 10, 50, 100, 200, or 400 ng/mL of CCL5 simultaneously for 2 h. In the time course, SHR VSMCs were untreated or treated with Ang II (0.1 μmol/L) and/or CCL5 (100 ng/mL) for the indicated times. After total RNAs were isolated from SHR VSMCs, real-time PCR was performed. Relative transcription levels of DDAH-1 were normalized with the signal intensity of the untreated SHR VSMCs. β-actin was used as an internal control. Bars represent the means ± SEM of three independent experiments. [*] $p < 0.05$ vs. SHR VSMCs treated with Ang II, [**] $p < 0.01$ vs. SHR VSMCs treated with Ang II, and [***] $p < 0.001$ vs. SHR VSMCs treated with Ang II.

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in a humidified incubator at 37 °C and 5% CO_2 in the presence or absence of stimuli for the indicated times.

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

Total RNA was extracted using Trizol reagent according to the manufacturer's instructions. The quantity of total RNA was determined by measuring the optical density (OD) at 260 and 280 nm. One microgram of total RNA per sample was reverse-transcribed using Moloney murine leukemia virus reverse transcriptase (Perkin Elmer, Norwalk, CT, USA) with oligo dT priming at 42 °C for 15 min according to the manufacturer's instructions.

DDAH-1, DDAH-2, AT_2 R, 12-LO, or ET-1 was amplified by real-time PCR using LightCycler (Roche, Germany). RNA was reverse-transcribed to cDNA from 1 μg of total RNA and then subjected to real-time PCR. PCR reactions were performed in triplicate. The total PCR volume was 20 μL, and each PCR reaction consisted of LightCycler FastStart DNA SYBR Green I mix (Roche, Germany), 2 μL 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. β-actin expression levels were used for sample normalization. Results for each gene were expressed as the relative expression level compared with β-actin. Primers used for PCR were as follows: DDAH-1 (181 bp) sense, 5′-cgcaatagggtccagtgaat-3′, antisense, 5′-ttgcgctttctgggtactct-3′; DDAH-2 (336 bp) sense, 5′-gcaacgactaggtctgcagcttc-3′, antisense, 5′-ttctcatcccccatctccacaat-3′; AT_2 R (65 bp) sense, 5′-ccgtgaccaagtcttgaagatg-3′, antisense, 5′-agggaagccagcaaatgatg-3′; 12-LO (312 bp) sense, 5′-tggggcaactg-gaagg-3′, antisense, 5′-agagcgcttcagcaccat-3′; ET-1 (370 bp) sense, 5′-ctcctccttgatggacaagg-3′, antisense, 5′-cttgatgctgttgctgatgg-3′; and β-actin (101 bp) sense, 5′-tactgccctggctcctagca-3′, antisense, 5′-tggacagtgaggccaggatag-3′. The mRNA levels of DDAH-1, DDAH-2, AT_2 R, 12-LO, and ET-1 were determined by comparing experimental levels to standard curves and were expressed as relative fold expression levels.

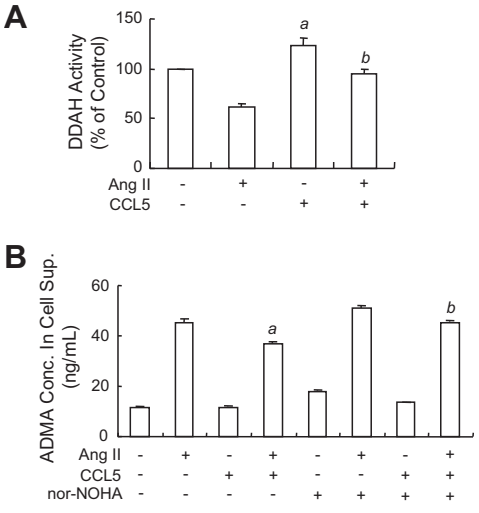

Fig. 2. CCL5 reduces Ang II-induced ADMA production through DDAH-1 activity. (A) SHR VSMCs were untreated or treated with Ang II (0.1 μmol/L) and/or CCL5 (100 ng/mL) for 2 h, DDAH activity was measured by converting ADMA to L-citrulline. Bars represent the means ± SEM of three independent experiments. [a] $p < 0.05$ vs. untreated SHR VSMCs and [b] $p < 0.01$ vs. SHR VSMCs treated with Ang II. (B) SHR VSMCs were untreated or treated with Ang II (0.1 μmol/L) and/or CCL5 (100 ng/mL) in the presence or absence of nor-NOHA (an inhibitor of DDAH-1 activity, 50 μmol/L) for 2 h. ELISA was performed to measure ADMA production. Bars represent the means ± SEM of three independent experiments. [a] $p < 0.01$ vs. SHR VSMCs treated with Ang II and [b] $p < 0.05$ vs. SHR VSMCs treated with Ang II and CCL5 simultaneously (Ang II/CCL5).

2.5. Measurement of DDAH Activity

DDAH activity was assayed as described by Ueda et al. [16]. Equal amounts of protein (20 μg) were 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 reaction was stopped by the addition of an equal volume of 4% sulfosalicylic acid, the supernatants (100 μL) were boiled with diacetyl monoxime (0.8% wt/vol in 5% acetic acid) and antipyrine (0.5% wt/vol in 50% sulfuric acid). The amounts of L-citrulline formed were determined with the spectrophotometric analysis at 466 nm (UV–Visible spectrophotometer, shimadzu UV-160, kyoto, Japan).

2.6. Western blotting

Total lysates were prepared in PRO-PREP buffer (iNtRON, Seoul, Korea). Protein concentrations were determined by Bradford assay (Bio-Rad, Hercules, CA, USA) using bovine serum albumin as a standard. 20 μg of protein samples were separated by 10% SDS-polyacrylamide gel electrophoresis and then transferred to nitrocellulose membranes. 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 DDAH-1, 12-LO, ET-1, and γ-tubulin at 4 °C. Membranes were then washed three times with TBST for 10 min and incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Finally, membranes were rinsed three times with TBST for 10 min, after which antigen–antibody complexes were detected using an enhanced chemiluminescence detection system (LAS-3000; Fujifilm, Tokyo, Japan).

2.7. Enzyme-linked immunosorbent assay (ELISA)

ADMA levels in cell culture supernatants were determined using an ELISA kit obtained from Uscn Life Science Inc. (Wuhan, China). All procedures were performed in accordance with the manufacturer's instructions.

2.8. Small interfering RNA (siRNA)

VSMCs were plated on 24-well plates and grown to 90% confluence. VSMCs were then transfected with DDAH-1, and AT_2 R siRNA oligomers (50 nmol/L) using lipofectamine 2000 in accordance with the manufacturer's instructions. After 24 h of incubation, VSMCs were placed in growth medium for 24 h before the experiments. Cells were then cultured in the presence or absence of stimuli for 2 h. Sense and antisense oligonucleotides used in these experiments were as follows: DDAH-1 siRNA sense, 5′-ucagagaga cugagucacu-3′, antisense, 5′-agugacucagucucucuga-3′; and AT_2 R siRNA sense, 5′-gaguguugauagguaccaa-3′, antisense, 5′-uugguac cuaucaacacuc-3′.

2.9. Statistical analysis

Results were expressed as the means ± SEM of at least three or four independent experiments. Statistical significance was determined by Student's *t*-test or one-way analysis of variance (ANOVA) followed by a Bonferroni test. A *P* value less than 0.05 was considered as statistically significant.

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3. Results

3.1. CCL5 increases DDAH-1 expression and attenuates Ang II-induced DDAH-1 inhibition in SHR VSMCs

We first compared the constitutive mRNA expression levels of DDAH-1 and DDAH-2 in SHR thoracic aorta tissues and VSMCs with those in WKY thoracic aorta tissues and VSMCs. Expression levels of DDAH-1 in both SHR thoracic aorta tissues and VSMCs were higher than those in WKY thoracic aorta tissues and VSMCs. However, expression levels of DDAH-2 were not significantly different between SHR and WKY thoracic aorta tissues or VSMCs (Fig. 1A). Next, the effects of CCL5 on DDAH-1 expression in SHR and WKY VSMCs were compared with those of Ang II in SHR and WKY VSMCs. Ang II has been shown to reduce DDAH activity in human umbilical vein endothelial cells [4]. In this study, whereas Ang II inhibited DDAH-1 expression in SHR VSMCs, CCL5 increased the mRNA level of DDAH-1. However, in WKY VSMCs, both Ang II and CCL5 showed no effects on DDAH-1 expression (Fig. 1B).

We next examined the direct effect of CCL5 on Ang II-induced DDAH-1 inhibition in SHR VSMCs. CCL5 attenuated the inhibitory effect of Ang II on DDAH-1 expression in SHR VSMCs (Fig. 1C). At the protein level, CCL5 also attenuated Ang II-induced inhibition of DDAH-1 protein production (Fig. 1C). In addition, we examined the dose response and time course of Ang II-induced DDAH-1 inhibition in response to CCL5 treatment. 10 ng/mL of CCL5 attenuated Ang II-induced DDAH-1 inhibition, and doses ranging from 10 ng/mL to 400 ng/mL similarly elevated DDAH-1 expression compared to SHR VSMCs treated with Ang II alone (Fig. 1D). The time course of Ang II-induced DDAH-1 inhibition in response to CCL5 treatment was determined over a 16 h time period. Attenuation of Ang II-induced DDAH-1 inhibition by CCL5 was detected 1 h after Ang II/CCL5 treatment and was sustained for up to 16 h. Increased DDAH-1 expression remained almost constant from 1 to 16 h upon Ang II/CCL5 treatment in SHR VSMCs (Fig. 1D).

3.2. CCL5 induces DDAH activity and decreases Ang II-induced ADMA production in SHR VSMCs

Although CCL5 was shown to induce DDAH-1 mRNA expression and protein production in SHR VSMCs, gene expression and protein production of DDAH-1 may not always be correlated with DDAH activity. Therefore, we confirmed DDAH activity in SHR VSMCs. DDAH activity was detected in SHR VSMCs treated with CCL5, and CCL5 increased DDAH activity in SHR VSMCs treated with Ang II (Fig. 2A).

Ang II directly stimulates the production of ADMA [17], which is metabolized by DDAH. Therefore, we examined whether or not

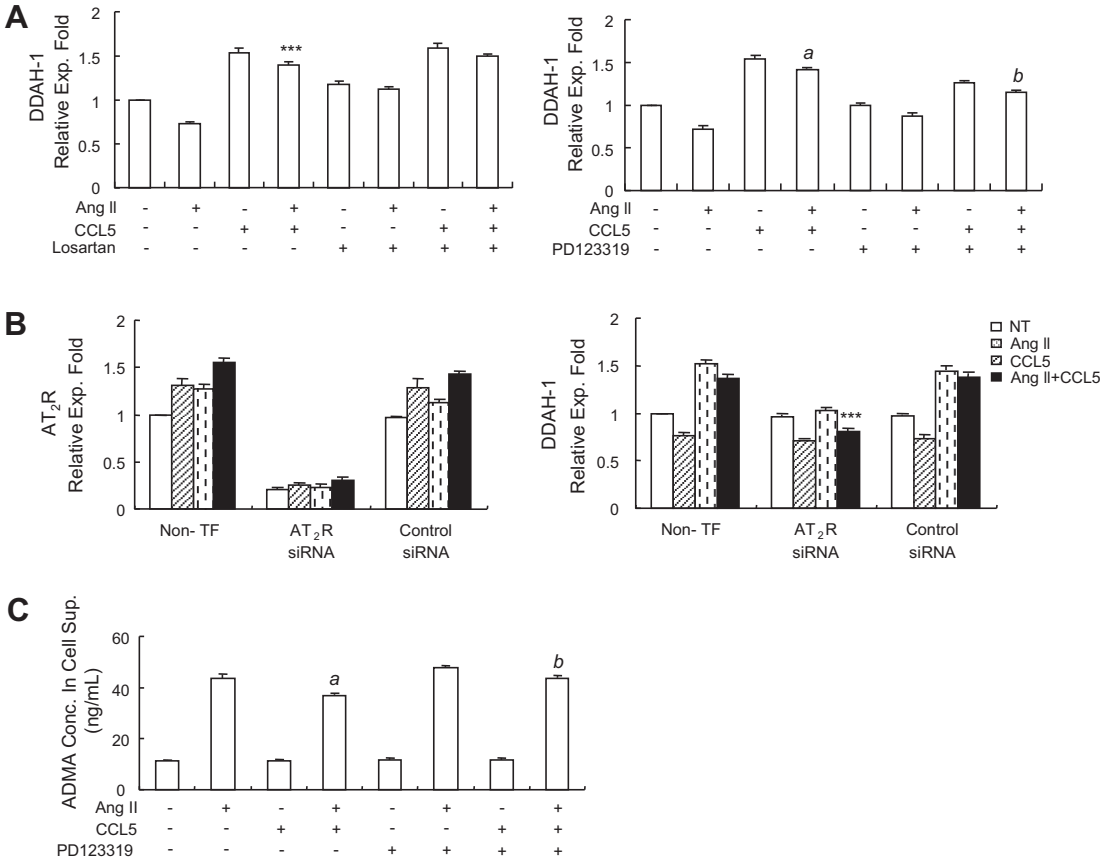

Fig. 3. Attenuation of Ang II-induced DDAH-1 inhibition by CCL5 is mediated through AT_2 R in SHR VSMCs. (A) SHR VSMCs were untreated or treated with Ang II (0.1 μmol/L) and/or CCL5 (100 ng/mL) in the presence or absence of losartan (AT_1 R antagonist, 10 μmol/L) or PD123319 (AT_2 R antagonist, 10 μmol/L) for 2 h. Relative transcription levels of DDAH-1 were normalized with the signal intensity of the untreated SHR VSMCs. β-actin was used as an internal control. Bars represent the means ± SEM of three independent experiments. *** $p < 0.001$ vs. SHR VSMCs treated with Ang II, [a] $p < 0.001$ vs. SHR VSMCs treated with Ang II and [b] $p < 0.01$ vs. SHR VSMCs treated with Ang II/CCL5. (B) SHR VSMCs were transfected with AT_2 R siRNA oligomers (50 nmol/L). Real-time PCR was performed on samples treated with AT_2 R-directed siRNA. Successful transfection of AT_2 R siRNA into SHR VSMCs was also confirmed by real-time PCR. Non-TF: non-transfected SHR VSMCs, TF: siRNA-transfected SHR VSMCs. Bars represent the means ± SEM of three independent experiments. *** $p < 0.001$ vs. control siRNA-transfected SHR VSMCs treated with Ang II/CCL5. (C) SHR VSMCs were untreated or treated with Ang II (0.1 μmol/L) and/or CCL5 (100 ng/mL) in the presence or absence of PD123319 (AT_2 R antagonist, 10 μmol/L) for 2 h. ELISA was performed to measure ADMA production. Bars represent the means ± SEM of three independent experiments. [a] $p < 0.01$ vs. SHR VSMCs treated with Ang II and [b] $p < 0.001$ vs. SHR VSMCs treated with Ang II/CCL5.

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attenuation of Ang II-induced DDAH-1 inhibition by CCL5 leads to reduction of Ang II-induced ADMA production. For this, SHR VSMCs were untreated or treated with Ang II (0.1 μmol/L) and/or CCL5 in the presence or absence of nor-NOHA (an inhibitor of DDAH-1 activity, 50 μmol/L) for 2 h, after which cell supernatants were isolated from cultures of SHR VSMCs and ADMA production measured by ELISA. CCL5 itself had no effect on ADMA production in SHR VSMCs. However, CCL5 attenuated Ang II-induced ADMA production, and nor-NOHA prevented the inhibitory effect of CCL5 on Ang II-induced ADMA production (36.6 ± 0.1 ng/mL in cells treated with Ang II/CCL5 vs. 45.5 ± 0.3 ng/mL in cells treated with Ang II/CCL5 plus nor-NOHA; $p < 0.01$, Fig. 2B).

3.3. Action mechanism of CCL5 on Ang II-induced DDAH-1 inhibition in SHR VSMCs

We also examined whether or not attenuation of Ang II-induced DDAH-1 inhibition by CCL5 is mediated through AT_1 R or AT_2 R. Attenuation of Ang II-induced DDAH-1 inhibition by CCL5 was reduced by the AT_2 R antagonist PD123319 but not the AT_1 R antagonist losartan (Fig. 3A). Further, losartan blocked Ang II-induced inhibition of DDAH-1 expression, whereas it had no effect on CCL5 or Ang II/CCL5-induced DDAH-1 mRNA expression. On the other hand, the inducible effect of CCL5 alone on DDAH-1 expression was inhibited by PD123319 (Fig. 3A). To confirm these results, real-time PCR was performed on samples treated with AT_2 R-directed siRNA. In VSMCs transfected with AT_2 R siRNA, attenuation of Ang II-induced DDAH-1 inhibition by CCL5 was remarkably reduced (Fig. 3B). We also observed the effect of PD123319 on attenuation of Ang II-induced ADMA production by CCL5. Whereas PD123319 had no effect on Ang II-induced ADMA production, it reversed the attenuation of Ang II-induced ADMA production by CCL5 (Fig. 3C).

3.4. Inhibitory effects of CCL5 on Ang II-induced hypertensive mediators are related to DDAH-1 activity induced by CCL5

We next investigated the relationship between the inhibitory effects of CCL5 on Ang II-induced hypertensive mediators (12-LO and ET-1) and DDAH-1 activity stimulated by CCL5 in SHR VSMCs. Although an inhibitor of DDAH-1 activity, nor-NOHA only weakly attenuated the inhibitory effect of CCL5 on Ang II-induced 12-LO mRNA expression, the change was still statistically significant (relative expression fold of 12-LO: 2.55 ± 0.03 in cells treated with Ang II/CCL5 with nor-NOHA vs. 2.24 ± 0.02 in cells treated with Ang II/CCL5; $p < 0.05$, Fig. 4A). Additionally, the rate of reduction of Ang II-induced 12-LO expression by CCL5 in DDAH-1 siRNA-transfected SHR VSMCs was reduced to 26% ± 1% compared to 36% ± 1% in non-transfected SHR VSMCs (Fig. 4B). In case of ET-1 expression, CCL5 itself did not affect ET-1 mRNA expression or protein production. On the other hand, CCL5 significantly inhibited Ang II-induced ET-1 mRNA expression. Further, the inhibitory effect of CCL5 on

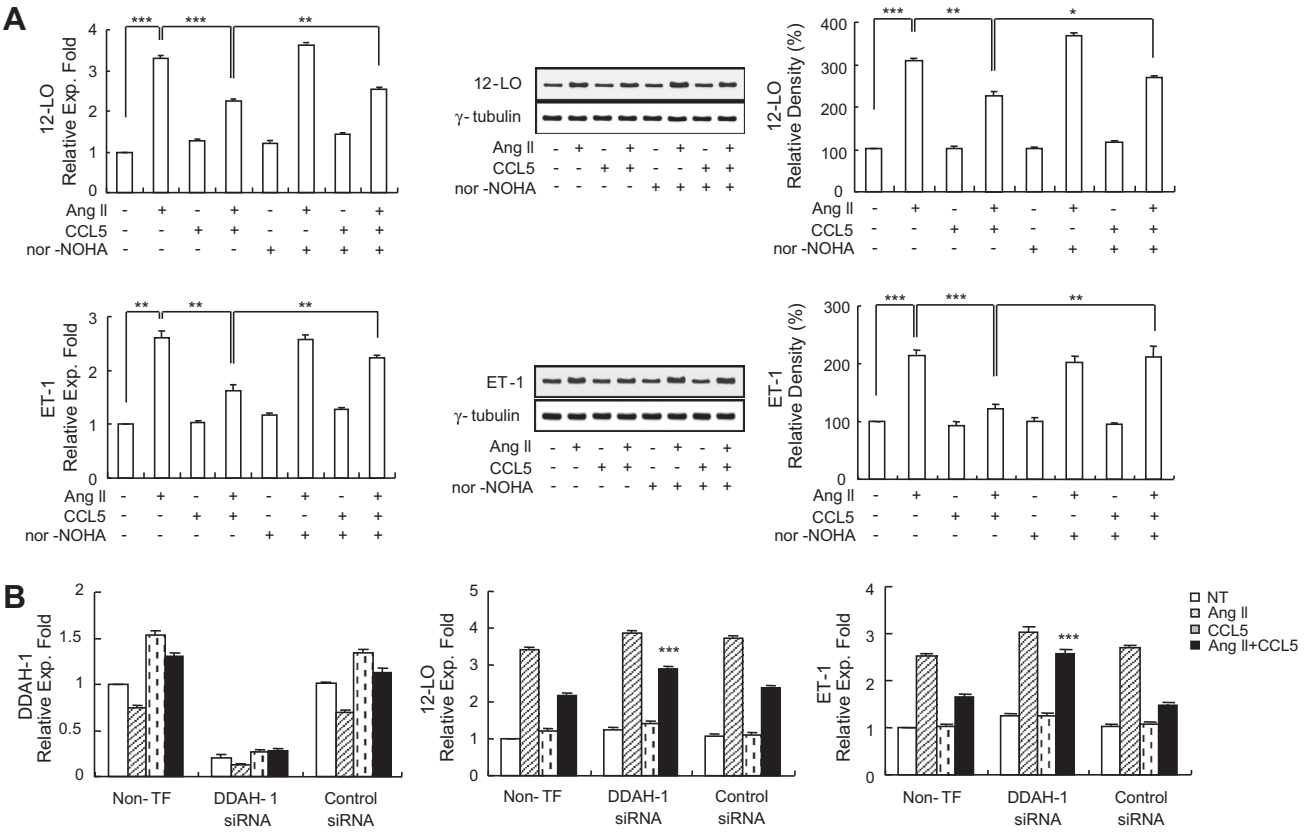

Fig. 4. DDAH-1 induction by CCL5 reduces Ang II-induced 12-LO and ET-1 expression in SHR VSMCs. (A) SHR VSMCs were untreated or treated with Ang II (0.1 μmol/L) and/or CCL5 (100 ng/mL) in the presence or absence of nor-NOHA (an inhibitor of DDAH-1 activity, 50 μmol/L) for 2 h. Real-time PCR and immunoblotting were performed. Data are representative of three independent experiments. Relative transcription levels of 12-LO or ET-1 were normalized with the signal intensity of the un-treated SHR VSMCs. β-actin was used as an internal control Bars represent the means ± SEM of three independent experiments. * $p < 0.05$ vs. SHR VSMCs treated with Ang II/CCL5, ** $p < 0.01$ vs. untreated SHR VSMCs, or SHR VSMCs treated with Ang II or Ang II/CCL5, and *** $p < 0.001$ vs. untreated SHR VSMCs or SHR VSMCs treated with Ang II. In Western blotting analysis, each ratios were normalized by the ratio of untreated SHR VSMCs with γ-tubulin. γ-tubulin was used as an internal control. (B) SHR VSMCs were transfected with DDAH-1 siRNA oligomers (50 nmol/L). Successful transfection of DDAH-1 siRNA into SHR VSMCs was also confirmed by real-time PCR. Non-TF: non-transfected VSMCs, TF: siRNA-transfected VSMCs. Bars represent the means ± SEM of three independent experiments. *** $p < 0.001$ vs. control siRNA-transfected SHR VSMCs treated with Ang II/CCL5.

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Ang II-induced ET-1 expression was attenuated by nor-NOHA (Fig. 4A). An additional experiment involving siRNA-mediated down-regulation of DDAH-1 confirmed this result. The rate of reduction of Ang II-induced ET-1 expression by CCL5 in DDAH-1 siRNA-transfected SHR VSMCs was reduced to 16 ± 2% compared to 40 ± 1% in non-transfected SHR VSMCs (Fig. 4B).

4. Discussion

DDAH-1 is widely expressed in the aorta, forebrain, pancreas besides liver, and kidney at sites of NOS expression [18]. DDAH-2 is expressed predominantly in blood vessels and the endothelium at sites of eNOS expression, in addition to the heart, placenta, and kidney [18]. In the present study, expression of DDAH-1 in SHR thoracic aorta tissues and VSMCs was up-regulated compared to that in WKY thoracic aorta tissues and VSMCs. In contrast to DDAH-1, expression of DDAH-2 was not significantly different between SHR and WKY thoracic aorta tissues and VSMCs. In addition, CCL5 directly increased expression of DDAH-1 but not DDAH-2 (data not shown) in SHR VSMCs. Therefore, it can be speculated that DDAH-1 rather than DDAH-2 plays an active role in SHR VSMCs. High expression of DDAH-1 in SHR thoracic aorta tissues and VSMCs might compensate for reduced ADMA degradation in the SHR vasculature.

Inhibition of DDAH activity results in ADMA accumulation and vascular damage [3,4]. In our results, CCL5 induced DDAH activity in SHR VSMCs and increased DDAH activity in SHR VSMCs treated with Ang II. Additionally, the inhibitory effect of CCL5 on Ang II-induced ADMA production was attenuated by nor-NOHA. These results indicate that DDAH activity induced by CCL5 attenuates Ang II-induced ADMA production.

Ang II has two subtype receptors, AT_1 R and AT_2 R, with the density of AT_2 R lower than that of AT_1 R in VSMCs [19]. AT_1 R mediates the major stimulatory actions of Ang II, including vasoconstriction, cell proliferation, aldosterone secretion, and sodium retention [20]. In contrast, AT_2 R has been reported to antagonize the vascular actions of AT_1 R [19,21]. In our study, Ang II-induced DDAH-1 inhibition was mediated by the AT_1 R pathway, not the AT_2 R pathway. On the other hand, CCL5-induced DDAH-1 expression was mediated by the AT_2 R pathway. The attenuating effect of CCL5 on Ang II-induced DDAH-1 inhibition was reversed by PD123319, not by losartan. In our previous study, the inhibitory effect of CCL5 on Ang II-induced 12-LO mRNA expression was also mediated via the AT_2 R pathway, and CCL5 significantly increased AT_2 R expression in SHR VSMCs [11]. Therefore, the AT_2 R pathway is thought to play a major role in the interaction between CCL5 and Ang II in SHR VSMCs.

The activities of both 12-LO and ET-1 have been linked to the development of hypertension [22,23]. ET-1 is characterized as a potent vasoconstrictor secreted by the endothelium, and it participates in the regulation of vascular tone [24]. Proinflammatory chemokine CXCL8 increases ET-1 expression in SHR VSMCs and mouse peritoneal macrophages [25]. In this study, CCL5 remarkably inhibited Ang II-induced ET-1 expression and this inhibitory effect of CCL5 was associated with increased DDAH-1 activity. Additionally, the inhibitory effect of CCL5 on Ang II-induced 12-LO expression was partly associated with DDAH-1 activity induced by CCL5. Therefore, although CCL5 plays functional roles in acute and chronic inflammatory responses in the progression of various diseases [9,10], it is obvious that CCL5 more likely plays an attenuating role in Ang II-induced vascular hypertension.

There has been little research demonstrating the effects of CCL5 on DDAH activity or Ang II-induced hypertensive mediators in SHR VSMCs. This study provides the first evidence that CCL5 directly stimulates DDAH-1 production and attenuates the inhibitory effect of Ang II on DDAH-1 expression, which is mediated by the AT_2 R pathway. Further, CCL5-induced DDAH-1 activity inhibits Ang II-induced expression of 12-LO and ET-1 in SHR VSMCs. Although further *in vivo* studies should be performed, these findings combined with our previous results suggest that CCL5 is a potential down-regulatory factor in Ang II-induced vascular hypertension.

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