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IL-10 up-regulates CCL5 expression in vascular smooth muscle cells from spontaneously hypertensive rats

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ABSTRACT

An anti-inflammatory cytokine, interleukin-10 (IL-10) exerts inhibitory effects on vascular inflammation. Chemokines promote vascular inflammation and play a pathogenic role in the development and maintenance of hypertension. However, chemokine CCL5 has down-regulatory effects on angiotensin II (Ang II)-induced hypertensive mediators. In the present study, IL-10 increased CCL5 expression and attenuated Ang II-induced CCL5 inhibition significantly in vascular smooth muscle cells (VSMCs) from spontaneously hypertensive rats (SHR), whereas TGF-β had no effect on CCL5 expression or Ang II-induced CCL5 inhibition. Increased CCL5 expression due to IL-10 was mediated mainly through AT_2 R activation. Additionally, IL-10 increased activation of AMP-activated protein kinase (AMPK), which further mediated the up-regulatory effect of IL-10 on CCL5 expression. Attenuation of Ang II-induced CCL5 inhibition by IL-10 was associated with suppression of NF-κB activation, and IL-10 inhibited both Ang II-induced IκB-α and IκB-β degradation in SHR VSMCs. Moreover, IL-10 partially mediated the inhibitory effects of CCL5 on Ang II-induced 12-lipoxygenase (LO) and endothelin (ET)-1 expression in SHR VSMCs. Taken together, this study provides novel evidence that IL-10 plays an up-regulatory role in the anti-hypertensive activity of CCL5 in SHR VSMCs.

1. Introduction

Along with transforming growth factor-β (TGF-β) and interleukin-1 (IL-1) receptor antagonist (IL-1ra), inhibitory cytokine IL-10 has important inhibitory effects on vascular inflammatory responses [1,2]. IL-10 is produced by various types of immune cells, including TH_2 cells, and plays an important role in immunoregulation. IL-10 is also well known to have potent down-regulatory effects on the expression of adhesion molecules and cytokines in vascular cells and macrophages [3–5]. In hypertension studies, IL-10 is shown to prevent Ang II-induced vasoconstriction by decreasing expression of NADPH oxidase [6] as well as regulate vascular function by down-regulating proinflammatory cytokine expression and superoxide production in vascular walls [7,8]. Moreover, endogenous IL-10 inhibits Ang II-induced proinflammatory cytokine expression and vascular dysfunction [9].

Both proinflammatory cytokines and chemokines are present at sites of vascular inflammation, and the involvement of vascular inflammation in the pathological conditions of hypertension has been well demonstrated. Chemokines mediate the migration and infiltration of inflammatory cells into vascular walls, which in combination with oxidative stress, contribute to hypertension-related pathogenesis [10–12]. Whereas increased activation of chemokines CCL2 and CXCL8 occurs in arterial walls of hypertensive animals, suppression of chemokine-induced inflammatory cell infiltration has been shown to ameliorate hypertension in experimental animal models [12–15]. Therefore, inhibition of chemokine production is important in the regulation of inflammatory reactions in hypertensive vascular walls.

Inflammatory chemokine CCL5 (regulated upon activation, normally T-cell expressed, and presumably secreted; RANTES) is a potent chemoattractant for memory T lymphocytes and monocytes/macrophages, and it plays functional roles in both acute and chronic inflammatory responses during atherosclerosis, renal disease progression, and vascular wall remodeling during pulmonary arterial hypertension [16–19]. Overproduction of CCL5 is associated with diverse disease progression, and Ang II has been shown to increase CCL5 expression in rat glomerular endothelial cells and the renal cortex [17,18]. On the other hand, 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 the inhibitory effect of Ang II on CCL5 expression in SHR VSMCs [20]. In addition, CCL5 has been shown to inhibit Ang II-induced hypertensive mediators,

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12-lipoxygenase (LO), and endothelin (ET)-1 production [21,22]. Moreover, CCL5 was shown to up-regulate the production of dimethylarginine dimethylaminohydrolase (DDAH)-1, an important regulator of NO bioavailability, and attenuate Ang II-induced DDAH-1 inhibition in SHR VSMCs [22]. 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, which is in contrast to the up-regulatory roles of chemokine CCL2 and CXCL8 in hypertension development and maintenance [10,23–25].

Although both IL-10 and CCL5 have down-regulatory effects on Ang II-induced vascular dysfunction, the potential interactive role between the two remains unknown. Therefore, the present study investigated the effect of IL-10 on CCL5 expression as well as the mechanisms underlying its action 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). PD123319, losartan, and Compound C were obtained from Sigma–Aldrich Co. (St. Louis, MO, USA). CCL5, IL-10, and TGF-β1 were obtained from R&D systems (Minneapolis, MN). Bay11-7082 was obtained from Merck (Merck KGaA, Darmstadt, Germany). LightCycler FastStart DNA SYBR Green I Mix was obtained from Roche (Mannheim, Germany). Goat anti-human CCL5 polyclonal antibody was purchased from Santa Cruz Biotechnology (California, USA). Mouse anti-human AT_1 R monoclonal antibody and rabbit anti-human AT_2 R polyclonal were purchased from Abcam (Cambridge, UK). Rabbit anti-human p-AMPK, AMPK, IκB-α, and IκB-β polyclonal antibodies were purchased from Cell Signaling (Cambridge, UK). Primer sequences for CCL5, CXCL8, CCL2, AT_1 R, AT_2 R, 12-LO, ET-1, and β-actin were synthesized at Bionics (Daejeon, South Korea). Rat IL-10, 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. Animals and experimental protocols

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.

2.3. Preparation of VSMCs

VSMCs were obtained from thoracic aortas of 13-week-old SHR and WKY rats following the explant method as described by Kim et al. [25]. 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.

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

Total RNA was extracted using the 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.

CCL5, CXCL8, CCL2, 12-LO, ET-1, AT_1 R, or AT_2 R 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: CCL5 (110 bp) sense, 5′-cgtgaaggagtatttttacaccagc-3′, antisense, 5′-cttgaacccacttcttctctggg-3′; CXCL8 (365 bp) sense, 5′-gaagatagattgcaccga-3′, antisense, 5′-catagcctctcacacatttc-3′; CCL2 (396 bp) sense, 5′-cctgttgttcacagttgctgcc-3′, antisense, 5′-tctacagaagtgcttgaggtggttg-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′; AT_1 R (445 bp) sense, 5′-cacctatgtaagatcgcttc-3′, antisense, 5′-gcacaatcgccataattatcc-3′; AT_2 R (65 bp) sense, 5′-ccgtgaccaagtcttgaagatg-3′, antisense, 5′-agggaagccagcaaatgatg-3′; and β-actin (101 bp) sense, 5′-tactgccctggctcctagca-3′, antisense, 5′-tggacagtgaggccaggatag-3′. The mRNA levels of CCL5, CXCL8, CCL2, 12-LO, ET-1, AT_1 R, and AT_2 R were determined by comparing experimental levels to standard curves and were expressed as relative fold expression levels.

2.5. 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 protein samples were 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 CCL5, AT_1 R, AT_2 R, IκB-α, IκB-β, p-AMPK, AMPK, 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).

2.6. Enzyme-linked immunosorbent assay (ELISA)

CCL5 levels in cell supernatants were determined using a Quantikine ELISA kit obtained from R&D systems (Minneapolis, USA) was performed. All procedures were performed in accordance with the manufacturer's instructions.

2.7. Small interfering RNA (siRNA)

VSMCs were plated on 24-well plates and grown to 90% confluence. VSMCs were then transfected with IL-10, AMPK, AT_1 R, 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 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′-uugguaccuaucaacacuc-3′; IL-10 siRNA sesnse, 5′-cagguugcuccuuccauga-3′, antisense, 5′-ucauggaaggagcaaccug-3′; and AMPK siRNA sense, 5′-cgugugaagaucggacacu-3′, antisense, 5′-aguguccgaucuucacacg-3′.

2.8. Electrophoretic mobility shift assay (EMSA)

Cells were washed three times with cold PBS, scraped, and then harvested by centrifugation. Cell pellets were next resuspended and incubated on ice for 15 min in 400 μL of hypotonic buffer A (10 mmol/L of HEPES, 10 mmol/L of KCl, 1.5 mmol/L of $MgCl_2$, 0.5 mmol/L of DTT, 0.1 mmol/L of PMSF, 10 μg/mL of pepstatin, 10 μg/mL of leupeptin, 10 μg/mL of autipain, and 10 μg/mL of aprotinin). Nonidet P-40 was then added to a final concentration of 2.5%, after which the cells were vortexed for 10 s. Nuclei were separated from the cytosol by centrifugation at 12,000*g* for 15 s. Pellets were resuspended in 40 μL of hypotonic buffer C (20 mmol/L of HEPES, 25% glycerol, 0.4 mol/L of NaCl, 1 mmol/L of EDTA, 1 mmol/L of EGTA, 0.5 mmol/L of DTT, 0.1 mmol/L of PMSF, 10 μg/mL of pepstatin, 10 μg/mL of leupeptin, 10 μg/mL of autipain, and 10 μg/mL of aprotinin). Samples were sonicated for 2–3 s and then centrifuged for 10 min at 4 °C. The nuclear protein concentration was measured using Bradford assay (Bio-Rad, Richmond, CA). The consensus sequence of the NF-κB DNA-binding site (5′-AGTTGAGGGGACTTTCCCAGGC-3′) (sc-2505; Santa Cruz Biotechnology, Santa Cruz, CA) was labeled with [α-^{32}P]dCTP using a random-primed DNA labeling kit (Roche). The mutant NF-κB binding sequence (5′-AGTTGAGGCGACTTTCCCAGGC-3′) was identical to sc-2505 except for a "G" → "C" substitution in the NF-κB DNA-binding motif (sc-2511; Santa Cruz Biotechnology). The labeled DNA was purified over an S-200 HR column (Pharmacia, Piscataway, NJ) to remove any unbound nucleotides. Nuclear protein extracts were incubated at room temperature for 20 min with approximately 50,000 cpm of labeled oligonucleotides suspended in binding buffer [200 mmol/L of HEPES, 500 mmol/L of KCl, 10 mmol/L of EDTA, 50% glycerol, 10 mmol/L of DTT, 1 mg/mL of BSA, and 1 μg/μL of poly(dI-dC)]. Following this incubation step, the samples were resolved by 4% polyacrylamide gel electrophoresis at 140 V and exposed to film.

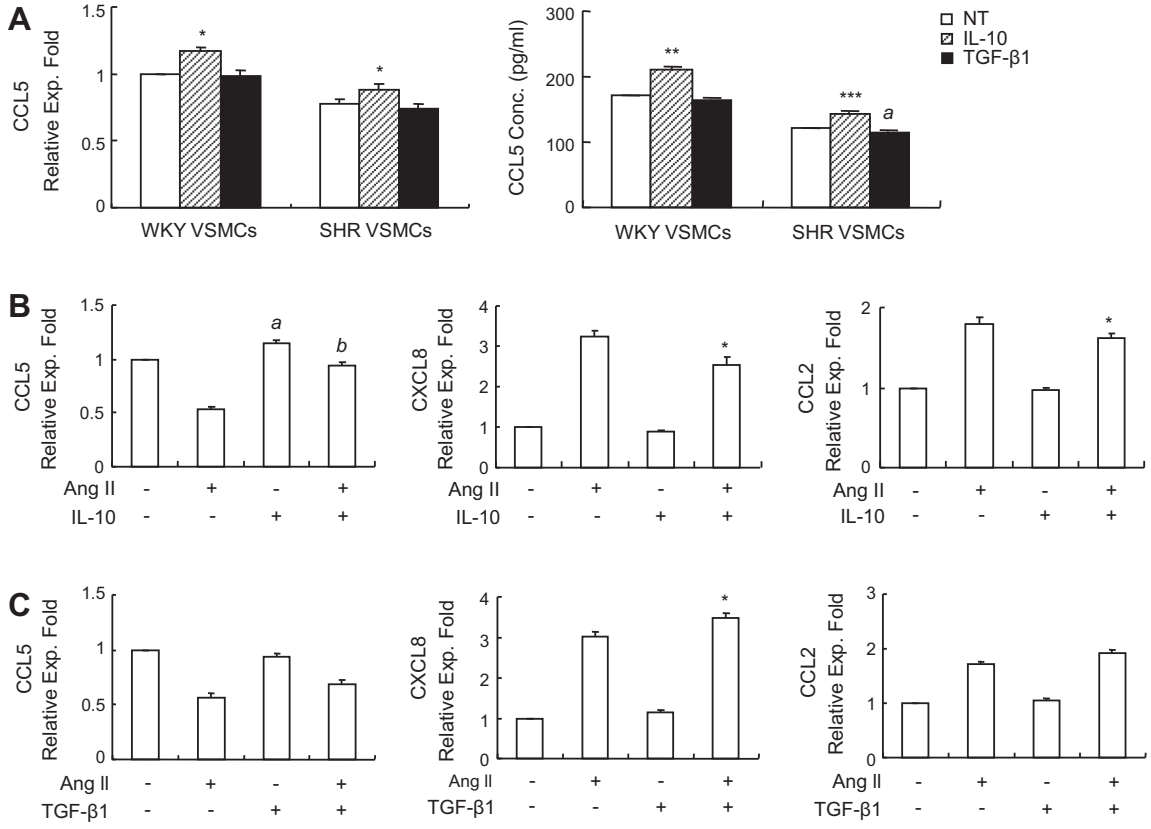

Fig. 1. IL-10 but not TGF-β increases CCL5 mRNA expression with different effects from TGF-β on Ang II-induced chemokine expression in SHR VSMCs. (A) SHR and WKY VSMCs were treated with or without IL-10 (25 ng/mL) or TGF-β (5 ng/mL) for 1 h. After total RNAs and cell supernatants were isolated, real-time PCR and ELISA for CCL5 were performed. Bars represent the means ± SEM of three independent experiments. $^{*}p < 0.05$ vs. untreated SHR or WKY VSMCs. $^{**}p < 0.01$ vs. untreated WKY VSMCs. $^{***}p < 0.001$ vs. untreated SHR VSMCs. $^{a}p < 0.01$ vs. untreated SHR VSMCs. (B and C) SHR VSMCs were treated with or without Ang II (0.1 μmol/L) and/or IL-10 (25 ng/mL or TGF-β, 5 ng/mL) for 1 h. After total RNAs were prepared, real-time PCR was performed. Bars represent the means ± SEM of three independent experiments. $^{a}p < 0.05$ vs. untreated SHR VSMCs. $^{b}p < 0.01$ vs. SHR VSMCs treated with Ang II. $^{*}p < 0.05$ vs. SHR VSMCs treated with Ang II.

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.

3. Results

3.1. IL-10 up-regulates CCL5 expression and attenuates Ang II-induced CCL5 inhibition in SHR VSMCs

TGF-β as well as IL-10 mediate anti-inflammatory, inhibitory effects on vascular cells [1,2]. Therefore, we first compared the direct effect of IL-10 on CCL5 mRNA expression with that of TGF-β in SHR and WKY VSMCs. TGF-β had no effect on CCL5 mRNA expression in both SHR and WKY VSMCs, whereas IL-10 increased CCL5 mRNA expression in both SHR and WKY VSMCs, and the protein levels of CCL5 correlated to the mRNA levels (Fig. 1A). Ang II increases chemokine CXCL8 and CCL2 expression, whereas it inhibits the expression of CCL5 in SHR VSMCs [20,23,24]. Thus, we additionally compared the effect of IL-10 with that of TGF-β on Ang II-induced expression of CCL5 as well as CXCL8 and CCL2 in SHR VSMCs. IL-10 inhibited Ang II-induced CXCL8 and CCL2 mRNA expression in SHR VSMCs, whereas it attenuated Ang II-induced CCL5 inhibition (Fig. 1B). On the other hand, TGF-β had no effect on Ang II-induced CCL2 mRNA expression or Ang II-induced CCL5 mRNA inhibition, and expression of Ang II-induced CXCL8 mRNA was rather increased by TGF-β (Fig. 1C).

We next compared the effect of IL-10 on Ang II-induced CCL5 inhibition between SHR and WKY VSMCs. First, Ang II had no statistically significant effect on CCL5 expression in WKY VSMCs, whereas Ang II inhibited CCL5 expression in SHR VSMCs. IL-10-induced CCL5 expression in WKY VSMCs was not significantly different from that in WKY VSMCs treated with Ang II and IL-10 simultaneously (Ang II/IL-10) (Fig. 2A). Up-regulation of CCL5 protein production and attenuation of Ang II-induced CCL5 protein inhibition by IL-10 were also detected in SHR VSMCs (Fig. 2B). Further, we observed a dose-dependent response of Ang II-induced CCL5 mRNA inhibition in response to IL-10 treatment. Doses of IL-10 ranging from 25 ng/mL to 100 ng/mL elevated CCL5 mRNA expression close to the level of untreated SHR VSMCs (Fig. 2C). The time course of Ang II-induced CCL5 mRNA inhibition in response to IL-10 treatment was also determined over a 16-h time period. Attenuation of Ang II-induced CCL5 mRNA inhibition by IL-10 was detected at 1 h after Ang II/IL-10 treatment and was sustained for up to 16 h. Increased CCL5 mRNA expression remained almost constant from 1 to 16 h upon Ang II/IL-10 treatment in SHR VSMCs (Fig. 2D).

3.2. Action mechanism of IL-10 on CCL5 expression in SHR VSMCs

We examined whether or not the IL-10-induced increase in CCL5 expression is mediated through AT_1 R or AT_2 R. First, we determined the effect of IL-10 on Ang II-induced AT_1 R and AT_2 R expression. IL-10 alone had no effect on either AT_1 R or AT_2 R expression (Fig. 3A). However, IL-10 reduced Ang II-induced AT_1 R mRNA and protein expression in SHR VSMCs, whereas it increased Ang II-induced AT_2 R mRNA and protein expression (Fig. 3A). Next, 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 the AT_1 R antagonist losartan (10 μmol/L) or the AT_2 R antagonist PD123319 (10 μmol/L) for 1 h, after which total RNAs were analyzed by real-time PCR. Losartan blocked Ang II-induced inhibition of CCL5 mRNA expression, whereas it had no effect on IL-10-induced CCL5 mRNA expression. Therefore, Ang II-induced inhibition of CCL5 mRNA expression was mediated by AT_1 R activation, whereas attenuation of Ang II-induced CCL5 mRNA inhibition by IL-10 was not (Fig. 3B). On the other hand, PD123319 inhibited IL-10-induced CCL5 expression. Expression of IL-10-induced CCL5

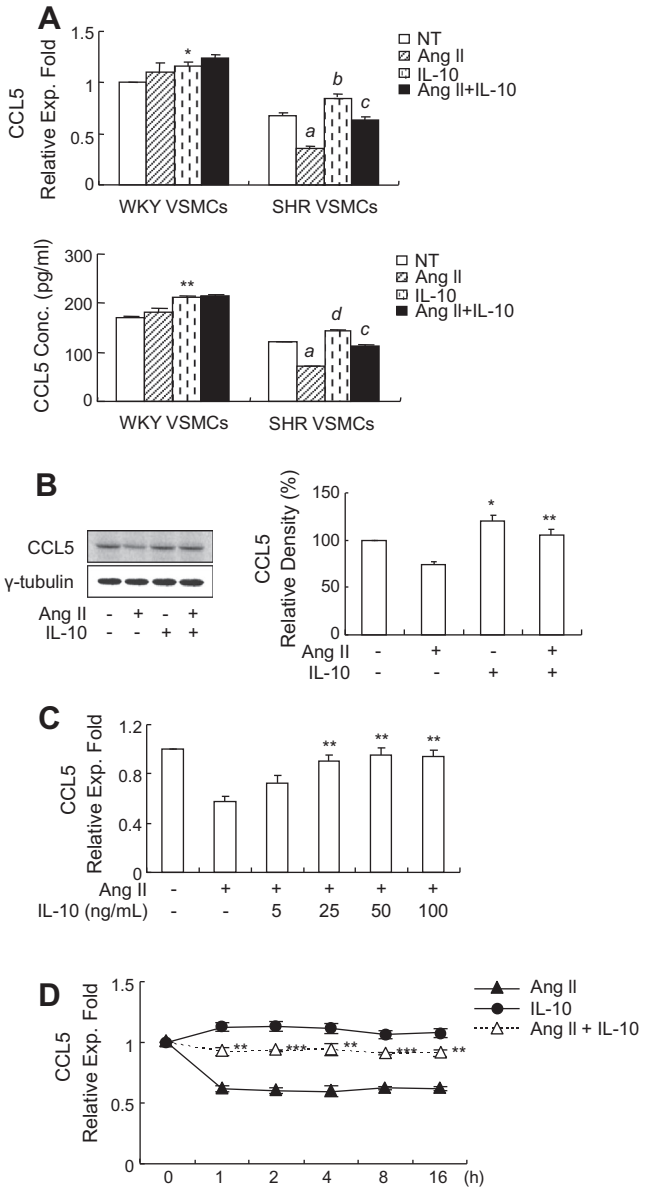

Fig. 2. IL-10 increases CCL5 expression and attenuates Ang II-induced CCL5 inhibition in SHR VSMCs. (A) SHR and WKY VSMCs were treated with or without Ang II (0.1 μmol/L) and/or IL-10 (25 ng/mL) for 1 h. After total RNAs and cell supernatants were isolated, real-time PCR and ELISA for CCL5 were performed. Bars represent the means ± SEM of three independent experiments. $^{*}p < 0.05$ vs. untreated WKY VSMCs. $^{**}p < 0.01$ vs. untreated WKY VSMCs. $^{a}p < 0.01$ vs. untreated SHR VSMCs. $^{b}p < 0.05$ vs. untreated SHR VSMCs. $^{c}p < 0.01$ vs. SHR VSMCs treated with Ang II. $^{d}p < 0.01$ vs. untreated SHR VSMCs. (B) SHR VSMCs were treated with or without Ang II (0.1 μmol/L) and/or IL-10 (25 ng/mL) for 1 h. After cell lysates were prepared, immunoblotting was performed. 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. SHR VSMCs treated with Ang II. (C) SHR VSMCs were treated with or without Ang II (0.1 μmol/L) and 0, 5, 25, 50, or 100 ng/mL of IL-10 simultaneously (Ang II/IL-10) for 1 h. (D) 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.

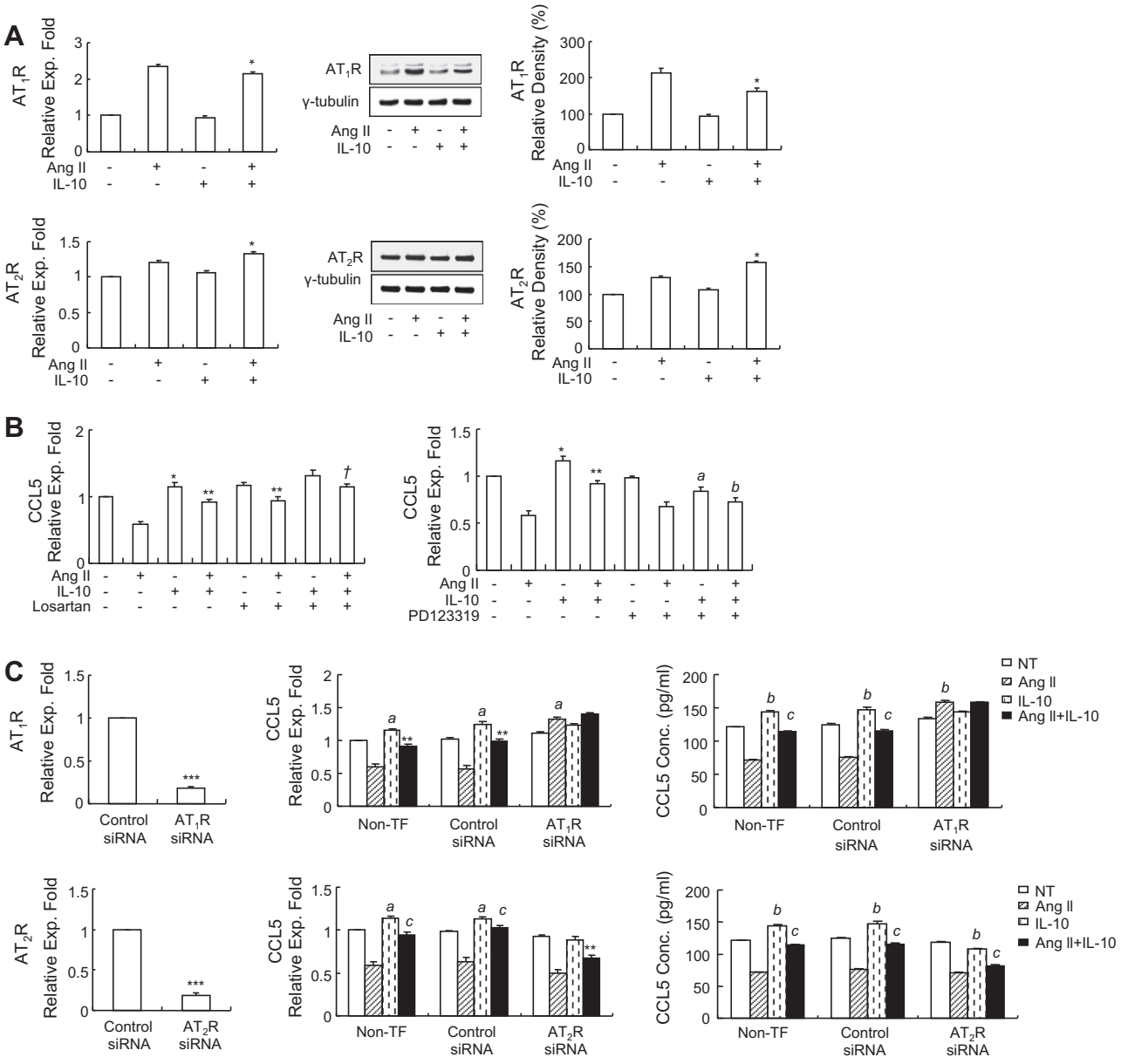

Fig. 3. IL-10-induced elevation of CCL5 expression is mediated by AT_2 R activation in SHR VSMCs. (A) SHR VSMCs were treated with or without Ang II (0.1 μmol/L) and/or IL-10 (25 ng/mL) for 1 h. After total RNAs and cell lysates were prepared, real-time PCR and immunoblotting were performed. Cell lysates were separated by 10% SDS-polyacrylamide gel electrophoresis and then immunoblotted with anti-AT_1 R or anti-AT_2 R antibody. 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. (B) 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 losartan (AT_1 R antagonist, 10 μmol/L) or PD123319 (AT_2 R antagonist, 10 μmol/L) for 1 h, after which total RNAs were analyzed by real-time PCR. Bars represent the means ± SEM of three independent experiments. $^{*}p < 0.05$ vs. untreated SHR VSMCs. $^{**}p < 0.01$ vs. SHR VSMCs treated with Ang II. $^{\dagger}p < 0.01$ vs. SHR VSMCs treated with Ang II/IL-10. $^{a}p < 0.01$ vs. SHR VSMCs treated with IL-10. $^{b}p < 0.05$ vs. SHR VSMCs treated with Ang II/IL-10. (C) For AT_1 R or AT_2 R siRNA transfection, SHR VSMCs were plated on 24-well plates, grown to 90% confluence, and transfected with AT_1 R siRNA, AT_2 R siRNA, or control siRNA siRNA oligomers (50 nmol/L). Total RNAs were analyzed by real-time PCR to confirm successful transfection. Additionally, VSMCs were treated with or without Ang II (0.1 μmol/L) and/or IL-10 (25 ng/mL) for 1 h, and CCL5 expression was verified by real-time PCR and ELISA. Non-TF: non-transfected SHR VSMCs. Bars represent the means ± SEM of three independent experiments. Data are representative of three independent experiments. $^{**}p < 0.01$ vs. SHR VSMCs treated with Ang II. $^{***}p < 0.001$ vs. non-transfected SHR VSMCs. $^{a}p < 0.01$ vs. untreated SHR VSMCs. $^{b}p < 0.001$ vs. untreated SHR VSMCs. $^{c}p < 0.001$ vs. SHR VSMCs treated with Ang II.

mRNA by PD123319 was reduced to a level below that in untreated SHR VSMCs. Attenuation of Ang II-induced CCL5 inhibition by IL-10 was also significantly reduced by PD123319 (Fig. 3B). To confirm these results, real-time PCR was performed on samples treated with AT_1 R or AT_2 R-directed siRNA. In SHR VSMCs transfected with AT_1 R siRNA, IL-10-induced CCL5 mRNA expression was sustained, whereas Ang II-induced CCL5 mRNA inhibition was not detected, and the protein levels of CCL5 correlated to the mRNA levels (Fig. 3C). On the other hand, in SHR VSMCs transfected with AT_2 R siRNA, IL-10-induced CCL5 mRNA expression was not detected, and attenuation of Ang II-induced CCL5 mRNA inhibition by IL-10 was significantly reduced, and the protein levels of CCL5 correlated to the mRNA levels. Further, the rate of reduction of Ang II-induced CCL5 mRNA inhibition by IL-10 in AT_2 R siRNA-transfected SHR VSMCs was reduced to 37.3 ± 5.1% compared to 62.9 ± 9.0% in control siRNA-transfected SHR VSMCs (Fig. 3C).

AMPK controls cellular metabolism [26,27]. Moreover, activation of AMPK has been implicated in the improvement of vascular abnormalities [26,28,29]. Therefore, we examined whether or not the up-regulatory effect of IL-10 on CCL5 expression is mediated

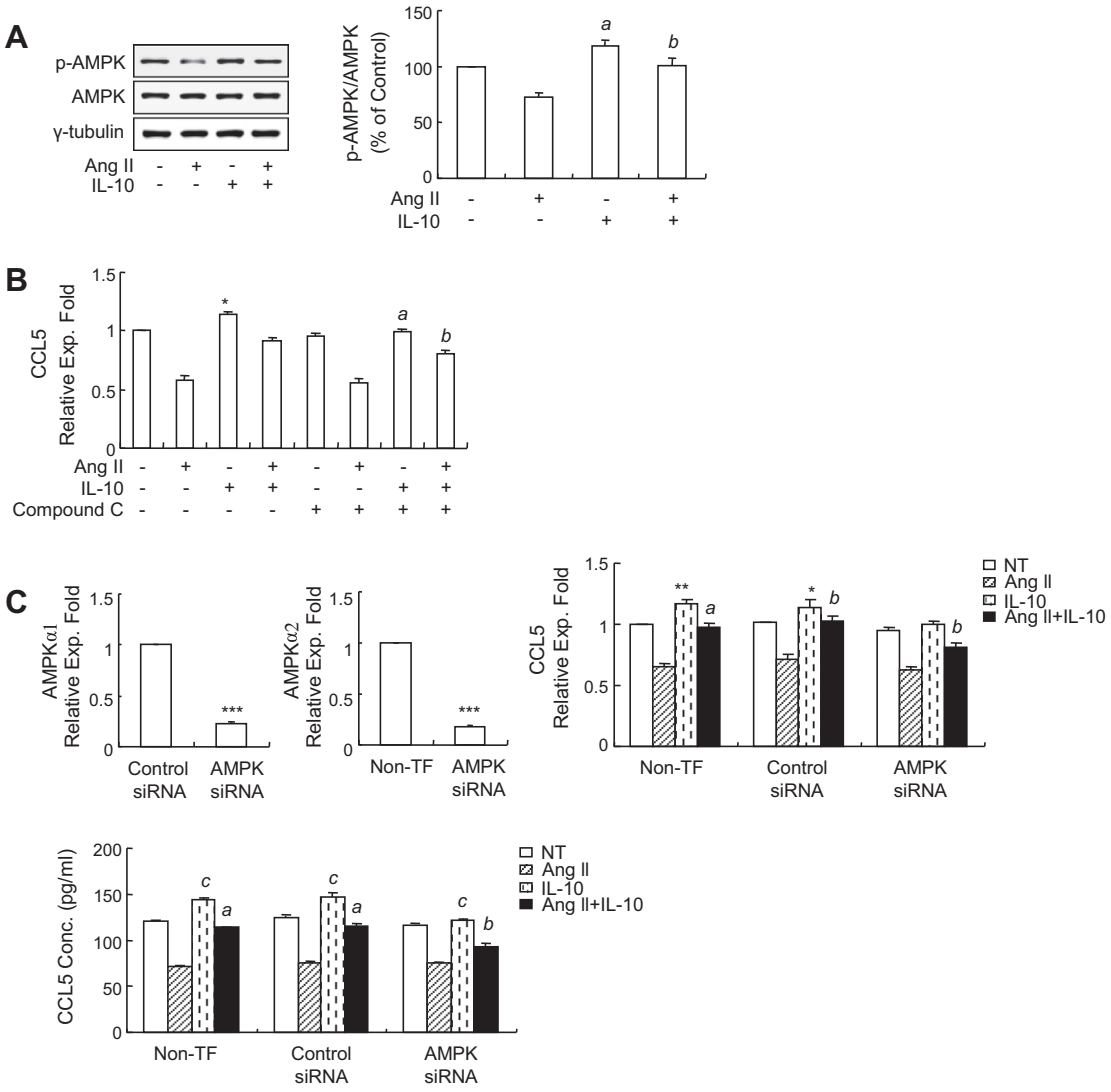

Fig. 4. IL-10 increases CCL5 expression through activation of AMPK in SHR VSMCs. (A) SHR VSMCs were treated with or without Ang II (0.1 μmol/L) and/or IL-10 (25 ng/mL) for 1 h. After cell lysates were prepared, immunoblotting was performed. Cell lysates were separated by 10% SDS-polyacrylamide gel electrophoresis and then immunoblotted with anti-p-AMPK or anti-AMPK antibody. 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. $^{b}p < 0.05$ vs. SHR VSMCs treated with Ang II. (B) 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 AMPK inhibitor, 10 μmol/L) for 1 h, after which total RNAs were analyzed by real-time PCR. $^{*}p < 0.05$ vs. untreated SHR VSMCs. $^{a}p < 0.05$ vs. SHR VSMCs treated with IL-10. $^{b}p < 0.05$ vs. SHR VSMCs treated with Ang II/IL-10. (C) For AMPK siRNA transfection, SHR VSMCs were plated on 24-well plates, grown to 90% confluence, and transfected with AMPK siRNA or control siRNA oligomers (50 nmol/L). Total RNAs were analyzed by real-time PCR to confirm successful transfection. Additionally, VSMCs were treated with or without Ang II (0.1 μmol/L) and/or IL-10 (25 ng/mL) for 1 h, and CCL5 expression was verified by real-time PCR and ELISA. Non-TF: non-transfected SHR VSMCs. Bars represent the means ± SEM of three independent experiments. Data are representative of three independent experiments. $^{*}p < 0.05$ vs. untreated SHR VSMCs. $^{**}p < 0.01$ vs. untreated SHR VSMCs. $^{***}p < 0.001$ vs. non-transfected SHR VSMCs. $^{a}p < 0.001$ vs. SHR VSMCs treated with Ang II. $^{b}p < 0.01$ vs. SHR VSMCs treated with Ang II. $^{c}p < 0.001$ vs. untreated SHR VSMCs.

through AMPK activation in SHR VSMCs. First, IL-10 itself increased AMPK activation and attenuated Ang II-induced p-AMPK inhibition in SHR VSMCs (Fig. 4A). Compound C, an AMPK inhibitor, inhibited the up-regulatory effect of IL-10 on CCL5 mRNA expression in SHR VSMCs. Specifically, IL-10-induced CCL5 mRNA expression upon Compound C treatment was reduced to the level of untreated SHR VSMCs, whereas it had no effect on Ang II-induced CCL5 inhibition. Attenuation of Ang II-induced CCL5 inhibition by IL-10 was also significantly reduced by Compound C (Fig. 4B). To confirm these results, real-time PCR was performed on samples transfected with AMPK-directed siRNA. In SHR VSMCs transfected with AMPK siRNA, IL-10-induced CCL5 mRNA expression was not detected, and attenuation of Ang II-induced CCL5 mRNA inhibition by IL-10 was reduced, and the protein levels of CCL5 correlated to the mRNA levels. The rate of reduction of Ang II-induced CCL5 inhibition by IL-10 in AMPK siRNA-transfected SHR VSMCs was reduced to 30.3 ± 6.2% compared to 43.6 ± 2.9% in control siRNA-transfected SHR VSMCs (Fig. 4C).

3.3. Attenuation of Ang II-induced CCL5 inhibition by IL-10 is mediated through nuclear factor-κB (NF-κB) inactivation in SHR VSMCs

To determine how IL-10 attenuates Ang II-induced CCL5 inhibition in SHR VSMCs, the role of NF-κB activation was examined. SHR VSMCs were treated with or without Ang II (0.1 μmol/L) and/or IL-10 (25 ng/mL) in the absence or presence of Bay11-7082 (10 μM, a selective inhibitor of IκB-α) for 1 h. Increased CCL5 mRNA expression by IL-10 was not associated with NF-κB activation, whereas the inhibitory effect of Ang II on CCL5 mRNA expression was dependent on NF-κB activation. Therefore, Bay11-7082 increased

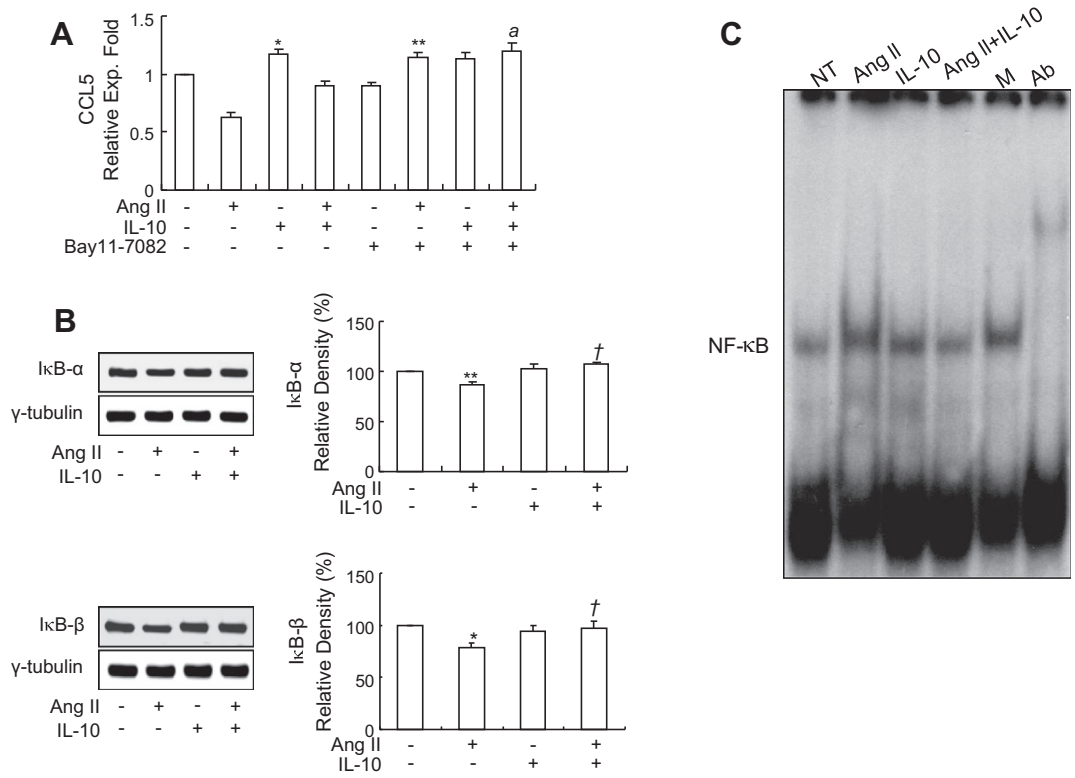

Fig. 5. Attenuation of Ang II-induced CCL5 inhibition by IL-10 is associated with NF-κB inactivation in SHR VSMCs. (A) SHR VSMCs were treated with or without Ang II (0.1 μmol/L) and/or IL-10 (25 ng/mL) in the absence or presence of Bay11-7082 (a selective inhibitor of IκB-α, 10 μM) for 1 h. After total RNAs were isolated, real-time PCR was performed. Bars represent the means ± SEM of three independent experiments. $^{*}p < 0.05$ vs. untreated SHR VSMCs. $^{**}p < 0.01$ vs. SHR VSMCs treated with Ang II. $^{a}p < 0.05$ vs. SHR VSMCs treated with Ang II/IL-10. (B) SHR VSMCs were treated with or without Ang II (0.1 μmol/L) and/or IL-10 (25 ng/mL) for 1 h. After cell lysates were prepared, immunoblotting was performed. Cell lysates were separated by 10% SDS-polyacrylamide gel electrophoresis and then immunoblotted with anti-IκB-α or anti-IκB-β antibody. 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. $^{\dagger}p < 0.01$ vs. SHR VSMCs treated with Ang II. (C) Specific binding activity of NF-κB from nuclear extracts was assessed by EMSA. SHR VSMCs were treated with or without Ang II (0.1 μmol/L) and/or IL-10 (25 ng/mL) for 1 h. Aliquots of nuclear extract were incubated with 100-fold excess of mutant probe (M) or 2 μg of anti-NF-κB antibody (Ab) before EMSA. Data shown are representative of three independent experiments.

CCL5 mRNA expression in SHR VSMCs treated with Ang II/IL-10 (Fig. 5A). To elucidate this result, we examined IκB-α and IκB-β degradation patterns as well as NF-κB activities in SHR VSMCs treated with Ang II/IL-10 compared with those in SHR VSMCs treated with Ang II or IL-10 alone. IL-10 alone did not affect either IκB-α or IκB-β degradation in SHR VSMCs, whereas IL-10 attenuated Ang II-induced IκB-α and IκB-β degradation (Fig. 5B). Additionally, inhibition of Ang II-induced NF-κB activation by IL-10 was detected (Fig. 5C).

3.4. IL-10 partially mediates the inhibitory effect of CCL5 on Ang II-induced hypertensive mediators in SHR VSMCs

CCL5 reduces Ang II-induced 12-LO and ET-1 expression in SHR VSMCs [21,22]. Therefore, we investigated whether or not IL-10 mediates the inhibitory actions of CCL5 in SHR VSMCs. First, we examined the effect of IL-10 on 12-LO and ET-1 mRNA expression in SHR VSMCs. IL-10 alone did not affect 12-LO and ET-1 mRNA expression. However, IL-10 had an inhibitory effect on Ang II-induced 12-LO and ET-1 mRNA expression. In addition, IL-10 and CCL5 synergistically inhibited 12-LO and ET-1 mRNA expression as well as Ang II-induced 12-LO and ET-1 mRNA expression (Fig. 6A). Next, an experiment involving siRNA-mediated down-regulation of IL-10 was performed. The rate of reduction of Ang II-induced 12-LO expression by CCL5 in IL-10 siRNA-transfected SHR VSMCs was reduced to 14.9 ± 1.8% compared to 32.9 ± 1.0% in control siRNA-transfected SHR VSMCs, and the protein levels of 12-LO and ET-1 correlated to the mRNA levels. In the case of ET-1 expression, the rate of reduction of Ang II-induced ET-1 expression by CCL5 in IL-10 siRNA-transfected SHR VSMCs was reduced to 11.3 ± 4.3% compared to 33.1 ± 1.1% in control siRNA-transfected SHR VSMCs, and the protein levels of 12-LO and ET-1 correlated to the mRNA levels (Fig. 6B).

4. Discussion

IL-10 has been suggested to be an important mediator of vascular protection during atherosclerosis [30] and prevents impairment of Ang II-induced endothelium-dependent vasorelaxation by suppressing NADPH oxidase expression and Ang II-induced inflammation [9,31]. In an *in vivo* study, IL-10 was shown to mediate a feedback loop for the continued inhibition of proinflammatory cytokine production [1]. However, several *in vitro* studies have shown that IL-10 has no anti-inflammatory effect on adhesion molecule and chemokine production in endothelial cells or TNF-α-induced IL-8 and MCP-1 expression in human VSMCs [32,33]. It has been suggested that these negative effects of IL-10 on vascular cells can be attributed to a lack of IL-10 receptor or impairment of the intracellular IL-10 signaling pathway [32]. In the present study, IL-10 up-regulated CCL5 expression in SHR VSMCs. Although the increase in CCL5 expression induced by IL-10 was low, it was statistically significant, and the attenuation of Ang II-induced CCL5

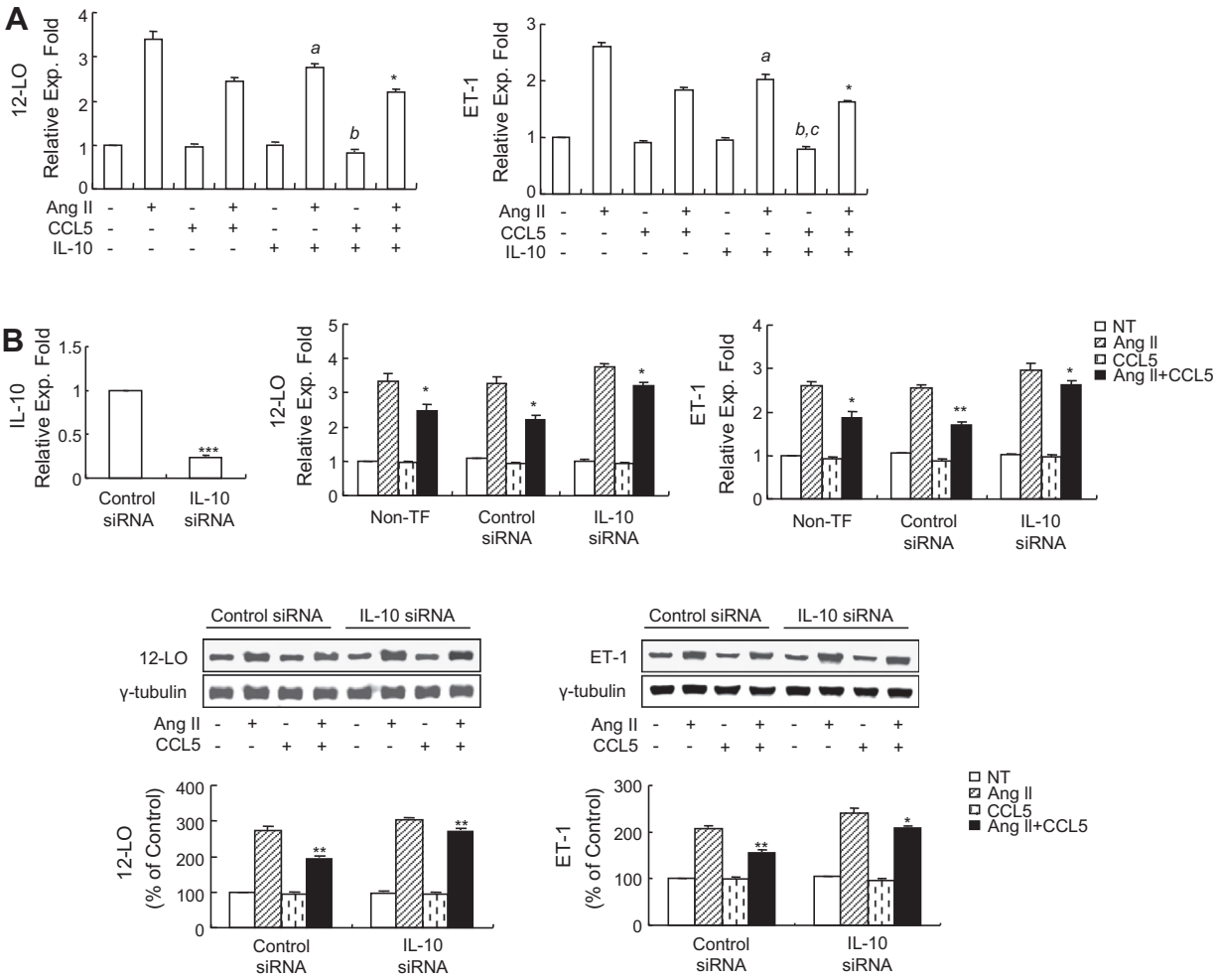

Fig. 6. IL-10 partially mediates inhibitory effects of CCL5 on Ang II-induced 12-LO and ET-1 expression in SHR VSMCs. (A) SHR VSMCs were treated with or without Ang II (0.1 μmol/L) and/or CCL5 (100 ng/mL) in the absence or presence of IL-10 (25 ng/mL) for 2 h. After total RNAs were prepared, real-time PCR was performed. Bars represent the means ± SEM of three independent experiments. [a]$p < 0.05$ vs. SHR VSMCs treated with Ang II. [b]$p < 0.05$ vs. SHR VSMCs treated with CCL5 or IL-10. [c]$p < 0.01$ vs. SHR VSMCs treated with IL-10. *$p < 0.05$ vs. SHR VSMCs treated with Ang II/CCL5. (B) For IL-10 siRNA transfection, SHR VSMCs were plated on 24-well plates, grown to 90% confluence, and transfected with IL-10 siRNA or control siRNA oligomers (50 nmol/L). Total RNAs were analyzed by real-time PCR to confirm successful transfection. Additionally, VSMCs were treated with or without Ang II (0.1 μmol/L) and/or CCL5 (100 ng/mL) for 2 h, after which 12-LO and ET-1 expression were verified by real-time PCR and immunoblotting. Non-TF: non-transfected SHR VSMCs. Bars represent the means ± SEM of three independent experiments. Data are representative 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. non-transfected SHR VSMCs.

inhibition by IL-10 was high. Moreover, contrary to IL-10, TGF-β had no effect on CCL5 mRNA expression or Ang II-induced CCL5 inhibition. It is well known that although both IL-10 and TGF-β exert inhibitory effects on vascular inflammatory responses and have potent anti-inflammatory effects on vascular cells, TGF-β mainly down-regulates cytokine-induced adhesion molecule expression and neutrophil migration. On the other hand, IL-10 directly inhibits proinflammatory cytokine production in macrophages [1,2] Therefore, IL-10 and TGF-β are likely to differentially affect SHR VSMCs, and IL-10 instead of TGF-β may play an up-regulatory role in CCL5 activity in SHR VSMCs.

Ang II inhibits the expression of CCL5 in SHR VSMCs [20]. In this study, Ang II had no effect on CCL5 expression in WKY VSMCs. Although a new action mechanism for Ang II in CCL5 expression must be elucidated between SHR and WKY VSMCs, this result indicates the pleiotropism of Ang II as a cytokine. Additionally, in our *in vivo* study (data not shown), CCL5 expression in thoracic aorta tissues from C57BL/6 IL-10 knockout mice was elevated compared with that in wild type mice. This result suggests that CCL5 is likely to function as both a proinflammatory chemokine in a normotensive state and as an anti-hypertensive mediator in a hypertensive state.

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 [34]. AT_1 R plays up-regulatory roles in Ang II-induced proinflammatory cytokine production and reactive oxygen species (ROS) release via NADPH oxidase. 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 has been reported to antagonize the vascular actions of AT_1 R. Many studies have suggested that AT_2 R has growth inhibitory and proapoptotic effects in VSMCs [35,37,38]. However, several studies have reported growth and proinflammatory actions of AT_2 R in VSMCs [34,38]. In addition, AT_2 R has been demonstrated to play a positive role in Ang II-induced CCL5 expression in rat glomerular endothelial cells and the rat renal cortex [17,18]. However, studies on the action of IL-10 on AT_1 R or AT_2 R activation cannot be found. In this study, IL-10 alone had no effect on either AT_1 R or AT_2 R mRNA expression in SHR VSMCs. However, IL-10 reduced Ang II-induced AT_1 R expression and elevated Ang II-induced AT_2 R expression in SHR VSMCs. This effect of IL-10 on Ang II receptors may suggest a positive regulatory role in vascular hypertensive walls. Moreover, the increase in IL-10-induced

CCL5 mRNA expression was mediated by AT_2 R activation. In our previous studies, the attenuating effects of CCL5 on Ang II-induced 12-LO expression as well as Ang II-induced DDAH-1 inhibition were shown to be mediated via AT_2 R activity in SHR VSMCs [21,22]. Therefore, although the actions of AT_2 R are still controversial, AT_2 R activation is thought to have up-regulatory effects on the activities of CCL5 and IL-10 in Ang II-treated SHR VSMCs.

The AMP-activated protein kinase (AMPK) signaling system plays a crucial role in cellular metabolic homeostasis [26,27]. Although AMPK is known for its effects on cellular metabolism, the AMPK pathway involves inhibition of NF-κB signaling and suppression of inflammation [29]. Sag et al. [39] reported that IL-10 stimulates the phosphorylation and activation of AMPK in macrophages. In addition, AMPK activation exhibits beneficial effects on vascular function, such as improvement of endothelial function or inhibition of VSMC proliferation [40]. In SHR VSMCs, we also observed that IL-10 stimulated AMPK activation, and the effect of IL-10 on CCL5 expression was dependent on AMPK activation.

Many studies have reported that NF-κB activation plays a pivotal role in the vascular inflammatory process of hypertension [41–43]. Higher mRNA expression of NF-κB and lower expression of IκB in SHR aortic tissue compared to WKY aortic tissue has been documented, and increased inflammatory mediators in SHR are associated with up-regulation of NF-κB activity [43]. Moreover, Ang II is a proinflammatory mediator that plays an important role in vascular and cardiac tissue inflammation associated with hypertension, and this role involves activation of the NF-κB system [41,43–46]. Interaction between Ang II and AT_1 R activates NF-κB [44]. In our previous study, we demonstrated that Ang II-induced inhibition of CCL5 expression in SHR VSMCs is mediated by activation of NF-κB. In this study, IL-10-induced up-regulation of CCL5 mRNA expression was not related to NF-κB activity. Further, IL-10 alone did not affect IκB-α or IκB-β degradation in SHR VSMCs. However, IL-10 inhibited Ang II-induced IκB-α and IκB-β degradation in SHR VSMCs. We also detected inhibition of NF-κB activity in SHR VSMCs treated with Ang II/IL-10 by EMSA. These results coincide with several IL-10 studies on NF-κB activity [46–48]. Activation of NF-κB is attenuated upon inhibition of IκB degradation in proteasomes. IL-10 blocks stimuli-induced NF-κB activity via both inhibition of IκB kinase activity (IκB degradation) and NF-κB DNA-binding activity [1,47]. Schottelius et al. [47] has provided evidence that IL-10 inhibits TNF-induced NF-κB activation by blocking inhibition of IκB degradation and NF-κB binding to DNA in human monocytic cell lines. They also showed that IL-10 alone has no effect on IκB degradation or NF-κB DNA-binding activity. Salminen et al. [29] suggested that AMPK inhibits NF-kB signaling indirectly via its downstream mediators (SIRT1, FoxO, and PGC-1α). Thus, along with the direct inhibitory effect of IL-10 on IkB degradation, the inhibition of NF-kB activity due to IL-10-induced AMPK activation may play a role in the reversal of Ang II-induced CCL5 inhibition by IL-10.

Activities of both 12-LO and ET-1 have been linked to the development of hypertension [49,50]. Ang II is a potent positive regulator of 12-LO activation, and activity of 12-LO 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 [50]. CCL5 remarkably inhibits Ang II-induced 12-LO and ET-1 expression [21,22] Therefore, we examined whether or not IL-10 can alter the anti-hypertensive effects of CCL5 in SHR VSMCs. IL-10 increased not only CCL5 expression but also the inhibitory effects of CCL5 on Ang II-induced 12-LO and ET-1 expression. Moreover, IL-10 partially mediated the inhibitory effects of CCL5 on Ang II-induced 12-LO and ET-1 expression. This result suggests that IL-10 most likely plays an anti-hypertensive synergistic role along with CCL5 in Ang II-induced hypertensive vascular environments.

In conclusion, IL-10 increases CCL5 expression and attenuates the inhibitory effect of Ang II on CCL5 expression in SHR VSMCs. IL-10-induced elevation of CCL5 expression is mediated mainly through AT_2 R and AMPK activation, whereas the attenuating effect of IL-10 on Ang II-induced inhibition of CCL5 expression is mediated mainly via NF-κB inactivation. Moreover, IL-10 partially mediates the inhibitory effects of CCL5 on Ang II-induced 12-LO and ET-1 expression in SHR VSMCs. Until now, there has been no direct evidence of a role for IL-10 in CCL5 activity in Ang II-induced vascular hypertension. Thus, this study provides the first evidence that IL-10 acts in an up-regulatory role in CCL5 activity in SHR VSMCs. Although further *in vivo* studies should be performed, it is highly possible that IL-10 plays a cooperative role in the anti-hypertensive activity of CCL5 during Ang II-induced vascular hypertension.

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