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# Dimethylarginine dimethylaminohydrolase-1 mediates inhibitory effect of interleukin-10 on angiotensin II-induced hypertensive effects in vascular smooth muscle cells of spontaneously hypertensive rats

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ABSTRACT

In hypertension studies, anti-inflammatory cytokine interleukin-10 (IL-10) has been shown to prevent angiotensin II (Ang II)-induced vasoconstriction and regulate vascular function by down-regulating pro-inflammatory cytokine and superoxide production in vascular cells. However, little is known about the mechanism behind the down-regulatory effect of IL-10 on Ang II-induced hypertensive mediators. In this study, we demonstrated the effects of IL-10 on expression of dimethylarginine dimethylaminohydrolase (DDAH)-1, a regulator of NO bioavailability, as well as the down-regulatory mechanism of action of IL-10 in relation to Ang II-induced hypertensive mediator expression and cell proliferation in vascular smooth muscle cells (VSMCs) from spontaneously hypertensive rats (SHR). IL-10 increased DDAH-1 but not DDAH-2 expression and increased DDAH activity. Additionally, IL-10 attenuated Ang II-induced DDAH-1 inhibition in SHR VSMCs. Increased DDAH activity due to IL-10 was mediated mainly through Ang II subtype II receptor ($AT_2$ R) and AMP-activated protein kinase (AMPK) activation. DDAH-1 induced by IL-10 partially mediated the inhibitory action of IL-10 on Ang II-induced 12-lipoxygenase (LO) and endothelin (ET)-1 expression in SHR VSMCs. In addition, the inhibitory effect of IL-10 on proliferation of Ang II-induced VSMCs was mediated partially via DDAH-1 activity. These results suggest that DDAH-1 plays a potentially important role in the anti-hypertensive activity of IL-10 during Ang II-induced hypertension.



## 1. Introduction

Interleukin-10 (IL-10) is known to have potent, broad down-regulatory effects on expression of pro-inflammatory cytokines in various immune and vascular cells [3–5] and plays an important role in immune homeostasis [1–4]. Vascular inflammation is involved in vascular hypertension and is potently inhibited by IL-10 [3,4]. Specifically, IL-10 inhibits elevation of vascular superoxide levels and activation of NF-kB, and many of its anti-inflammatory effects are mediated via this mechanism [5]. Studies have shown that plasma levels of IL-10 are reduced in hypertensive rats, and IL-10 knockout mice show vascular structure damage and remodeling [6,7]. Additionally, IL-10 knockout mice with Ang II-induced hypertension show increased vascular dysfunction [4]. In experimental hypertensive rats, IL-10 prevents Ang II-induced inflammation and improves microvascular endothelial function via inhibition of NADPH oxidase activity and elevation of endothelial nitric oxide synthase (eNOS) activity [4,8,9].

Dimethylarginine dimethylaminohydrolase (DDAH) is a key enzyme metabolizing plasma asymmetric ($N^G$, $N^G$) dimethylarginine (ADMA), an inhibitor of eNOS [10]. Plasma levels of ADMA are elevated in patients with hypertension [11]. Inhibition of DDAH activity induces accumulation of ADMA, resulting in vascular damage and increased blood pressure through inhibition of NO-mediated blood vessel relaxation [12]. DDAH exists as two isoforms, DDAH-1 and DDAH-2 [13]. DDAH-1 is widely expressed in the liver, kidney, aorta, and pancreas at sites of NOS expression, whereas DDAH-2 predominates in the vascular endothelium and is widely expressed in the heart, placenta, and immune tissues [10,14]. Although DDAH-1 expression has been observed in the endothelium, DDAH-2 is the predominant isoform expressed in blood vessels. Plasma levels of ADMA are regulated by DDAH-1, whereas DDAH-2 acts more importantly to preserve endothelial function in blood vessel resistance. DDAH-1 +/− mice show reduced DDAH-1 expression along with unaltered DDAH-2 expression, leading to pulmonary hypertension [10].

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Although IL-10 has been shown to play protective and regulatory roles in vascular hypertension, its function is mainly anti-inflammatory in the vascular system through inhibition of pro-inflammatory cytokines and chemokines. Additionally, although both IL-10 and DDAH-1 have down-regulatory effects on vascular dysfunction, the potential interactive synergy between the two remains unknown. Therefore, the present study focused on the effect of IL-10 on DDAH-1 as well as mechanisms underlying its down-regulatory action in SHR VSMCs.

## 2. Materials and methods

### 2.1. Reagents

Total RNA extraction kit was purchased from iNtRON (Biotechnology, Seoul, Korea). Ang II was obtained from Calbiochem (San Diego, CA, USA). PD123319 and Compound C were obtained from Sigma–Aldrich Co. (St. Louis, MO, USA). IL-10 was obtained from R&D systems (Minneapolis, MN, USA). Nor-NOHA was obtained from Cayman Chemical (Ann Arbor, Michigan, USA). LightCycler FastStart DNA SYBR Green I Mix was obtained from Roche (Mannheim, Germany). Lipofectamine 2000 was obtained from Invitrogen (Carlsbad, CA, USA). Primer sequences for Ang II subtype I receptor ($AT_1$ R), Ang II subtype II receptor ($AT_2$ R), DDAH-1, DDAH-2, 12-LO, ET-1, AMP-activated protein kinase (AMPK) α1, AMPKα2, and β-actin were synthesized at Bionics (Daejeon, South Korea). Goat anti-DDAH-1 antibody was purchased from Santa Cruz Biotechnology (California, USA). Monoclonal anti-γ-tubulin antibody was purchased from Sigma–Aldrich (St. Louis, MO, USA). Rat $AT_1$ R, $AT_2$ R, AMPK, and DDAH-1 siRNA sequences were purchased from Bioneer Technology (Daejeon, South Korea).

### 2.2. Animals

Specific pathogen-free male inbred spontaneously hypertensive rats (SHR), all aged 13-weeks, were purchased from Japan SLC Inc. (Shizuka, Japan). All experimental animals were fed autoclaved food and received bedding in order to minimize exposure to 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 isolated from thoracic aortas of 13-week-old male SHR rats following the explant method [15]. VSMCs were cultured in DMEM supplemented with 10% FBS and 1% penicillin–streptomycin. Cells were detached with 0.25% trypsin/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 cultures 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 isolated using an easy-BLUE total RNA extraction kit (iNtRON Biotechnology, Seoul, Korea). $AT_1$ R, $AT_2$ R, DDAH-1, DDAH-2, 12-LO, ET-1, AMPKα1, and AMPKα2 cDNAs were obtained from VSMCs by reverse transcription of 1 μg of total RNA. cDNAs were then subjected to real-time PCR using a LightCycler with a FastStart DNA Master SYBR Green kit. PCR amplification was performed as described by Kim et al. [15]. The primers used for PCR were as follows: $AT_1$ R (445 bp) sense, 5′-cacctatgtaagatcgcttc-3′, antisense, 5′-gcacaatcgccataattatcc-3′; $AT_2$ R (65 bp) sense, 5′-ccg tgaccaagtcttgaagatg-3′, antisense, 5′-agggaagccagcaaatgatg-3′; DDAH-1 (181 bp) sense, 5′-cgcaatagggtccagtgaat-3′, antisense, 5′-ttgcgctttctgggtactct-3′; DDAH-2 (336 bp) sense, 5′-gcaacgac taggtctgcagcttc-3′, antisense, 5′-ttctcatcccccatctccacaat-3′; 12-LO (312 bp) sense, 5′-tggggcaactggaagg-3′, antisense, 5′-agagcgcttcag caccat-3′; ET-1 (370 bp) sense, 5′-ctcctccttgatggacaagg-3′, antisense, 5′-cttgatgctgttgctcatgg-3′; AMPKα1 (180 bp) sense, 5′-gcaga gagatccagaacctg-3′, antisense, 5′-ctccttttcgtccaaccttcc-3′; AMPKα2 (222 bp) sense, 5′-gctctcgatcgccaaattat-3′, antisense, 5′-gcatcagca gagtggcaata-3′; and β-actin (101 bp) sense, 5′-tactgccctggctcc tagca-3′, antisense, 5′-tggacagtgaggccaggatag-3′. mRNA level of each sample was normalized to the mRNA level of β-actin, a housekeeping gene.

### 2.5. Measurement of DDAH activity

DDAH activity was measured according to the instructions of Ueda et al. [16]. Equal amounts of protein (20 μg) were incubated with 4 mmol/L of ADMA-0.1 mol/L of 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 addition of an equal volume of 4% sulfosalicylic acid, 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 by spectrophotometric analysis at 466 nm (UV–Visible spectrophotometer, Shimadzu UV-160, kyoto, Japan).

### 2.6. Enzyme-linked immunosorbent assay (ELISA)

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

### 2.7. Western blotting

Western blots were performed on cytoplasmic protein extracts from VSMCs using polyclonal antibody against DDAH-1 (dilution 1/200) and monoclonal antibody against γ-tubulin (dilution 1/2000) as described by Kim et al. [15].

### 2.8. Small interfering RNA (siRNA)

VSMCs were plated on 6-well plates and grown to 90% confluence. VSMCs were then transfected with $AT_1$ R, $AT_2$ R, AMPK, and DDAH-1 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 1 h. Sense and antisense oligonucleotides used in these experiments were as follows: $AT_1$ R siRNA sense, 5′-gucacuguuacuacaccua-3′, antisense, 5′-uagguguaguaacagugac-3′; $AT_2$ R siRNA sense, 5′-gaguguugauagguaccaa-3′, antisense, 5′-uug guaccuaucaacacuc-3′; AMPK siRNA sense, 5′-cgugugaagaucgga cacu-3′, antisense, 5′-aguguccgaucuucacacg-3′; and DDAH-1 siRNA sense, 5′-ucagagagacugagucacu-3′, antisense, 5′-agugacucagucucu cuga-3′.

### 2.9. VSMCs proliferation

VSMCs were plated on 24-well plates with serum-free medium for 24 h and then exposed to the stimulant. [$^3$H]-thymidine (1 μCi/mL) (Perkin Elmer precisely, Boston, MA, USA) was added to plates during the last 24 h of incubation. Cells were subsequently washed

three times with cold PBS. [$^3$H]-thymidine-labeled cells were collected with 0.1% SDS, and radioactivity was counted using a Packard scintillation counter (Packard Instrument Company, Meriden, CT, USA).

### 2.10. 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. A *P* value less than 0.05 was considered as statistically significant. Statistical analysis was performed using SPSS v. 22.0 (IBM Corp., Armonk, NY, USA).

## 3. Results

### 3.1. IL-10 up-regulates DDAH-1 expression and DDAH activity as well as attenuates Ang II-induced DDAH-1 inhibition in SHR VSMCs

We first examined the direct effect of IL-10 on DDAH-1 expression in SHR VSMCs. IL-10 increased DDAH-1 mRNA expression but had no effect on DDAH-2 mRNA expression (Fig. 1A). Production of DDAH-1 protein by IL-10 was also detected (Fig. 1B). Although IL-10 was shown to increase DDAH-1 mRNA expression and protein production in SHR VSMCs, expression of DDAH-1 may not always be correlated with DDAH activity. Therefore, we confirmed DDAH activity in SHR VSMCs. Increased DDAH activity was detected in SHR VSMCs treated with IL-10 (Fig. 1C). Next, we examined the effect of IL-10 on Ang II-induced DDAH-1 inhibition in SHR VSMCs. IL-10 attenuated the inhibitory effect of Ang II on DDAH-1 mRNA expression and elevated DDAH activity in SHR VSMCs treated with Ang II (Fig. 2A). To confirm this result, we examined whether or not attenuation of Ang II-induced DDAH-1 inhibition by IL-10 corresponds with reduction of Ang II-induced ADMA production. ADMA is an endogenous inhibitor of endothelial nitric oxide synthase (eNOS) that is metabolized by DDAH, and its production is directly stimulated by Ang II [17]. IL-10 had no effect on ADMA production in SHR VSMCs. However, Ang II-induced ADMA production was reduced by IL-10 (Fig. 2B). In addition, we examined the dose response and time course of Ang II-induced DDAH-1 inhibition in response to IL-10 stimulation. Treatment with IL-10 (25 ng/mL) attenuated Ang II-induced DDAH-1 inhibition, and doses ranging from 25 to 100 ng/mL similarly elevated DDAH-1 expression compared to SHR VSMCs treated with Ang II alone (Fig. 2C). The time course of Ang II-induced DDAH-1 inhibition in response to IL-10 stimulation was observed over a 16 h time period. The increased DDAH-1 expression level in SHR VSMCs treated with IL-10 remained almost constant from 1 to 16 h upon IL-10 treatment in SHR VSMCs. Attenuation of Ang II-induced DDAH-1 inhibition by IL-10 was also detected 1 h after Ang II/IL-10 treatment and was sustained for up to 16 h (Fig. 2C).

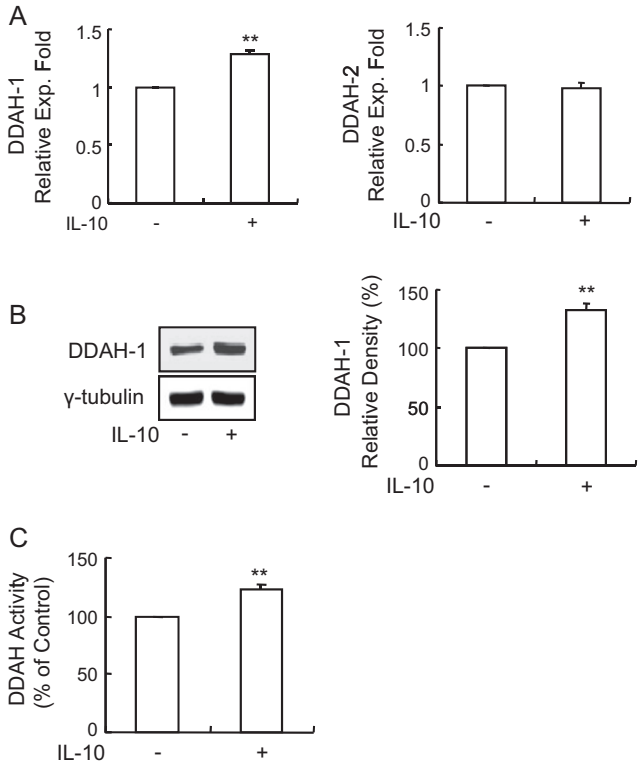


**Fig. 1.** IL-10 increases DDAH-1 expression in SHR VSMCs. SHR VSMCs were treated with or without IL-10 (25 ng/mL) for 1 h. After total RNAs and cell lysates were prepared, real-time PCR (A), immunoblotting (B), and measurement of DDAH activity (C) were performed. DDAH activity was measured by converting ADMA to L-citrulline. Data shown are representative of three independent experiments. Bars represent the means ± SEM of three independent experiments. $^{**}p < 0.01$ vs. untreated SHR VSMCs.

### 3.2. Mechanism of action of IL-10 in relation to DDAH-1 expression in SHR VSMCs

We next examined whether or not attenuation of Ang II-induced DDAH-1 inhibition by IL-10 is mediated by $AT_1$ R or $AT_2$ R. Real-time PCR was performed on samples treated with $AT_1$ R or $AT_2$ R-directed siRNA. Attenuation of Ang II-induced DDAH-1 inhibition by IL-10 was not reduced in VSMCs transfected with $AT_1$ R siRNA. However, attenuation of Ang II-induced DDAH-1 inhibition by IL-10 was remarkably reduced in SHR VSMCs transfected with $AT_2$ R siRNA. Elevation of DDAH-1 expression by IL-10 was also inhibited (Fig. 3A). We also investigated the effect of PD123319, an antagonist of $AT_2$ R, on attenuation of Ang II-induced inhibition of DDAH activity by IL-10. Although PD123319 had no effect on Ang II-induced inhibition of DDAH activity, it reduced elevation of IL-10-induced DDAH activity as well as attenuation of Ang II-induced inhibition of DDAH activity by IL-10 in SHR VSMCs (Fig. 3B). Additionally, the effect of PD123319 on reduction of Ang II-induced ADMA production by IL-10 was examined. PD123319 had no effect on Ang II-induced ADMA production, whereas it reduced the inhibitory effect of IL-10 on Ang II-induced ADMA production (Fig. 3C).

Activation of AMPK results in improvement of vascular abnormalities [17]. Therefore, we examined whether or not the up-regulatory effect of IL-10 on DDAH-1 expression is mediated through AMPK activation in SHR VSMCs. For this, real-time PCR was performed on samples transfected with AMPK-directed siRNA. In SHR VSMCs transfected with AMPK siRNA, IL-10-induced DDAH-1 mRNA expression as well as attenuation of Ang II-induced DDAH-1 mRNA inhibition by IL-10 were remarkably reduced (Fig. 4A). In the case of DDAH activity, the AMPK inhibitor Compound C inhibited the up-regulatory effect of IL-10 on DDAH activity in SHR VSMCs, whereas it had no effect on Ang II-induced inhibition of DDAH activity. Attenuation of Ang II-induced inhibition of DDAH activity by IL-10 was also significantly reduced by Compound C (Fig. 4B). Lastly, Compound C reduced the inhibitory effect of IL-10 on Ang II-induced ADMA production (Fig. 4C).

### 3.3. Inhibitory effects of IL-10 on Ang II-induced hypertensive mediators are related to induction of DDAH-1

Activities of 12-LO and ET-1 are related to the development of hypertension [18,19]. IL-10 is known to reduce Ang II-induced 12-LO and ET-1 expression in SHR VSMCs [20]. Therefore, we

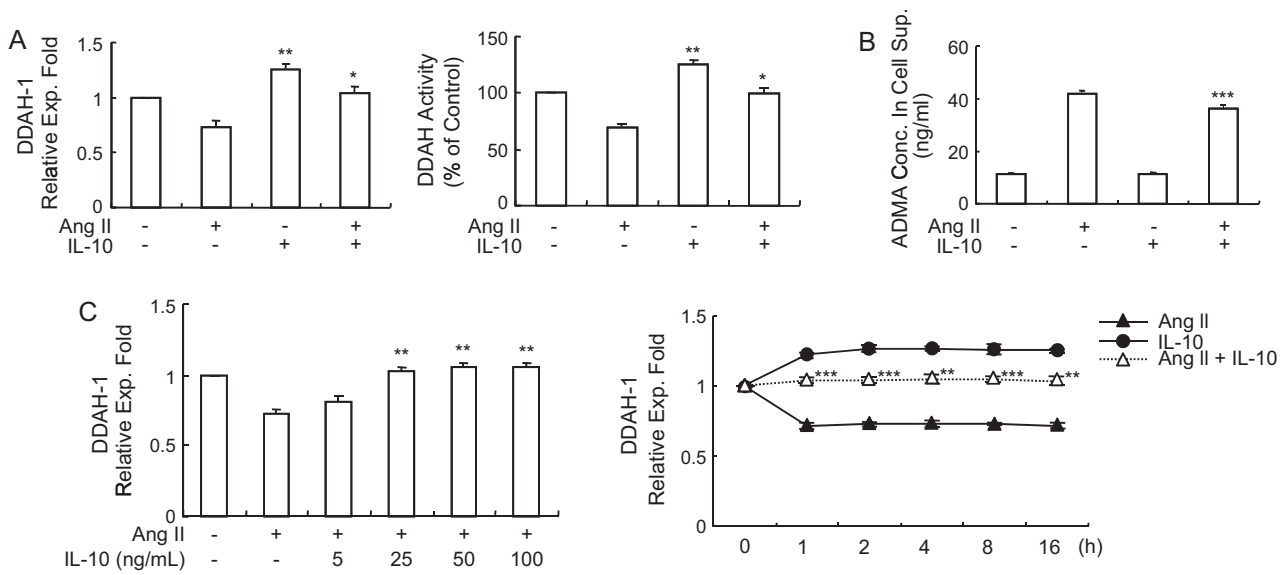


**Fig. 2.** IL-10 attenuates Ang II-induced DDAH-1 inhibition in SHR VSMCs. (A) SHR VSMCs were treated with or without Ang II (0.1 μmol/L) and/or IL-10 (25 ng/mL) for 1 h. After total RNAs and cell lysates were prepared, real-time PCR and measurement of DDAH activity were performed. $^{*}p < 0.05$ vs. SHR VSMCs treated with Ang II. $^{**}p < 0.01$ vs. untreated SHR VSMCs. (B) SHR VSMCs were untreated or treated with Ang II (0.1 μmol/L) and/or IL-10 (25 ng/mL) for 1 h, after which ADMA levels in cell culture supernatants were determined by ELISA. $^{***}p < 0.001$ vs. SHR VSMCs treated with Ang II. (C) SHR VSMCs were treated with or without Ang II (0.1 μmol/L) and 5, 25, 50, and 100 ng/mL of IL-10 simultaneously (Ang II/IL-10) for 1 h. After total RNAs were prepared, real-time PCR was performed. For the time course reaction, SHR VSMCs were treated with or without Ang II (0.1 μmol/L) and/or IL-10 (25 ng/mL) for the indicated times. After total RNAs were isolated, real-time PCR was performed. Bars represent the means ± SEM of three independent experiments. $^{**}p < 0.01$ vs. SHR VSMCs treated with Ang II. $^{***}p < 0.001$ vs. SHR VSMCs treated with Ang II.

investigated whether or not DDAH-1 mediates the inhibitory effect of IL-10 on Ang II-induced 12-LO and ET-1 expression in SHR VSMCs. nor-NOHA, an inhibitor of DDAH-1 activity, reduced the inhibitory effect of IL-10 on Ang II-induced 12-LO and ET-1 mRNA expression (Fig. 5A). Additionally, the rate of reduction of Ang II-induced 12-LO expression by IL-10 in DDAH-1 siRNA-transfected SHR VSMCs was reduced to 12.1% ± 1.3% compared to 28.0% ± 2.0% in control siRNA-transfected SHR VSMCs (Fig. 5B). In the case of ET-1 expression, the rate of reduction of Ang II-induced ET-1 expression by IL-10 in DDAH-1 siRNA-transfected SHR VSMCs was reduced to 9.2% ± 0.7% compared to 17.8% ± 1.5% in control siRNA-transfected SHR VSMCs (Fig. 5B).

### *3.4. DDAH-1 partially mediates inhibitory effect of IL-10 on Ang II-induced VSMC proliferation*

IL-10 inhibits smooth muscle cell migration and proliferation of VSMCs [21,22]. Thus, we also examined whether or not DDAH-1 activation mediates the inhibitory effect of IL-10 on Ang II-induced VSMC proliferation. IL-10 alone did not affect VSMC proliferation. However, IL-10 reduced Ang II-induced VSMC proliferation, and this was significantly reversed by nor-NOHA (Fig. 6A). In the case of SHR VSMCs transfected with DDAH-1 siRNA, the rate of reduction of Ang II-induced VSMC proliferation by IL-10 in DDAH-1 siRNA-transfected SHR VSMCs was reduced to 13.8% ± 1.3% compared to 30.1% ± 6.8% in control siRNA-transfected SHR VSMCs (Fig. 6B).

## 4. Discussion

DDAH-1, not DDAH-2, regulates plasma ADMA levels [10]. Expression of DDAH-1 in thoracic aorta tissues and VSMCs from hypertensive rats is up-regulated compared to that in normotensive rats [23]. In contrast to DDAH-1, expression of DDAH-2 is not significantly different between SHR and WKY thoracic aorta tissues and VSMCs [23]. Moreover, IL-10 directly increases expression of DDAH-1 but not DDAH-2 in SHR VSMCs. Therefore, in the present study, we focused on expression of DDAH-1, not DDAH-2, in SHR VSMCs.

In general, the interaction between a cytokine and its receptor at the cell wall is the most important determinant of the outcomes exerted by the cytokine. Ang II plays a major role in the regulation of blood pressure via two subtype receptors, $AT_1$ R and $AT_2$ R. The density of $AT_1$ R is higher than that of $AT_2$ R in VSMCs [24]. $AT_1$ R mediates the major effective actions of Ang II, including inflammatory responses, vasoconstriction, cell proliferation, and sodium retention [25]. Ang II stimulates pro-inflammatory cytokine and chemokine production through $AT_1$ R [26]. In contrast, $AT_2$ R is known to antagonize the vascular actions of $AT_1$ R [24]. Activation of $AT_2$ R plays a defensive role in the development of deoxycorticosterone acetate/salt-induced hypertension [27]. However, $AT_2$ R activation does not have a lowering effect on blood pressure in male SHR [28]. Specifically, $AT_2$ R plays a sex-specific role in regulating blood pressure, with a positive effect in females [29]. Therefore, the diverse actions of $AT_2$ R are still controversial. In the present study, Ang II-induced DDAH-1 inhibition was not mediated by either the $AT_1$ R or $AT_2$ R pathway. On the other hand, IL-10-induced DDAH-1 expression was mediated by the $AT_2$ R pathway, and attenuation of Ang II-induced inhibition of DDAH activity by IL-10 was inhibited by PD123319. The ability of $AT_2$ R to stimulate IL-10-induced DDAH-1 expression in hypertensive rats has not been investigated to date. However, the down-regulatory effects of CCL5 on Ang II-induced DDAH-1 inhibition and Ang II-induced hypertensive mediator expression are also known to be mediated via the $AT_2$ R pathway [23]. In addition, THP-1 macrophages treated with lipopolysaccharide and $AT_2$ R agonist have been shown to display increased IL-10 production [26]. Therefore, the $AT_2$ R pathway is thought to play an important role in the anti-hypertensive actions of IL-10.

The relationship between DDAH-1 and AMPK activation has not been well evaluated in hypertensive rats. AMPK is known to play a central role in cellular energy homeostasis [30]. Although AMPK

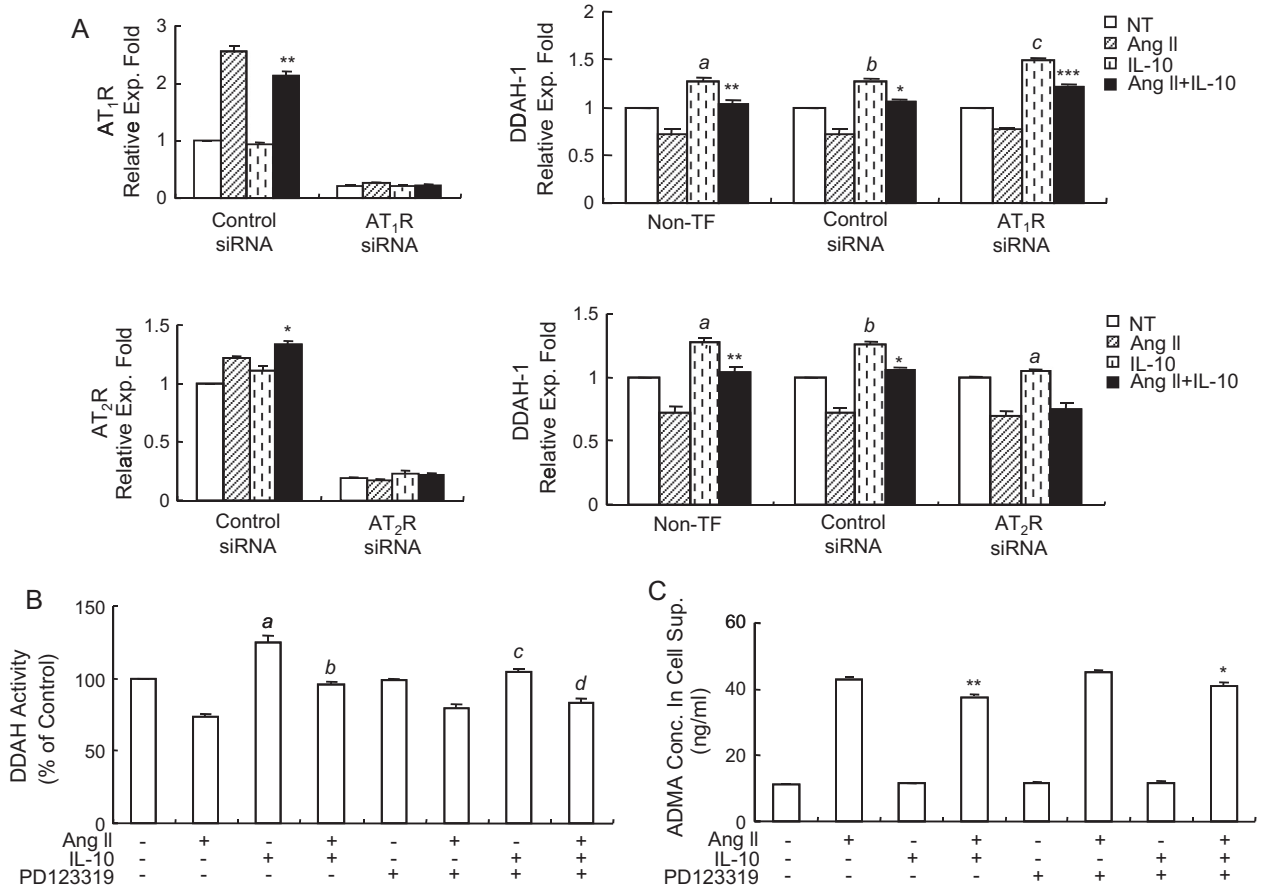


**Fig. 3.** IL-10-induced elevation of DDAH-1 mRNA expression and DDAH activity is mediated by $AT_2$ R in SHR VSMCs. (A) For $AT_1$ R or $AT_2$ R siRNA transfection, SHR VSMCs were plated on 6-well plates, grown to 90% confluence, and transfected with $AT_1$ R, $AT_2$ R or control siRNA oligomers (50 nmol/L). Additionally, transfected VSMCs were treated with or without Ang II (0.1 μmol/L) and/or IL-10 (25 ng/mL) for 1 h, and DDAH-1 expression was determined by real-time PCR. Non-TF: non-transfected SHR VSMCs. 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. $^{***}p < 0.001$ vs. SHR VSMCs treated with Ang II. $^{a}p < 0.05$ vs. untreated SHR VSMCs. $^{b}p < 0.01$ vs. untreated SHR VSMCs. $^{c}p < 0.001$ vs. untreated SHR VSMCs. (B, C) SHR VSMCs were treated with or without Ang II (0.1 μmol/L) and/or IL-10 (25 ng/mL) in the presence or absence of PD123319 ($AT_2$ R antagonist, 10 μmol/L) for 1 h, after which DDAH activity and ADMA concentration were measured. Bars represent the means ± SEM of three independent experiments. $^{a}p < 0.01$ vs. untreated SHR VSMCs. $^{b}p < 0.001$ vs. SHR VSMCs treated with Ang II. $^{c}p < 0.05$ vs. SHR VSMCs treated with IL-10. $^{d}p < 0.01$ vs. SHR VSMCs treated with Ang II/IL-10. $^{*}p < 0.05$ vs. SHR VSMCs treated with Ang II/IL-10. $^{**}p < 0.01$ vs. SHR VSMCs treated with Ang II.

plays critical roles in homeostasis, recent studies suggest that AMPK plays protective roles in hypertension [17]. AMPK activation induces down-regulation of blood pressure, improvement of endothelial function, and inhibition of VSMC proliferation [31,32]. IL-10 up-regulates AMPK activity in SHR VSMCs [20]. Therefore, we hypothesized that DDAH-1 activity induced by IL-10 may be related to AMPK activation. In SHR VSMCs transfected with AMPK siRNA, IL-10-induced DDAH-1 mRNA expression as well as attenuation of Ang II-induced DDAH-1 mRNA inhibition by IL-10 were reduced. Additionally, the AMPK inhibitor Compound C reduced IL-10-induced DDAH activity as well as attenuation of Ang II-induced inhibition of DDAH activity by IL-10. This result indicates that AMPK activity mediates the up-regulatory effect of IL-10 on DDAH-1 activation in SHR VSMCs. Reversely, CCL5 has been shown to increase activation of AMPK via DDAH-1 activity in SHR VSMCs [33]. Therefore, interaction between DDAH-1 and AMPK activation obviously leads to up-regulation of anti-hypertensive state in SHR VSMCs.

The activities of 12-LO and ET-1 are major factors in the development and maintenance of hypertension [18,19]. Ang II is a potent inducer of 12-LO activation, and elevation of 12-LO activity is observed in SHR. ET-1 produced by endothelial cells regulates vascular tone [34]. Although, IL-10 alone does not affect expression of 12-LO or ET-1 in SHR VSMCs, IL-10 inhibits Ang II-induced 12-LO and ET-1 expression in SHR VSMCs [20]. Therefore, we hypothesized that DDAH-1 induction may be related to the inhibitory effect of IL-10 on Ang II-induced 12-LO and ET-1 expression. As shown in the results, DDAH-1 partially mediated the inhibitory effect of IL-10 on Ang II-induced 12-LO and ET-1 expression. Therefore, DDAH-1 more likely plays diverse down-regulatory roles in Ang II-induced vascular hypertension.

The pathogenesis of hypertension includes VSMC proliferation, and the renin–angiotensin system is a critical player modulating the proliferation of vascular cells [35]. IL-10 alone does not affect SHR VSMC proliferation but is known to inhibit Ang II-induced VSMC proliferation in SHR, resulting in reduction of intimal hyperplasia [22]. DDAH-1 regulates endothelial cell proliferation by degrading ADMA, thereby increasing NO production with consequent inhibition of VSMC proliferation [36]. However, the direct effect of DDAH-1 on VSMC proliferation in hypertensive rats has not been well studied. Therefore, we examined whether or not DDAH-1 production mediates the inhibitory effect of IL-10 on Ang II-induced VSMC proliferation in SHR. The inhibitory effect of IL-10 on Ang II-induced VSMC proliferation was reduced in

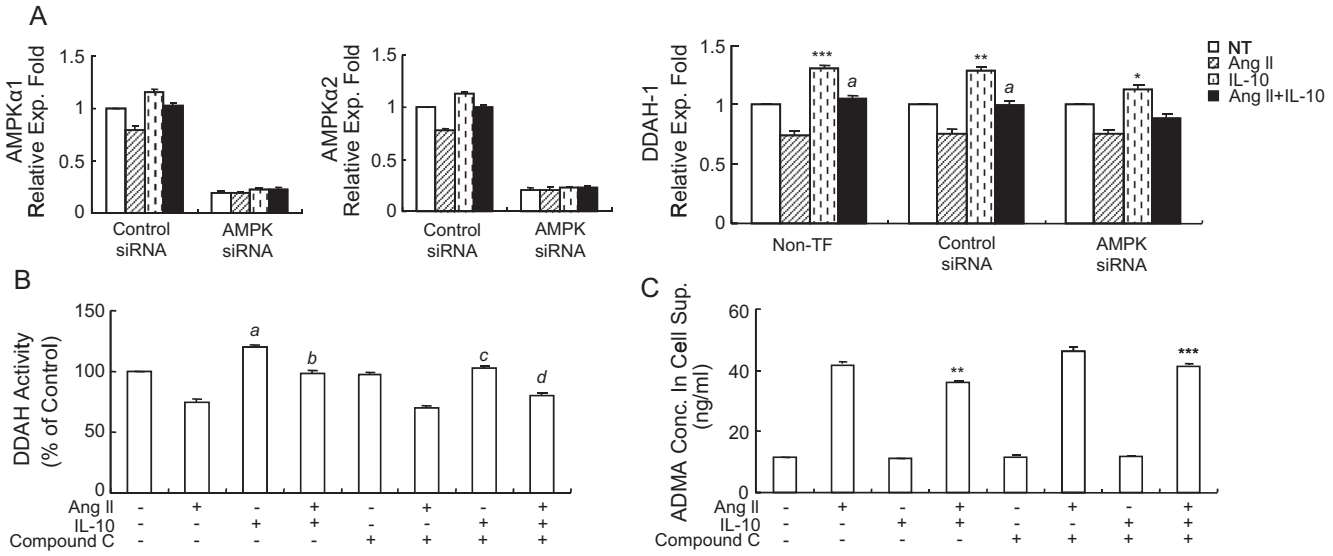


**Fig. 4.** AMPK activation partially mediates IL-10-induced DDAH-1 expression in SHR VSMCs. (A) For AMPK siRNA transfection, SHR VSMCs were plated on 6-well plates, grown to 90% confluence, and transfected AMPK or control siRNA oligomers (50 nmol/L). Additionally, transfected VSMCs were treated with or without Ang II (0.1 μmol/L) and/or IL-10 (25 ng/mL) for 1 h, and DDAH-1 expression was determined by real-time PCR. Non-TF: non-transfected SHR VSMCs. Bars represent the means ± SEM of three independent experiments. $^{*}p < 0.05$ vs. untreated SHR VSMCs. $^{**}p < 0.01$ vs. untreated SHR VSMCs. $^{***}p < 0.001$ vs. untreated SHR VSMCs. $^{a}p < 0.01$ vs. SHR VSMCs treated with Ang II. (B, C) SHR VSMCs were treated with or without Ang II (0.1 μmol/L) and/or IL-10 (25 ng/mL) in the presence or absence of Compound C (an inhibitor of AMPK activity, 10 μmol/L) for 1 h, after which DDAH activity and ADMA concentration were measured. Bars represent the means ± SEM of three independent experiments. $^{a}p < 0.001$ vs. untreated SHR VSMCs. $^{b}p < 0.001$ vs. SHR VSMCs treated with Ang II. $^{c}p < 0.001$ vs. SHR VSMCs treated with IL-10. $^{d}p < 0.01$ vs. SHR VSMCs treated with Ang II/IL-10. $^{**}p < 0.01$ vs. SHR VSMCs treated with Ang II. $^{***}p < 0.001$ vs. SHR VSMCs treated with Ang II/IL-10.

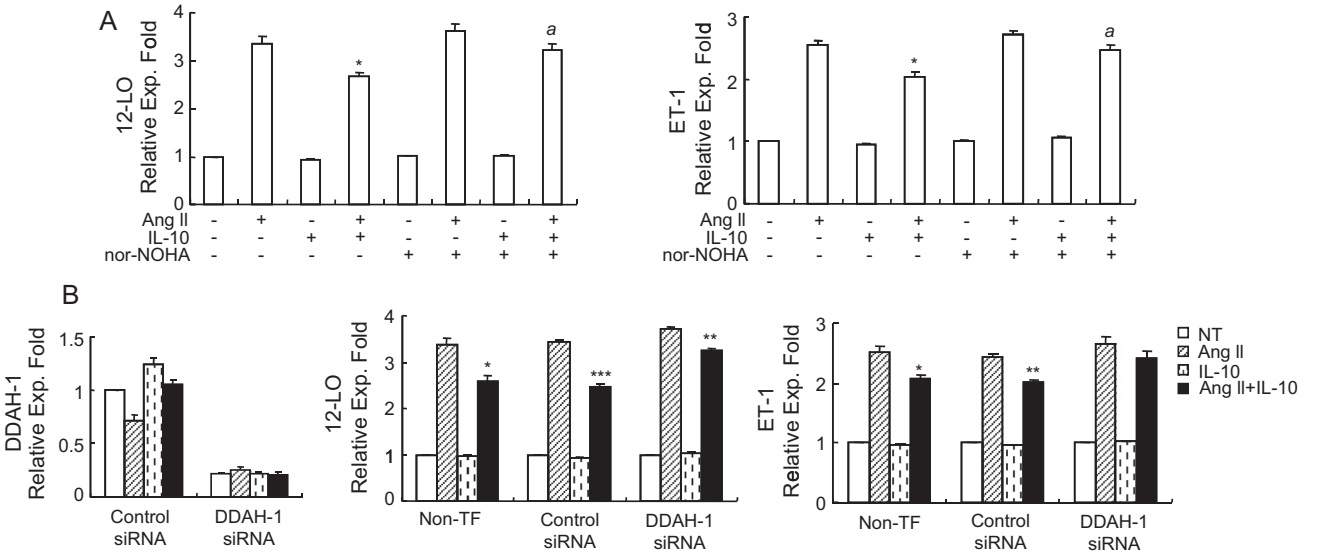


**Fig. 5.** DDAH-1 partially mediates inhibitory effects of IL-10 on Ang II-induced 12-LO and ET-1 expression in SHR VSMCs. (A) SHR VSMCs were untreated or treated with Ang II (0.1 μmol/L) and/or IL-10 (25 ng/mL) in the presence or absence of nor-NOHA (an inhibitor of DDAH-1 activity, 50 μmol/L) for 1 h. Expression of 12-LO or ET-1 was determined by real-time PCR. Bars represent the means ± SEM of three independent experiments. $^{*}p < 0.05$ vs. SHR VSMCs treated with Ang II. $^{a}p < 0.05$ vs. SHR VSMCs treated with Ang II/IL-10. (B) SHR VSMCs were transfected with DDAH-1 or control siRNA oligomers (50 nmol/L). Additionally, transfected VSMCs were treated with or without Ang II (0.1 μmol/L) and/or IL-10 (25 ng/mL) for 1 h. Expression of 12-LO or ET-1 was determined by real-time PCR. Non-TF: non-transfected VSMCs. 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. $^{***}p < 0.001$ vs. SHR VSMCs treated with Ang II.

SHR VSMCs transfected with DDAH-1 siRNA. Lastly, DDAH-1 activation by IL-10 reduced Ang II-induced VSMC proliferation in SHR.

These combined results indicate that IL-10 increases expression of DDAH-1 via $AT_2$ R and AMPK activation, and DDAH-1 is partially responsible for the inhibitory effects of IL-10 on Ang II-induced 12-LO and ET-1 expression as well as VSMC proliferation in SHR VSMCs (Fig. 7). Until now, there has been no direct evidence of a relationship between DDAH-1 and IL-10 in Ang II-induced hypertension. Thus, this study provides the first evidence that DDAH-1 induced by IL-10 exerts an important influence on the anti-hypertensive effects of IL-10 in SHR VSMCs.

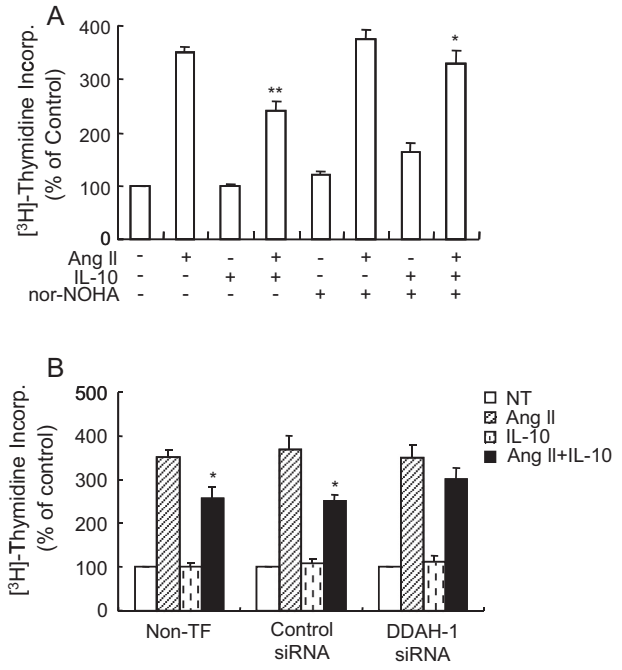


**Fig. 6.** DDAH-1 partially mediates inhibitory effect of IL-10 on Ang II-induced VSMC proliferation. (A) SHR VSMCs were untreated or treated with Ang II (0.1 μmol/L) and/or IL-10 (25 ng/mL) in the presence or absence of nor-NOHA (an inhibitor of DDAH-1 activity, 50 μmol/L) for 24 h in medium containing [$^3$H]-thymidine (1 μCi/mL). [$^3$H]-thymidine incorporation is shown on the *Y*-axis. Bars represent means ± SEM of four independent experiments. $^*p < 0.05$ vs. SHR VSMCs treated with Ang II/IL-10. $^{**}p < 0.01$ vs. SHR VSMCs treated with Ang II. (B) SHR VSMCs were plated on 24-well plates, grown to 90% confluence, and then transfected with DDAH-1 or control siRNA oligomers (50 nmol/L). Transfected VSMCs were then untreated or treated with Ang II (0.1 μmol/L) and/or IL-10 (25 ng/mL) for 24 h in medium containing [$^3$H]-thymidine (1 μCi/mL). [$^3$H]-thymidine incorporation is shown on the *Y*-axis. Bars represent means ± SEM of four independent experiments. $^*p < 0.05$ vs. SHR VSMCs treated with Ang II.

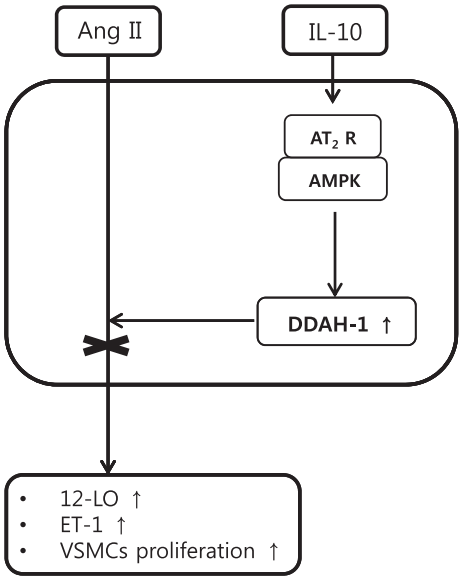


**Fig. 7.** Flow diagram of the effect of IL-10 on Ang II-induced hypertensive mediators and VSMC proliferation in SHR VSMCs.

## Acknowledgment

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