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CCL5 upregulates activation of AMP-activated protein kinases in vascular smooth muscle cells of spontaneously hypertensive rats

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ABSTRACT

AMP-activated protein kinase (AMPK) activation plays a central role in cellular metabolic homeostasis. Although AMPK is known for its roles in energy homeostasis, numerous recent studies have suggested broader protective roles in inflammation and hypertension. Chemokine CCL5 has shown down-regulatory effects on angiotensin II (Ang II)-induced hypertensive mediators as well as VSMCs proliferation in spontaneously hypertensive rats (SHR) VSMCs. In the present study, we investigated the relationship between CCL5 and AMPK in the anti-hypertensive effects of CCL5 in SHR VSMCs. CCL5 increased AMPK phosphorylation and attenuated Ang II-induced AMPK inhibition. AMPK activation induced by CCL5 was mediated mainly through the AT_2 R pathway. Activation of dimethylarginine dimethylaminohydrolase (DDAH)-1 by CCL5 resulted in AMPK activation as well as attenuation of Ang II-induced AMPK inhibition. In addition, AMPK activation induced by CCL5 was partially responsible for the inhibitory effects of CCL5 on Ang II-induced 12-lipoxygenase (12-LO) and endothelin (ET)-1 expression, and the inhibitory effect of CCL5 on Ang II-induced VSMCs proliferation was also mediated via AMPK activation in SHR VSMCs. In conclusion, CCL5 induces activation of AMPK via DDAH-1 activity in SHR VSMCs, and activation of AMPK is partially responsible for the inhibitory effects of CCL5 on Ang II-induced hypertensive mediators. These results suggest that activation of AMPK by CCL5 potentially expands the anti-hypertensive role of CCL5 in SHR VSMCs.

1. Introduction

The AMP-activated protein kinase (AMPK) signaling system plays a central role in cellular metabolic homeostasis [1–3]. Although AMPK is known for its roles in energy homeostasis, numerous recent studies have suggested broader protective roles in inflammation and hypertension [4–8]. There have been various studies on the relationship between AMPK and regulation of blood pressure [5,6,9–11]. AMPK activators, such as metformin, AICAR, thiazolidinedione, and adiponectin have been reported to reduce blood pressure in experimental animals and humans. In addition, AMPK activation exhibits beneficial effects on vascular function, such as improvement of endothelial function and inhibition of VSMCs proliferation [12,13], as well as protective effects on the cardiovascular system [7]. AMPK can be activated by anti-inflammatory stimuli and inactivated by proinflammatory stimuli in macrophages [4]. AMPK is a heterotrimeric complex that consists of a catalytic α subunit (α1 and α2) and two regulatory β and γ subunits (β1, β, γ1, γ2, and γ3), and activation of AMPK involves phosphorylation of the Thr172 residue on AMPK located in the α subunit [1].

The involvement of vascular inflammation in the pathology of hypertension has been well demonstrated, and chemokines are present at sites of vascular inflammation. Increased activation of chemokines CCL2 and CXCL8 occurs in arterial walls of hypertensive animals, and the up-regulatory roles of CCL2 and CXCL8 in hypertension development and maintenance have been well studied [14–16]. Therefore, inhibition of chemokine production is important in the regulation of inflammatory reactions in hypertensive vascular walls [17,18]. Chemokine CCL5 also plays functional roles in both acute and chronic inflammatory responses during atherosclerosis, renal disease progression, and vascular wall remodeling during pulmonary arterial hypertension [19–21]. Therefore, overproduction of CCL5 is associated with diverse disease progression. On the other hand, CCL5 has down-regulatory effects on angiotensin II (Ang II)-induced hypertensive mediators, including 12-lipoxygenase (LO) and endothelin (ET)-1, in vascular smooth muscle cells (VSMCs) of spontaneously hypertensive rats (SHR) as well as proliferation of SHR VSMCs [22,23]. Additionally, expression of CCL5 in SHR VSMCs is inhibited compared to that

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in normotensive Wistar-Kyoto rats (WKY) VSMCs [22]. Moreover, CCL5 up-regulates the production of dimethylarginine dimethylaminohydrolase (DDAH)-1, an important regulator of NO bioavailability, and attenuates Ang II-induced DDAH-1 inhibition in SHR VSMCs [23]. Therefore, 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 and CXCL8 in hypertension [14–16].

Accordingly, we hypothesized that CCL5 may exert anti-hypertensive effects through the activation of AMPK in SHR VSMCs. Thus, we investigated the relationship between CCL5 and AMPK in the anti-hypertensive activity of CCL5 in SHR VSMCs.

2. Materials and methods

2.1. Reagents

Easy-blue total RNA extraction kit for total RNA isolation was purchased from iNtRON (Biotechnology, Seoul, Korea). 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). Compound C was obtained from Sigma–Aldrich Co. (St. Louis, MO, USA). nor-NOHA was obtained from Cayman Chemical (Ann Arbor, Michigan, USA). CCL5 was obtained from R&D systems (Minneapolis, MN). LightCycler FastStart DNA SYBR Green I Mix was obtained from Roche (Mannheim, Germany). Rabbit anti-human p-AMPK and AMPK polyclonal antibodies were purchased from Cell Signaling (Cambridge, UK). Primer sequences of AMPKα1, AMPKα2, 12-LO, ET-1, and β-actin were synthesized by Bionics (Seoul, South Korea). Rat DDAH-1, AMPK, AT_1 R, and AT_2 R siRNA sequences were purchased from Bioneer technology (Daejeon, South Korea). Negative control siRNA was purchased from Invitrogen (Carlsbad, CA, USA). All other reagents were pure-grade commercial preparations.

2.2. Preparation of SHR VSMCs and animal experimental protocol

Specific pathogen-free, male inbred SHR and WKY rats 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.

SHR VSMCs were obtained from thoracic aortas of 22-week-old SHR and WKY rats following the explant method as described by Kim et al. [16]. VSMCs were cultured in DMEM supplemented with 10% FBS and 1% penicillin–streptomycin. Cells were detached with 0.25% trypsin/EDTA and seeded onto 75-cm^2 tissue culture flasks at a density of 10^5 cells/mL. All experiments were conducted between cell passages three to seven. Prior to stimulation, 95% confluent VSMCs were serum-starved overnight by incubation in DMEM supplemented with 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 times.

SHR experimental protocol was reviewed and approved by the Committee on the Ethics of Animal Experiments, College of Medicine, Yeungnam University. The CCL5-treated group contained 6 SHR (SHR-I), where equal numbers of normal saline-treated SHR (SHR-C) served as control. Nineteen-week-old SHR received a subcutaneous injection of CCL5 (1.5 μg/kg) twice a day for 3 weeks. On the day after the final injection, both control and CCL5-treated rats were anesthetized via intraperitoneal injection of urethane (1.5 g/kg), and thoracic aorta tissues were collected.

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

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

One microgram of total RNA per sample was reverse-transcribed using a Maxime RT premix kit (iNtRON Biotechnology, Daejon, Korea) according to the manufacturer's instructions. cDNA synthesis was performed at 45 °C for 60 min, followed by RT inactivation at 95 °C for 5 min.

AMPKα1, AMPKα2, 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), 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: AMPKα1 (180 bp) sense, 5′-gcagagagatccagaacctg-3′, antisense, 5′-ctccttttcgtccaaccttcc-3′; AMPKα2 (222 bp) sense, 5′-gctctcgatcgccaaattat-3′, antisense, 5′-gcatcagcagagtggcaata-3′; 12-LO (312 bp) sense, 5′-tggggcaactggaagg-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 AMPKα1, AMPKα2, 12-LO, and ET-1 were determined by comparing experimental levels to standard curves and were expressed as relative fold expression levels.

2.4. 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. Twenty micrograms of each protein sample was separated by 10% SDS–polyacrylamide gel electrophoresis and then transferred onto 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 p-AMPKα (Thr172), AMPKα, 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).

2.5. 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 2 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′-uugguaccuaucaacacuc-3′; AMPK siRNA sense, 5′-cgugugaagaucggacacu-3′, antisense, 5′-aguguccgaucuucacacg-3′ and DDAH-1 siRNA sense, 5′-ucagagagacugagucacu-3′, antisense, 5′-agugacucagucucucuga-3′.

2.6. 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 the plates during the last 24 h of incubation. The 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).

2.7. 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.

3. Results

3.1. CCL5 up-regulates AMPK activation and attenuates Ang II-induced AMPK inhibition in SHR VSMCs

We first compared basal AMPK activation in SHR VSMCs with that in WKY VSMCs. Basal activation of AMPK (phosphorylation of AMPK activation site Thr172) in SHR VSMCs was reduced compared with that in WKY VSMCs (Fig. 1A). We next compared the effect of CCL5 and/or Ang II on AMPK phosphorylation between SHR and WKY VSMCs. CCL5 and Ang II had no statistically significant effect on AMPK phosphorylation in WKY VSMCs, whereas CCL5 induced phosphorylation of AMPK and Ang II inhibited AMPK phosphorylation in SHR VSMCs. Additionally, CCL5 attenuated Ang II-induced AMPK inhibition in SHR VSMCs, whereas Ang II or CCL5-induced AMPK activation in WKY VSMCs was not significantly different from that in WKY VSMCs treated with Ang II and CCL5 simultaneously (Ang II/CCL5) (Fig. 1B). Further, we observed a dose-dependent response of Ang II-induced AMPK inhibition in response to CCL5 treatment. Doses of CCL5 ranging from 10 ng/mL to 400 ng/mL elevated AMPK phosphorylation to levels close to or greater than that in untreated SHR VSMCs (Fig. 1C). The time course of Ang II-induced AMPK inhibition in response to CCL5 treatment was also determined over a 16 h time period. We examined the levels of AMPKα1 and AMPKα2 mRNA expression. Attenuation of Ang II-induced AMPKα1 or AMPKα2 mRNA inhibition by CCL5 was detected at 1 h after Ang II/CCL5 treatment and was sustained for up to 16 h. Increased AMPKα1 or AMPKα2 mRNA expression remained almost constant from 1 to 16 h upon Ang II/CCL5 treatment in SHR VSMCs (Fig. 1D).

Additionally, to confirm the effect of CCL5 on AMPK activation in SHR. SHR were treated with CCL5 (1.5 μg/kg) subcutaneously twice a day for 3 weeks. CCL5 treatment did not significantly influence body weight in SHR-I, while the rats showed an age-related increase in body weight (19 weeks of age: 376.30 ± 5.44 g, 20 weeks of age: 387.30 ± 5.3 g, 21 weeks of age: 395.80 ± 4.61 g, 22 weeks of age: 401.80 ± 4.56 g) (Fig. 2A). An elevated AMPK activity was observed in thoracic aorta tissues in SHR-I compared to that of SHR-C (Fig. 2B). The increase effect of CCL5 on AMPKα1 and AMPKα2 mRNA expression was also detected in VSMCs from SHR-I (Fig. 2C).

3.2. Action mechanism of CCL5 on AMPK activation in SHR VSMCs

We also examined whether or not the CCL5-induced increase in AMPK phosphorylation is mediated through AT_1 R or AT_2 R. Immunoblotting was performed on samples treated with AT_1 R or AT_2 R-directed siRNA. In SHR VSMCs transfected with AT_1 R siRNA, CCL5-induced AMPK phosphorylation was sustained, and attenuation of Ang II-induced AMPK inhibition was not reduced (Fig. 3A). On the other hand, in SHR VSMCs transfected with AT_2 R siRNA, both CCL5-induced AMPK phosphorylation and attenuation of Ang II-induced AMPK inhibition by CCL5 were significantly reduced (Fig. 3B). The rate of increase of CCL5-induced AMPK activation in AT_2 siRNA-transfected SHR VSMCs was reduced to 7.7 ± 0.1% compared to 21.3 ± 5.7% in control siRNA-transfected SHR VSMCs. Regarding the attenuation effect of CCL5 on Ang II-induced AMPK inhibition, the rate of reduction of Ang II-induced AMPK inhibition by CCL5 in AT_2 siRNA-transfected SHR VSMCs was reduced to 26.1 ± 4.9% compared to 47.2 ± 1.5% in control siRNA-transfected SHR VSMCs.

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 [24]. In our previous study, we demonstrated that CCL5 increases DDAH-1 expression, induces DDAH activity, and attenuates Ang II-induced DDAH-1 inhibition in SHR VSMCs [23]. Therefore, we examined whether or not the up-regulatory effect of CCL5 on AMPK phosphorylation is mediated through DDAH-1 activation in SHR VSMCs. An inhibitor of DDAH-1 activation, nor-NOHA, inhibited CCL5-induced AMPK activation to level close to that of the control. Additionally, attenuation of Ang II-induced AMPK inhibition by CCL5 was also reduced by nor-NOHA (Fig. 4A). To confirm this result, immunoblotting was performed on samples transfected with DDAH-1 siRNA. In SHR VSMCs transfected with DDAH-1 siRNA, CCL5-induced AMPK phosphorylation as well as attenuation of Ang II-induced AMPK inhibition by CCL5 were not detected (Fig. 4B).

3.3. AMPK activation partially mediates the inhibitory effect of CCL5 on Ang II-induced hypertensive mediators and proliferation in SHR VSMCs

Activities of both 12-LO and ET-1 have been linked to the development of hypertension [25,26]. CCL5 reduces Ang II-induced 12-LO and ET-1 expression in SHR VSMCs [22,23]. Therefore, we investigated whether or not AMPK activation mediates the inhibitory effect of CCL5 in SHR VSMCs. An inhibitor of AMPK activation, Compound C, elevated 12-LO and ET-1 mRNA expression in VSMCs treated with CCL5 as well as reduced the inhibitory effect of CCL5 on Ang II-induced 12-LO and ET-1 expression (Fig. 5A). To confirm this result, an experiment involving siRNA-mediated down-regulation of AMPK activation was performed. The rate of reduction of Ang II-induced 12-LO expression by CCL5 in AMPK siRNA-transfected SHR VSMCs was reduced to 12.5 ± 0.6% compared to 23.9 ± 0.1% in control siRNA-transfected SHR VSMCs. In the case of ET-1 expression, the rate of reduction of Ang II-induced ET-1 expression by CCL5 in AMPK siRNA-transfected SHR VSMCs was reduced to 9.1 ± 2.3% compared to 17.5 ± 1.3% in control siRNA-transfected SHR VSMCs (Fig. 5B).

As CCL5 reduces Ang II-induced VSMCs proliferation in SHR VSMCs [22], we also examined whether or not AMPK activation

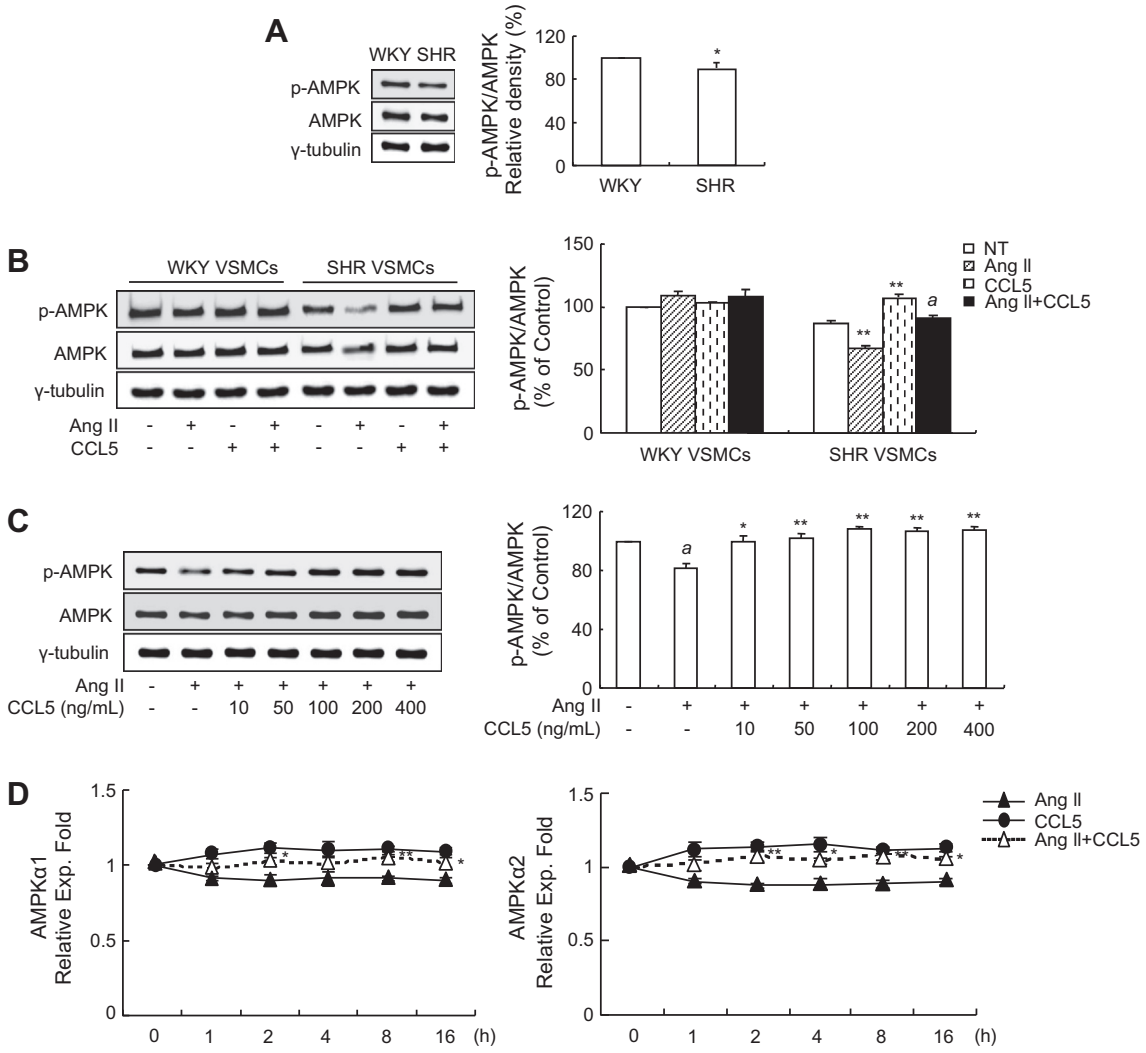

Fig. 1. CCL5 elevates AMPK phosphorylation and attenuates Ang II-induced AMPK inhibition in SHR VSMCs. (A) After total RNAs from SHR and WKY VSMCs were prepared, real-time PCR was performed. Bars represent the means ± SEM of three independent experiments. $^{*}p < 0.05$ vs. WKY VSMCs. (B) SHR and WKY VSMCs were treated with or without Ang II (0.1 μmol/L) and/or CCL5 (100 ng/mL) for 2 h. After cell lysates were prepared, immunoblotting was performed. AMPK phosphorylation of Thr172 (p-AMPK) was determined by Western blot and densitometric analyses. Data shown are representative of three independent experiments. Bars represent the means ± SEM of three independent experiments. $^{**}p < 0.01$ vs. untreated SHR VSMCs. $^{a}p < 0.01$ vs. SHR VSMCs treated with Ang II. (C) SHR VSMCs were treated with or without Ang II (0.1 μmol/L) and 0, 10, 50, 100, 200, or 400 ng/mL of CCL5 simultaneously (Ang II/CCL5) for 2 h. After cell lysates were prepared, Western blot and densitometeric analyses were performed. Data shown are representative of three independent experiments. Bars represent the means ± SEM of three independent experiments. $^{a}p < 0.05$ vs. untreated SHR VSMCs. $^{*}p < 0.05$ vs. SHR VSMCs treated with Ang II. $^{**}p < 0.01$ vs. SHR VSMCs treated with Ang II. (D) For the time course reaction, SHR VSMCs were treated with or without Ang II (0.1 μmol/L) and/or CCL5 (100 ng/mL) for the indicated times. After total RNAs were isolated, real-time PCR was performed. Data shown are representative of three independent experiments. Bars represent the means ± SEM of three independent experiments. $^{*}p < 0.05$ vs. SHR VSMCs treated with Ang II. vs. SHR VSMCs treated with Ang II.

mediates the inhibitory effect of CCL5 on Ang II-induced VSMCs proliferation. CCL5 alone weakly induced VSMCs proliferation. However, Compound C significantly increased CCL5-induced VSMCs proliferation as well as reduced the inhibitory effect of CCL5 on Ang II-induced VSMCs proliferation (Fig. 6A). An experiment involving siRNA-mediated down-regulation of AMPK activation was also performed. The rate of reduction of Ang II-induced VSMCs proliferation by CCL5 in AMPK siRNA-transfected SHR VSMCs was reduced to 8.0 ± 1.4% compared to 38.6 ± 9.3% in control siRNA-transfected SHR VSMCs (Fig. 6B).

4. Discussion

Activators of AMPK have been shown to lower blood pressure in SHR as well as ameliorate hypertension in an experimental rat model of preeclampsia [5,6,9–11]. Moreover, AMPK has anti-inflammatory effects, including down-regulation of NF-κB signaling as well as the expression of proinflammatory cytokines, inducible nitric oxide, and cyclooxygenase-2 in various cell types [8,27–30]. Therefore, this study investigated whether or not the anti-hypertensive mechanism of CCL5 is related to AMPK activation in SHR VSMCs.

Basal activation of AMPK is reduced by 50% in SHR thoracic aorta tissue compared to WKY thoracic aorta tissue [5]. In the case of SHR VSMCs, we also detected lower basal AMPK activation in SHR VSMCs than that in WKY VSMCs. However, basal activity of AMPK in SHR VSMCs was slightly lower compared to that in WKY VSMCs. The increase in AMPK activation induced by CCL5 was not prominent in SHR VSMCs, whereas CCL5 significantly attenuated Ang II-induced inhibition of AMPK activation. Although CCL5 has been reported to induce the AMPK signaling pathway in activated T cells in order to meet the energy demands of chemotaxis for efficient T cell migration [31], studies on the role of CCL5 in AMPK activation have not been performed in the hypertension field. Besides CCL5, IL-10 also has anti-hypertensive effects via inhibitory effects on

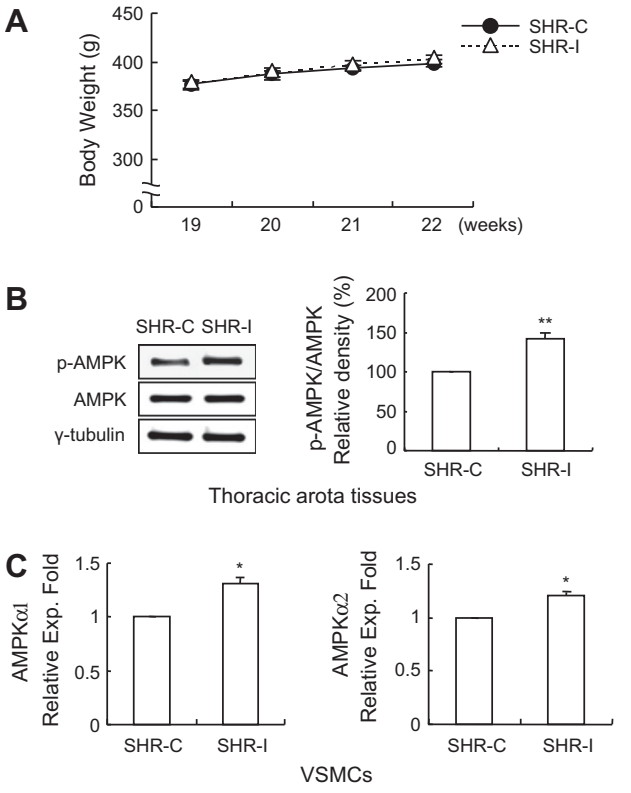

Fig. 2. CCL5 modulates the expression of AMPK in SHR thoracic aortas and VSMCs. (A) Body weight was measured before, every week during treatment and the day after final treatment with CCL5 (1.5 μg/kg, twice a day for 3 weeks) or normal saline. (B and C) After tissue lysates and total RNA were isolated from tissues and VSMCs from thoracic aorta of each rat group ($n = 6$ each), immunoblotting and real-time PCR were performed. Data shown are representative of three independent experiments. Bars represent the mean ± SEM. $^{*}p < 0.05$ vs. SHR-C. $^{**}p < 0.01$ vs. SHR-C. SHR-C: SHR treated with normal saline, SHR-I: SHR treated with CCL5.

vascular inflammation in hypertension [32,33]. In our IL-10 study, IL-10 also induced AMPK activation in SHR VSMCs, and the IL-10-induced increase in CCL5 expression was mediated via AMPK activation [data not shown]. Presumably, the anti-hypertensive effects of these two cytokines are related to the activity of AMPK.

Although a few studies have reported Ang II-induced AMPK phosphorylation [13,34] in normotensive rat or human VSMCs, Ang II has been shown to suppress AMPK activation in the kidney in salt-sensitive hypertension [9]. In SHR VSMCs, Ang II inhibited AMPK activation, although the specific mechanisms by which Ang II regulates AMPK remain unclear. Besides Ang II, regulation of AMPK activity may be influenced by various other metabolic factors in different cell types, and these factors may have the potential to act on AMPK activation differently according to cell type.

Ang II has two subtype receptors, AT_1 R and AT_2 R. AT_1 R mediates the major stimulatory actions of Ang II, including vasoconstriction, cell proliferation, vasopressin and aldosterone secretion, sympathetic activation, and sodium retention [35,36]. In contrast, AT_2 R is known to antagonize the vascular actions of AT_1 R. However, several studies have reported growth and proinflammatory activities for AT_2 R in VSMCs [37]. Nagata et al. reported that Ang II-induced AMPK activation is mediated by ROS production via the AT_1 R pathway and plays a negative feedback role in Ang II–induced VSMCS proliferation signaling [13]. In this study, the increase in CCL5-induced AMPK activation was mediated by AT_2 R activation, whereas attenuation of Ang II-induced AMPK inhibition by CCL5 was partially mediated via AT_2 R activation. In our previous studies, the inhibitory effects of CCL5 on Ang II-induced 12-LO expression as well as Ang II-induced DDAH-1 inhibition were also shown to be mediated via AT_2 R activation in SHR VSMCs [22,23]. Therefore, although the actions of AT_2 R are still controversial, AT_2 R is thought to be a positive regulator of the anti-hypertensive activities of CCL5 in Ang II-treated SHR VSMCs.

DDAH is an important regulator of plasma ADMA. Reduction of DDAH activity promotes onset of cardiovascular diseases, including hypertension accompanied by Ang II activity [24,38]. DDAH exists as two isoforms, DDAH-1 and DDAH-2 [24]. Plasma ADMA levels are regulated by DDAH-1, whereas DDAH-2 acts more importantly to preserve endothelial function in blood vessel resistance [24]. DDAH may modulate the expression levels of numerous genes to control ADMA concentrations and NO generation [24]. In our previous study, we demonstrated the up-regulatory effect of CCL5 on DDAH-1 production as well as the relationship between CCL5-

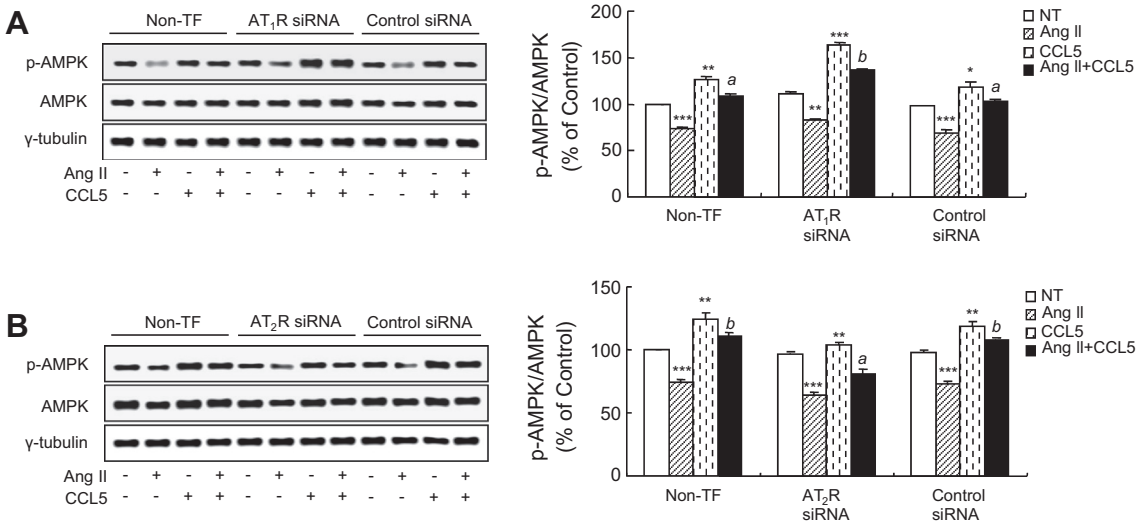

Fig. 3. CCL5-induced elevation of AMPK activation is mediated by AT_2 R in SHR VSMCs. For AT_1 R or AT_2 R siRNA transfection, SHR VSMCs were plated on 6-well plates, grown to 90% confluence, and transfected with AT_1 R, AT_2 R, or control siRNA oligomers (50 nmol/L). Additionally, transfected VSMCs were treated with or without Ang II (0.1 μmol/L) and/or CCL5 (100 ng/mL) for 2 h, and p-AMPK expression was determined by Western blot and densitometric analyses. Non-TF: non-transfected SHR VSMCs. Data shown are representative of three independent experiments. 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. $^{b}p < 0.001$ vs. SHR VSMCs treated with Ang II.

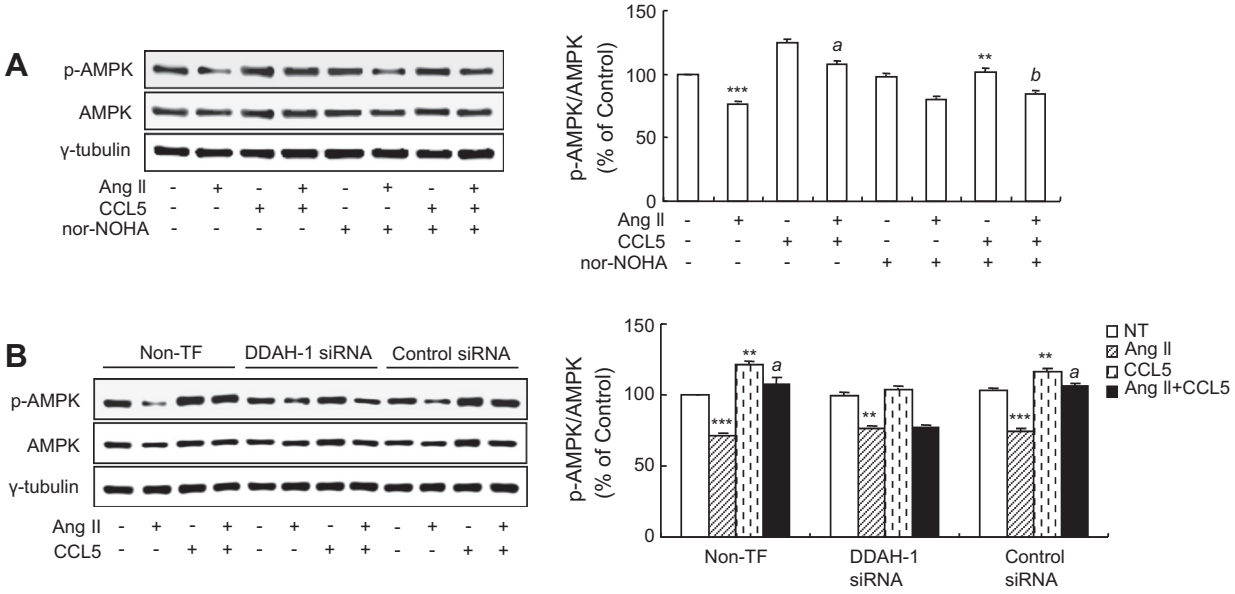

Fig. 4. DDAH-1 activation due to CCL5 mediates CCL5-induced AMPK activation in SHR VSMCs. (A) SHR VSMCs were untreated or treated with Ang II (0.1 μmol/L) and/or CCL5 (100 ng/mL) in the presence or absence of nor-NOHA (an inhibitor of DDAH-1 activity, 50 μmol/L) for 2 h. p-AMPK expression was determined by Western blot and densitometric analyses. Data shown are representative of three independent experiments. Bars represent the means ± SEM of three independent experiments. $^{**}p < 0.01$ vs. SHR VSMCs treated with CCL5. $^{***}p < 0.001$ vs. untreated SHR VSMCs. $^{a}p < 0.001$ vs. SHR VSMCs treated with Ang II. $^{b}p < 0.01$ vs. SHR VSMCs treated with Ang II/CCL5. (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 CCL5 (100 ng/mL) for 2 h, and p-AMPK expression was determined by Western blot and densitometric analyses. Non-TF: non-transfected VSMCs. Data shown are representative of three independent experiments. Bars represent the means ± SEM of three independent experiments. $^{**}p < 0.01$ vs. untreated SHR VSMCs. $^{***}p < 0.001$ vs. untreated SHR VSMCs. $^{a}p < 0.001$ vs. SHR VSMCs treated with Ang II.

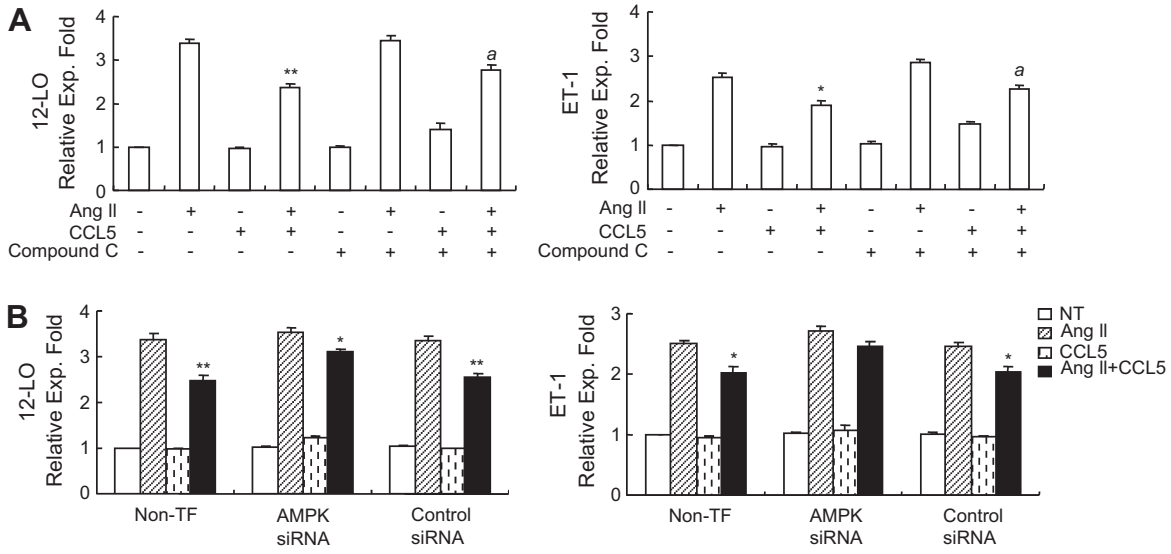

Fig. 5. AMPK activation by CCL5 partially mediates the inhibitory effects of CCL5 on Ang II-induced 12-LO and ET-1 expression in SHR VSMCs. (A) SHR VSMCs were untreated or treated with Ang II (0.1 μmol/L) and/or CCL5 (100 ng/mL) in the presence or absence of Compound C (an inhibitor of AMPK activity, 10 μmol/L) for 2 h. Real-time PCR was performed. Relative transcription levels of 12-LO or ET-1 were normalized to the signal intensity of the untreated SHR VSMCs. β-actin was used as an internal control. Data shown are representative of three independent experiments. 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. $^{a}p < 0.05$ vs. SHR VSMCs treated with Ang II/CCL5. (B) SHR VSMCs were transfected with AMPK or control siRNA oligomers (50 nmol/L). Additionally, transfected VSMCs were treated with or without Ang II (0.1 μmol/L) and/or CCL5 (100 ng/mL) for 2 h. Expression of 12-LO or ET-1 was determined by real-time PCR. Non-TF: non-transfected VSMCs. Data shown are representative of three independent experiments. 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.

induced DDAH activity and attenuation of Ang II-induced 12-LO and ET-1 production by CCL5 in SHR VSMCs [23]. Therefore, we hypothesized that DDAH-1 activity induced by CCL5 may be related to the effect of CCL5 on AMPK activation. In SHR VSMCs transfected with DDAH-1 siRNA, CCL5-induced AMPK phosphorylation as well as the attenuation effect of CCL5 on Ang II-induced AMPK inhibition were not detected. This result indicates that DDAH-1 activity mediates CCL5-induced AMPK activation in SHR VSMCs. However, the relationship between DDAH and AMPK activation has not been evaluated yet in hypertensive rats.

In the previous study, we demonstrated that Ang II inhibited CCL5 expression in SHR VSMCs, and the inhibition of CCL5 by

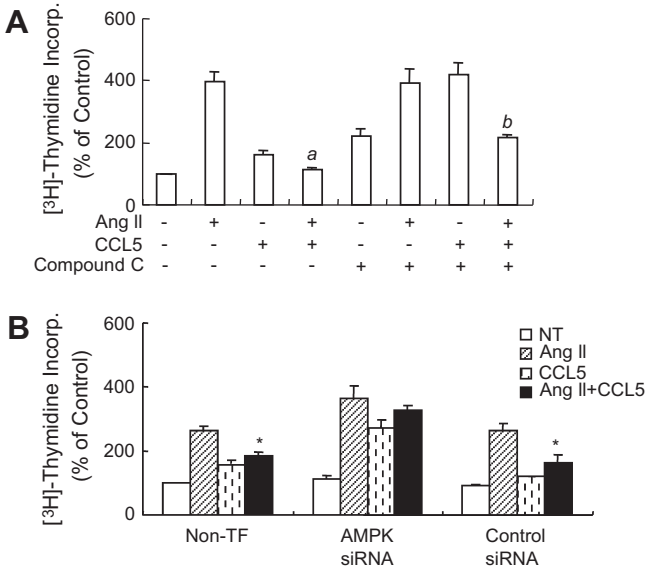

Fig. 6. AMPK activation by CCL5 mediates the inhibitory effect of CCL5 on Ang II-induced VSMCs proliferation. (A) SHR VSMCs were untreated or treated with Ang II (0.1 μmol/L) and/or CCL5 (100 ng/mL) in the presence or absence of Compound C (an inhibitor of AMPK activity, 10 μmol/L) for 24 h in medium containing [^{3}H]-thymidine (1 μCi/mL). [^{3}H]-thymidine incorporation is shown on the *Y*-axis. Data shown are representative of four independent experiments. Bars represent means ± SEM from four independent experiments. [a]$p < 0.001$ vs. SHR VSMCs treated with Ang II. [b]$p < 0.001$ vs. SHR VSMCs treated with Ang II/CCL5. (B) SHR VSMCs were plated on 24-well plates, grown to 90% confluence, and then transfected with AMPK or control siRNA oligomers (50 nmol/L). Transfected VSMCs were then untreated or treated with Ang II (0.1 μmol/L) and/or CCL5 (100 ng/mL) for 24 h in medium containing [^{3}H]-thymidine (1 μCi/mL). [^{3}H]-thymidine incorporation is shown on the *Y*-axis. Data shown are representative of four independent experiments. Bars represent means ± SEM from four independent experiments. [*]$p < 0.05$ vs. SHR VSMCs treated with Ang II.

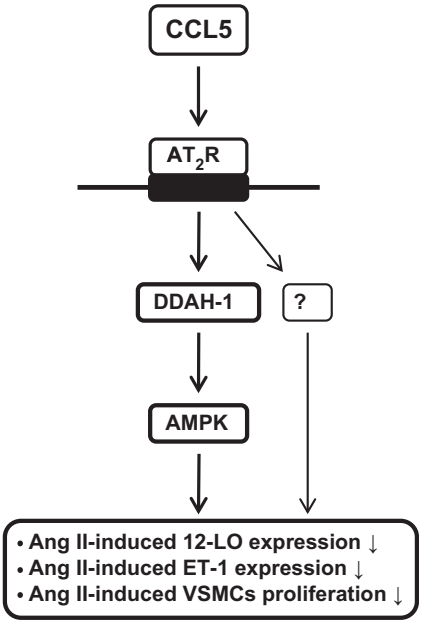

Fig. 7. Flow diagram of the action of CCL5 on Ang II-induced hypertensive mediators and VSMCs proliferation in SHR VSMCs.

Ang II was mediated by both AT_1 R and AT_2 R activation. Moreover, the inhibitory effect of Ang II on CCL5 expression was mediated by the 12-LO pathway [39]. Activities of both 12-LO and ET-1 have been linked to the development of hypertension [25,26]. Ang II is a potent positive regulator of 12-LO activation, and 12-LO activity is elevated in SHR. ET-1 is characterized as a potent vasoconstrictor secreted by the endothelium, and it participates in the regulation of vascular tone [26]. CCL5 significantly inhibits Ang II-induced 12-LO and ET-1 expression [22,23]. Moreover, CCL5 inhibits Ang II-induced VSMCs proliferation in SHR VSMCs [22]. Therefore, we examined whether or not AMPK activation can alter the anti-hypertensive effects of CCL5 in SHR VSMCs. Although AMPK activation did not significantly alter the inhibition of Ang II-induced 12-LO and ET-1 expression by CCL5, activation of AMPK was partially responsible for the inhibitory effects of CCL5 on Ang II-induced 12-LO and ET-1 expression in SHR VSMCs.

AMPK inhibits Ang II-induced VSMCs proliferation [13], and AMPK activators, such as metformin and AICAR, have shown inhibitory effects on VSMCs proliferation in hypertensive rats [30,40]. Moreover, CCL5 has an inhibitory effect on Ang II-induced VSMCs proliferation in SHR VSMCs [22]. Therefore, we examined whether or not AMPK activation due to CCL5 mediates the inhibitory effect of CCL5 on Ang II-induced VSMCs proliferation in SHR. In our study, AMPK activation by CCL5 inhibited Ang II-induced VSMCs proliferation in SHR. CCL5 alone also induced VSMCs proliferation. However, activation of AMPK by CCL5 suppressed CCL5-induced VSMCs proliferation. Namely, Compound C, an AMPK inhibitor, significantly increased CCL5-induced VSMCs proliferation and reduced the inhibitory effect of CCL5 on Ang II-induced VSMCs proliferation. Contrary to the inhibitory effect of AMPK on VSMCs proliferation [13,30,40], AMPK activation increases Ang II-induced proliferation in cardiac fibroblasts [41]. The mechanisms of action of AMPK activation in different cell types remain to be elucidated.

These combined results indicate that CCL5 induces activation of AMPK via DDAH-1 activity, and the activation of AMPK is partially responsible for the inhibitory effects of CCL5 on Ang II-induced hypertensive mediators and VSMCs proliferation in SHR VSMCs (Fig. 7). Until now, there has been no direct evidence of a relationship between AMPK activation and CCL5 in SHR VSMCs. Thus, this study provides the first evidence that activation of AMPK by CCL5 potentially expands the anti-hypertensive role of CCL5 in SHR VSMCs. Although further *in vivo* studies should be performed, it is highly possible that CCL5 plays anti-hypertensive roles in Ang II-induced vascular hypertension.

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