ORIGINAL ARTICLE

CCL5 upregulates IL-10 expression and partially mediates the antihypertensive effects of IL-10 in the vascular smooth muscle cells of spontaneously hypertensive rats

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Interleukin (IL)-10 inhibits angiotensin (Ang) II-induced vascular dysfunction and reduces blood pressure in hypertensive pregnant rats. The chemokine CCL5 has also been shown to downregulate Ang II-induced hypertensive mediators in spontaneously hypertensive rats (SHRs). This study investigated the effects of CCL5 on IL-10 expression, as well as its mechanisms of action in the vascular smooth muscle cells (VSMCs) of SHRs. CCL5 increased IL-10 expression in the VSMCs of SHRs; the s.c. injection of CCL5 ($1.5 \mu\text{g kg}^{-1}$, twice a day) for 3 weeks into SHRs with established hypertension upregulated IL-10 expression in both the thoracic aorta and the VSMCs and decreased systolic blood pressure. CCL5-induced the elevation of IL-10 expression, an effect mediated primarily via the activation of an Ang II subtype II receptor (AT_2 R). Dimethylarginine dimethylaminohydrolase (DDAH)-1 activity also contributed to the elevation of IL-10 expression via CCL5 in the VSMCs of SHRs. Moreover, CCL5 partially mediated the inhibitory effects of IL-10 on Ang II-induced 12-lipoxygenase (LO) and endothelin (ET)-1 expression in the VSMCs of SHRs. Taken together, this study provides novel evidence that CCL5 plays a role in the upregulation of IL-10 activity in the VSMCs of SHRs.

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INTRODUCTION

The involvement of vascular inflammation in the pathogenesis of hypertension has been well documented, as chemokines mediate the infiltration of inflammatory cells into vascular walls, contributing to the pathogenesis of hypertension.^{1–3} The increased activation of chemokines CCL2 and CXCL8 occurs within the arterial walls of hypertensive animals, and both the CCL2 and the CCR2 pathway are involved in inflammatory reactions associated with vascular injury in the setting of hypertension.^{4–6} Additionally, the suppression of chemokine-induced inflammatory cell infiltration and the blockade of chemokine receptors CXCR1 and CXCR2 has been shown to ameliorate hypertension in experimental animal models.^{1,4,7} Therefore, the inhibition of chemokine production may be important in the regulation of inflammatory reactions within hypertensive vascular walls. However, in our previous studies, the chemokine CCL5 downregulated the angiotensin (Ang) II-induced expression of the hypertensive mediators 12-lipoxygenase (LO) and endothelin (ET)-1, and also as upregulated dimethylarginine dimethylaminohydrolase (DDAH) activity, an important regulator of nitric oxide bioavailability, in the vascular smooth muscle cells (VSMCs) of spontaneously hypertensive rats (SHRs).^{8,9} Therefore, although CCL5 acts as an

inflammatory mediator in the pathogenesis of various diseases, it most likely plays a downregulatory role in the setting of Ang II-induced vascular hypertension, which contrasts with the upregulatory roles played by chemokines CCL2 and CXCL8 in hypertension development and maintenance.^{4–6,10}

Interleukin (IL)-10, an anti-inflammatory cytokine, exerts important inhibitory effects on vascular inflammatory responses.^{11,12} In previous hypertension studies, IL-10 has been shown to prevent Ang II-induced vasoconstriction by reducing the expression of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase; IL-10 also regulates vascular function by downregulating both proinflammatory cytokine expression and superoxide production within vascular walls.^{13–16} Additionally, exogenous IL-10 has been shown to reduce blood pressure in hypertensive pregnant rats.¹⁶ In a previous study, IL-10 increased CCL5 expression and attenuated Ang II-induced CCL5 inhibition significantly in the VSMCs of SHRs. Moreover, IL-10 partially mediated the inhibitory effects of CCL5 on both Ang II-induced 12-LO and ET-1 expression in the VSMCs of SHRs.¹⁷ These results suggested that IL-10 plays an upregulatory role in the antihypertensive activity of CCL5 in the VSMCs of SHRs. Therefore, we hypothesized that CCL5, which exerts an upregulatory effect on

antihypertensive mediators, also affects IL-10 activity in the VSMCs of SHRs.

Although both IL-10 and CCL5 have exhibited ameliorative effects on Ang II-induced vascular dysfunction, the potential effects of CCL5 on IL-10 expression in Ang II-induced vascular hypertension have not been evaluated. Therefore, the present study investigated the effects of CCL5 on IL-10 expression, as well as its mechanisms of action in the VSMCs of SHRs.

METHODS

Reagents

An easy-blue total RNA extraction kit for total RNA isolation was purchased from iNtRON Biotechnology (Seoul, Korea). CCL5 and IL-10 were purchased from R&D systems (Minneapolis, MN, USA). Losartan, PD123319 and Met-RANTES were purchased from Sigma-Aldrich Co. (St Louis, MO, USA). nor-NOHA was purchased from Cayman Chemical (Ann Arbor, MI, USA). The LightCycler FastStart DNA SYBR Green I Mix was obtained from Roche (Mannheim, Germany). The goat anti-human IL-10, 12-LO and ET-1 polyclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The primer sequences for IL-10, transforming growth factor (TGF)- β , the Ang II subtype I receptor (AT₁ R), the Ang II subtype II receptor (AT₂ R), DDAH-1, CCL5, 12-LO, ET-1, and β -actin were synthesized at Bionics (Daejeon, South Korea). The rat AT₁ R, AT₂ R, DDAH-1 and CCL5 small interfering RNA (siRNA) sequences were purchased from Bioneer Technology (Daejeon, South Korea). The negative control siRNA was purchased from Invitrogen (Carlsbad, CA, USA). All other reagents were pure-grade commercial preparations.

Animals and experimental protocols

Specific pathogen-free, male inbred SHR and Wistar-Kyoto rats (WKY) were purchased from Japan SLC Inc. (Shizuoka, Japan). All experimental animals received autoclaved food and bedding to minimize exposure to both viral and microbial pathogens. The rats were cared for in accordance with the Guide for the Care and Use of Experimental Animals of Yeungnam Medical Center. The 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 included six SHRs with established hypertension (eSHRi); an equal number of normal saline-treated SHRs (eSHRc) served as a control group. Nineteen-week-old SHRs received s.c. injections of CCL5 (1.5 $\mu\text{g kg}^{-1}$) twice a day for 3 weeks. Blood pressure was measured before treatment, every week during treatment and 1 day following the final injection of either CCL5 or normal saline. CCL5 treatment did not influence the body weights of the eSHRi rats. Both the eSHRi and eSHRc groups exhibited an age-related increase in body weight. One day following the final injection, both the control and the CCL5-treated rats were anesthetized via an i.p. injection of urethane (1.5 g kg^{-1}); thoracic aorta tissue specimens subsequently were collected. The VSMCs were then isolated from the thoracic aorta tissues, and the effects of CCL5 on IL-10 expression in both the thoracic aortas and the VSMCs were examined.

Measurement of blood pressure

Systolic and diastolic blood pressures were measured using a CODA high-throughput tail blood pressure system (Kent Scientific, Torrington, CT, USA). The rats were restrained inside clear Perspex rat restrainers and placed on a preheated base plate (38–40 °C) for ~35 min. The CODA tail-cuff blood pressure system utilizes volume pressure recording sensor technology to measure rat tail blood pressures. Several systolic and diastolic blood pressure readings were recorded for each rat, and four median systolic blood pressure readings were averaged. The average of the four median readings was used as the mean systolic and diastolic blood pressure.

Preparation of VSMCs

The VSMCs were obtained from the thoracic aortas of 22-week-old SHR and WKY rats via the explant method, as described by Kim *et al.*⁶ All experiments were conducted between cell passages three and seven. Prior to stimulation,

95% confluent VSMCs were serum-starved overnight via incubation in DMEM supplemented with 0.1% fetal bovine serum. The cell cultures were incubated in a humidified incubator at 37 °C and 5% CO₂ in either the presence or the absence of stimuli for the indicated times.

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

Total RNA was extracted using an easy-BLUE total RNA extraction kit (iNtRON Biotechnology) according to the manufacturer's instructions. Real-time PCR amplifications were performed as previously described.^{8,17} The primers used for PCR were as follows: IL-10 (245 bp) sense, 5'-tgccttcagtcag tgaagac-3', and antisense, 5'-aaactcattcatggccttgta-3'; TGF- β (205 bp) sense, 5'-tgtcttttgacgtcactggagtgt-3', and antisense, 5'-gggtggccatgaggacagg-3'; DDAH-1 (181 bp) sense, 5'-cgcaatagggtccagtgaat-3', and antisense, 5'-ttgcgctttctgggtactct-3'; CCL5 (110 bp) sense, 5'-cgtgaaggagtattttaccacagc-3', and antisense, 5'-cttgaaccactctctctctggg-3'; 12-LO (312 bp) sense, 5'-tggggcaac tgggaagg-3', and antisense, 5'-agagcgcttcagccatc-3'; ET-1 (370 bp) sense, 5'-ctcctccttgaggacaagg-3', and antisense, 5'-cttgatgctgtgctgatgg-3'; AT₁ R (445 bp) sense, 5'-caccatgtaagatcgcttc-3', and antisense, 5'-gcacaatcgccataatta tcc-3'; AT₂ R (65 bp) sense, 5'-ccgtgaccaagtcttgaagatg-3', and antisense, 5'-agg gaaggcagaatgatg-3'; and β -actin (101 bp) sense, 5'-tactgccttgcctctagca-3', and antisense, 5'-tggacagtggagccaggatag-3'. The mRNA levels of IL-10, TGF- β , DDAH-1, AT₁ R, AT₂ R, 12-LO and ET-1 were determined by comparing their experimental levels to standard curves, and were expressed as relative fold expression levels.

Western blotting

Total lysates were prepared in PRO-PREP buffer (iNtRON Biotechnology). The preparation of the cell lysates and the western blot analysis were performed as previously described, using the indicated antibodies.¹⁶

DDAH activity

DDAH activity was assayed as described by Ueda *et al.*¹⁸ Equal amounts of protein (20 μg) were incubated with 4 mmol l⁻¹ asymmetric (N^G, N^G) dimethylarginine (ADMA)-0.1 mol l⁻¹ 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 via the addition of an equal volume of 4% sulfosalicylic acid, the supernatants (100 μl) were boiled with diacetyl monoxime (0.8% wt/vol in 5% acetic acid) and antipyrine (0.5% wt/vol in 50% sulfuric acid). The amounts of L-citrulline formed were determined via a spectrophotometric analysis at 466 nm (UV-Visible spectrophotometer, Shimadzu UV-160, Kyoto, Japan).

Small interfering RNA (siRNA)

The SHR VSMCs were plated on 6-well plates and grown to 90% confluence. The VSMCs were then transfected with AT₁ R, AT₂ R, DDAH-1 and CCL5 siRNA oligomers (50 nmol l⁻¹) using lipofectamine 2000, according to the manufacturer's instructions (Invitrogen Life Technologies Inc., Gaithersburg, MD, USA). Following 24 h of incubation, the VSMCs were placed in growth medium for 24 h before the experiments. The cells were then cultured in either the presence or the absence of stimuli for 2 h. The sense and antisense oligonucleotides used in these experiments were as follows: AT₁ R siRNA sense, 5'-gucacuguuacuaacacua-3', and antisense, 5'-uagguuguaguacagugac-3'; AT₂ R siRNA sense, 5'-gaguguuguaguagguacaa-3', and antisense, 5'-uugguuacua caacacuc-3'; DDAH-1 siRNA sense, 5'-ucagagagacugagucacu-3', and antisense, 5'-agugacucagucucucuga-3'; and CCL5 siRNA sense, 5'-cagagaagaag ugguuacua-3', and antisense, 5'-ugaaccacuuucucucug-3'.

Statistical analysis

The results are expressed as the means $\pm$ s.e.m. of at least three or four independent experiments. Statistical significance was determined via either the Student's *t*-test or the one-way analysis of variance, followed by a Bonferroni test. A *P* value less than 0.05 was considered to be statistically significant.

Figure 1 CCL5 increases IL-10 expression in the thoracic aorta tissues and VSMCs of SHRs. (a) After the total RNAs were isolated from either the SHR or the WKY thoracic aorta tissues and VSMCs, real-time PCR was performed. * $P < 0.05$ vs. WKY thoracic aorta tissues or VSMCs. (b) The VSMCs of the SHRs were either untreated or treated with CCL5 (100 ng ml^{-1}) for 2 h, after which real-time PCR was performed. ** $P < 0.01$ vs. untreated SHR VSMCs. (c) The VSMCs of SHRs were either untreated or treated with either Ang II ($0.1 \mu\text{mol l}^{-1}$) or CCL5 (100 ng ml^{-1}) for 2 h. After the total RNAs and cell lysates were prepared, both real-time PCR and immunoblotting were performed. Additionally, the VSMCs of the SHRs were treated either with or without Ang II ($0.1 \mu\text{mol l}^{-1}$) and 0, 10, 50, 100, 200 or 400 ng ml^{-1} of CCL5 simultaneously for 2 h. For the time course reaction, the VSMCs of SHRs were treated either with or without Ang II ($0.1 \mu\text{mol l}^{-1}$) or CCL5 (100 ng ml^{-1}) for the indicated times. After the total RNAs were isolated, real-time PCR was performed. * $P < 0.05$ vs. untreated SHR VSMCs. ** $P < 0.01$ vs. untreated SHR VSMCs. *** $P < 0.001$ vs. untreated SHR VSMCs. ^a $P < 0.05$ vs. SHR VSMCs treated with Ang II. ^b $P < 0.01$ vs. SHR VSMCs treated with Ang II. ^c $P < 0.001$ vs. SHR VSMCs treated with Ang II. The bars represent the means $\pm$ s.e.m. of three independent experiments. The data are representative of three independent experiments.

RESULTS

CCL5 increases IL-10 expression in the VSMCs of SHRs

TGF- β , as well as IL-10, mediates the anti-inflammatory effects on vascular cells.^{11,12} Therefore, we first compared basal IL-10 mRNA expression in both SHR and WKY thoracic aorta tissues and VSMCs with TGF- β mRNA expression. The basal expression of IL-10 in the SHR thoracic aorta tissues and VSMCs was reduced compared with the WKY specimens. However, the basal expression of TGF- β was higher in the SHR thoracic aorta tissues and VSMCs compared with the WKY specimens (Figure 1a). We next examined the direct effects of CCL5 on IL-10 and TGF- β mRNA expression in the VSMCs of the SHRs. CCL5 increased IL-10 mRNA expression but exerted no statistically significant effects on TGF- β mRNA expression (Figure 1b). We also examined the effects of CCL5 on the Ang II-induced inhibition of IL-10 expression in the VSMCs of the SHRs. CCL5 attenuated the inhibitory effects of Ang II on IL-10 mRNA expression. CCL5 also upregulated IL-10 protein production and attenuated the

Ang II-induced inhibition of IL-10 protein expression in the VSMCs of the SHRs (Figure 1c). We also observed a dose-dependent response by IL-10 mRNA expression in response to CCL5. Doses of CCL5 ranging from 10 to 100 ng ml^{-1} gradually increased IL-10 mRNA expression; the increases in the level of IL-10 mRNA expression in response to 100–400 ng ml^{-1} of CCL5 were similar. Additionally, we observed a dose-dependent response in Ang II-induced IL-10 mRNA inhibition in response to CCL5 treatment. Doses of CCL5 ranging from 50 to 400 ng ml^{-1} increased IL-10 mRNA expression to a level close to that of the untreated SHR VSMCs (Figure 1c). The time courses of IL-10 mRNA expression and Ang II-induced IL-10 mRNA inhibition in response to CCL5 treatment were also determined over a 16 h time period. Increases in IL-10 expression were detected at 1 h following CCL5 treatment and were sustained as long as 16 h. The attenuation of the Ang II-induced IL-10 mRNA inhibition by CCL5 was also detected at 1 h following Ang II/CCL5 treatment and was sustained for as long as 16 h. The increased IL-10 mRNA expression

Figure 2 Three weeks of treatment with CCL5 increases the expression of IL-10 in SHR thoracic aorta tissues and VSMCs, and decreases blood pressure in SHRs. (a, b) Following the final treatment with either CCL5 ($1.5 \mu\text{g kg}^{-1}$, twice a day for 3 weeks) or normal saline, the tissue lysates and total RNA were isolated from the thoracic aorta tissues and VSMCs from each rat group ($n=6$ each), and both immunoblotting and real-time PCR were performed. $*P<0.05$ vs. eSHRc. $**P<0.01$ vs. eSHRc. $***P<0.001$ vs. eSHRc. (c) Blood pressure was measured before treatment, every week during treatment and 1 day following the final injection of either CCL5 ($1.5 \mu\text{g kg}^{-1}$, twice per day for 3 weeks) or normal saline, using the CODA high-throughput tail blood pressure system. The results are presented as the means $\pm$ s.e.m. ($n=6$, each). $*P<0.05$ vs. eSHRi aged 20 weeks. eSHRc, SHR treated with normal saline; eSHRi, SHR treated with CCL5.

remained almost constant between 1 and 16 h following Ang II/CCL5 treatment in the VSMCs of the SHRs (Figure 1c).

To confirm the upregulatory effects of CCL5 on IL-10 expression in the VSMCs of the SHRs, we also performed an *in vivo* study. The SHRs with established hypertension were treated with CCL5 ($1.5 \mu\text{g kg}^{-1}$) subcutaneously twice a day for 3 weeks (eSHRi). Following 3 weeks of treatment with CCL5, we first observed an increase in the level of CCL5 in the thoracic aorta tissues and VSMCs of the eSHRi group compared with the normal saline-treated SHRs (eSHRc) (Figure 2a). Elevated IL-10 protein production was observed in the thoracic aorta tissues of the eSHRi group compared with the eSHRc group. Elevated IL-10 mRNA expression was also detected in the VSMCs of the eSHRi rats (Figure 2b). Three weeks of treatment with CCL5 decreased the systolic blood pressures of these rats, whereas the systolic blood pressures of the rats in the eSHRc group increased progressively with age until 21 weeks of age. The systolic blood pressure values (means $\pm$ s.e.m.) in the eSHRc and eSHRi groups at 19 weeks of age were 197.70 ± 3.84 and 201.10 ± 4.32 mm Hg, respectively, and were 214.00 ± 1.74 and 190.30 ± 4.09 mm Hg, respectively, at 22 weeks of age (Figure 2c).

AT₂ R and DDAH-1 activity partially mediate effects of CCL5 on IL-10 expression in the VSMCs of SHRs

We subsequently examined whether the CCL5-induced elevation of IL-10 expression was mediated by either the AT₁ R or the AT₂ R

pathway. The VSMCs of the SHRs were treated either with or without CCL5 (100 ng ml^{-1}) in either the presence or the absence of the AT₁ R antagonist losartan ($10 \mu\text{mol l}^{-1}$) or the AT₂ R antagonist PD123319 ($10 \mu\text{mol l}^{-1}$) for 2 h. CCL5-induced IL-10 mRNA expression was not inhibited by losartan and therefore not mediated via AT₁ R activation. However, PD123319 reduced CCL5-induced IL-10 expression to the level observed in the untreated SHR VSMCs (Figure 3a). To confirm these results, either AT₁ R- or AT₂ R-directed siRNA was transfected into the VSMCs of the SHRs, followed by treatment either with or without CCL5 (100 ng ml^{-1}) for 2 h. In the VSMCs of the SHRs transfected with AT₁ R siRNA, CCL5-induced IL-10 mRNA expression was slightly elevated. The expression pattern of CCL5-induced IL-10 mRNA was similar to that noted in the VSMCs of the SHRs treated with CCL5 and losartan simultaneously. By contrast, the expression of CCL5-induced IL-10 mRNA was inhibited in the VSMCs of the SHRs transfected with AT₂ R siRNA (Figure 3b). The protein production of IL-10 induced by CCL5 was also inhibited in the AT₂ R siRNA-transfected VSMCs of the SHRs (Figure 3c).

DDAH is an important regulator of plasma ADMA, a major risk factor for cardiovascular disease and a regulator of nitric oxide bioavailability.^{19,20} CCL5 induces DDAH activity and decreases Ang II-induced ADMA production in the VSMCs of SHRs.⁸ Therefore, we determined whether DDAH activity is involved in the upregulatory effects exerted by CCL5 on IL-10 expression in the VSMCs of SHRs. First, we confirmed the inhibition of CCL5-induced DDAH activity

Figure 3 AT₂ R activation partially mediates the effects of CCL5 on IL-10 expression in the VSMCs of SHRs. **(a)** The VSMCs of the SHRs were treated either with or without CCL5 (100 ng ml⁻¹) in the either the presence or the absence of losartan (AT₁ R antagonist, 10 μmol l⁻¹) or PD123319 (AT₂ R antagonist, 10 μmol l⁻¹) for 2 h, after which the total RNAs were analyzed via real-time PCR. ***P*<0.01 vs. SHR VSMCs treated with CCL5. **(b, c)** For AT₁ R or AT₂ R siRNA transfection, the VSMCs of the SHRs were plated in 6-well plates, grown to 90% confluence, and transfected with AT₁ R siRNA, AT₂ R siRNA or control siRNA oligomers (50 nmol l⁻¹). The VSMCs of the SHRs were then treated either with or without CCL5 (100 ng ml⁻¹) for 2 h. Successful transfection and IL-10 mRNA expression were verified via real-time PCR **(b)**, and IL-10 protein expression was verified via immunoblotting **(c)**. Non-TF, non-transfected SHR VSMCs. ***P*<0.01 vs. untreated SHR VSMCs. ****P*<0.001 vs. untreated SHR VSMCs. The bars represent the means±s.e.m. of three independent experiments. The data are representative of three independent experiments.

via nor-NOHA, a DDAH inhibitor in the VSMCs of SHRs (Figure 4a). nor-NOHA reduced CCL5-induced IL-10 expression in the VSMCs of the SHRs (Figure 4b). To confirm this result, real-time PCR was performed on the samples transfected with DDAH-1-specific siRNA. CCL5-induced IL-10 mRNA expression was not detected, and CCL5-induced IL-10 protein production was also inhibited in the VSMCs of the SHRs transfected with DDAH-1 siRNA (Figure 4c).

Met-RANTES inhibits CCL5-induced IL-10 expression in the VSMCs of SHRs

CCL5 interacts with chemokine receptors, CCR1, CCR2 and CCR5. Met-RANTES is a CC receptor antagonist. Met-RANTES blocks CCL5/CCL5 receptor interactions. Therefore, we next observed the effects exerted by Met-RANTES on CCL5-induced IL-10 expression and compared it with the effects exerted by Met-RANTES and another agent on the inhibition of CCL5-induced IL-expression. Met-RANTES

inhibited CCL5-induced IL-10 expression in the VSMCs of the SHRs (Figures 5a and b). The inhibition of CCL5-induced IL-10 expression by Met-RANTES was stronger than the effects exerted by either AT₂ R or the DDAH-1 inhibitor. Moreover, treatment with either AT₂ R or the DDAH-1 inhibitor together with Met-RANTES (Met-RANTES/PD123319 or Met-RANTES/nor-NOHA) increased the inhibition of CCL5-induced IL-10 expression compared with the effects exerted by either AT₂ R or the DDAH-1 inhibitor alone. The levels of the inhibition of CCL5-induced IL-10 expression by Met-RANTES/PD123319 and Met-RANTES/nor-NOHA were almost the same as that mediated by Met-RANTES alone (Figures 5a and b).

CCL5 partially mediates the inhibitory effect of IL-10 on the expression of Ang II-induced hypertensive mediators in the VSMCs of SHRs

IL-10, as well as CCL5, reduced Ang II-induced IL-10 and ET-1 mRNA expression in the VSMCs of SHRs.¹⁷ Therefore, we

Figure 4 DDAH-1 activation due to CCL5 partially mediates CCL5-induced IL-10 expression in the VSMCs of SHRs. (a, b) The VSMCs of the SHRs were treated either with or without CCL5 (100 ng ml^{-1}) in either the presence or the absence of nor-NOHA (inhibitor of DDAH-1 activity, $50 \mu\text{mol l}^{-1}$) for 2 h. DDAH activity was measured by converting ADMA to L-citrulline (a), and IL-10 expression was determined via immunoblotting (b). $*P < 0.05$ vs. SHR VSMCs treated with CCL5. $***P < 0.001$ vs. untreated SHR VSMCs. (c) The VSMCs of the SHRs were transfected with either DDAH-1 or control siRNA oligomers (50 nmol l^{-1}); the transfected VSMCs were then treated either with or without CCL5 (100 ng ml^{-1}) for 2 h. Successful transfection and IL-10 mRNA expression were verified via real-time PCR, and IL-10 protein expression was verified via immunoblotting. Non-TF, non-transfected VSMCs. $*P < 0.05$ vs. untreated SHR VSMCs. $**P < 0.01$ vs. untreated SHR VSMCs. $***P < 0.001$ vs. untreated SHR VSMCs. The bars represent the means $\pm$ s.e.m. of three independent experiments. The data are representative of three independent experiments.

investigated whether CCL5 mediates the inhibitory effects exerted by IL-10 on these mediators in the VSMCs of SHRs. Real-time PCR was performed on the samples transfected with CCL5-specific siRNA. The rate of reduction of Ang II-induced 12-LO mRNA expression by IL-10 in the CCL5 siRNA-transfected VSMCs decreased to $10.8 \pm 1.6\%$ compared with $19.8 \pm 0.9\%$ in the control siRNA-transfected VSMCs (Figure 6a), and the protein level of Ang II-induced 12-LO by IL-10 correlated with the mRNA level noted in the CCL5 siRNA-transfected VSMCs (Figure 6b). In the case of ET-1 expression, the rate of reduction of Ang II-induced ET-1 mRNA expression by IL-10 in the CCL5 siRNA-transfected VSMCs decreased to $12.8 \pm 0.8\%$ compared with $21.1 \pm 1.5\%$ in the control siRNA-transfected VSMCs (Figure 6a), and the protein level of Ang II-induced ET-1 by IL-10 also correlated with the mRNA level noted in the CCL5 siRNA-transfected VSMCs (Figure 6b).

DISCUSSION

The involvement of chemokines and their receptors in the pathogenesis of hypertension is not fully understood. However, chemokines play an important role in the pathogenesis of hypertension. Chemokines stimulate the migration of inflammatory cells to the vascular walls in the setting of hypertension, and chemokine-induced vascular inflammation, oxidative stress and smooth muscle proliferation result in elevated blood pressure.²¹ CCL5 also plays a role in both acute and chronic inflammatory responses in the setting of vascular wall remodeling in hypertension.^{21–23} The overexpression of CCL5 has been observed within the pulmonary vascular walls in the setting of idiopathic pulmonary hypertension, and the CCL5/CCR5 pathway plays an important role in pulmonary vascular remodeling in the setting of pulmonary hypertension.²³ By contrast, CCL5 treatment in the vicinity of the nucleus tractus solitaries of SHRs decreases blood

Figure 5 Met-RANTES inhibits the effects of CCL5 on IL-10 expression in the VSMCs of SHRs. **(a, b)** The VSMCs of the SHRs were either untreated or pretreated with Met-RANTES (CCL5 antagonist, 20 nmol l⁻¹) for 30 min, before being treated either with or without CCL5 (100 ng ml⁻¹) in either the presence or the absence of PD123319 (AT₂ R antagonist, 10 μmol l⁻¹) or nor-NOHA (inhibitor of DDAH-1 activity, 50 μmol l⁻¹) for 2 h, after which the tissue lysates were isolated from the VSMCs of the SHRs, and immunoblotting was performed. ^a*P*<0.05 vs. SHR VSMCs treated with CCL5. ^b*P*<0.01 vs. SHR VSMCs treated with CCL5 and Met-RANTES. ^c*P*<0.01 vs. SHR VSMCs treated with CCL5. **P*<0.05 vs. SHR VSMCs treated with CCL5 and nor-NOHA. ***P*<0.01 vs. SHR VSMCs treated with CCL5 and PD123319. The bars represent the means±s.e.m. of three independent experiments. The data are representative of three independent experiments.

Figure 6 CCL5 partially mediates the inhibitory effects of IL-10 on Ang II-induced 12-LO and ET-1 expression in the VSMCs of SHRs. **(a, b)** For CCL5 siRNA transfection, the VSMCs of the SHRs were plated on 6-well plates, grown to 90% confluence, and transfected with either CCL5 siRNA or control siRNA oligomers (50 nmol l⁻¹), after which the VSMCs were treated either with or without Ang II (0.1 μmol l⁻¹) or IL-10 (25 ng ml⁻¹) for 2 h. Successful transfection, as well as 12-LO and ET-1 mRNA expression, were verified via real-time PCR **(a)**, and 12-LO and ET-1 protein expression were verified via immunoblotting **(b)**. Non-TF, non-transfected SHR VSMCs. **P*<0.05 vs. SHR VSMCs treated with Ang II. ***P*<0.01 vs. SHR VSMCs treated with Ang II. ****P*<0.01 vs. SHR VSMCs treated with Ang II. The bars represent the means±s.e.m. of three independent experiments. The data are representative of three independent experiments.

pressure.²⁴ Additionally, we observed decreased CCL5 expression in the VSMCs of the SHR than in the VSMCs of the normotensive WKY rats.⁸ Therefore, we have studied the downregulatory roles of CCL5 in vascular hypertension for the past 2 years.^{8,9,25}

Although both IL-10 and TGF- β exert potent anti-inflammatory effects on vascular cells, IL-10 directly inhibits proinflammatory cytokine production in macrophages, whereas TGF- β primarily down-regulates both cytokine-induced adhesion molecule expression and neutrophil migration.^{11,12} In this study, the basal expression of IL-10 in SHR thoracic aorta tissues and VSMCs was inhibited compared with the WKY rats. By contrast, the basal expression of TGF- β increased in the SHR thoracic aorta tissues and VSMCs. Moreover, CCL5 had no statistically significant effect on TGF- β expression in the VSMCs of the SHR. In a previous study, TGF- β had no effect on either CCL5 mRNA expression or Ang II-induced CCL5 inhibition.¹⁷ These results suggest that IL-10 is likely to have functions different from those of TGF- β in the setting of hypertension.

Although CCL5 exerted no significant effects on IL-10 expression in human umbilical vein endothelial cells (data not shown), CCL5 increased IL-10 expression in the VSMCs of the SHR, and also attenuated Ang II-induced IL-10 inhibition in the VSMCs of the SHR. By contrast, CCL5 had no effect on IL-10 expression, whereas Ang II increased IL-10 expression in the WKY VSMCs (data not shown). Furthermore, the renin-Ang system blockade by Ang-converting enzyme inhibitors increased IL-10 expression in the livers of bile duct-ligated rats.²⁶ In this study, Ang II directly inhibited IL-10 expression in the VSMCs of the SHR. The contrasting actions of CCL5 and Ang II according to cell type are indicators of pleiotropism.

The Ang II subtype receptor AT₁ R mediates the primary stimulatory actions of Ang II, including vasoconstriction, cell proliferation and sodium retention.²⁷ By contrast, AT₂ R antagonizes the vascular actions of AT₁ R.^{28,29} The density of the AT₂ R is lower than that of the AT₁ R in VSMCs.³⁰ Ang II increased AT₁ R expression in the VSMCs of the SHR but only slightly affected AT₂ R expression in the VSMCs of the SHR.⁶ In our previous study, IL-10 itself did not affect either AT₁ R or AT₂ R expression in the VSMCs of the SHR. However, IL-10 inhibited Ang II-induced AT₁ R expression and increased Ang II-induced AT₂ R expression. IL-10 also increased CCL5 expression via the AT₂ R pathway but not the AT₁ R pathway.¹⁷ CCL5 also inhibited Ang II-induced AT₁ R expression and increased Ang II-induced AT₂ R expression. By contrast, CCL5 directly increased AT₂ R expression.⁸ The inhibitory effects of CCL5 on Ang II-induced 12-LO expression, as well as DDAH-1 inhibition, were mediated via AT₂ R activation in the VSMCs of SHR.^{8,9} In this study, the AT₂ R pathway partially mediated CCL5-induced IL-10 expression. Although the functional roles played by AT₂ R remain controversial, the AT₂ R pathway is thought to mediate the upregulatory effects of CCL5 and IL-10 on hypertensive mediators in Ang II-treated SHR VSMCs.

DDAH is an important regulator of plasma ADMA. The reduction of DDAH activity promotes the onset of cardiovascular diseases, including hypertension, accompanied by Ang II activity.^{19,20} DDAH exists as two isoforms, DDAH-1 and DDAH-2.¹⁹ DDAH-1 is widely expressed in the aorta, forebrain, pancreas, liver and kidney at sites of nitric oxide synthase expression.³¹ DDAH-2 is expressed predominantly in blood vessels and the endothelium at sites of endothelial nitric oxide synthase expression.³¹ Plasma ADMA levels are regulated by DDAH-1, whereas DDAH-2 preserves endothelial function in the setting of blood vessel resistance.¹⁹ The expression of DDAH-1 in the SHR thoracic aorta tissues and VSMCs was elevated compared with the WKY thoracic aorta tissues and the VSMCs.⁹ In contrast to

DDAH-1, the expression of DDAH-2 was not significantly different between the SHR and WKY thoracic aorta tissues and VSMCs.⁷ Therefore, DDAH-1 rather than DDAH-2 exerted effects in the VSMCs of the SHR. CCL5-induced DDAH activity mediated the inhibition of Ang II-induced 12-LO and ET-1 expression via CCL5 in the VSMCs of the SHR.⁹ Moreover, CCL5 induced the activation of AMPK via DDAH-1 activity in the VSMCs of the SHR.²⁵ Therefore, we hypothesized that the DDAH-1 activity induced by CCL5 may be responsible for the upregulatory effects exerted by CCL5 on IL-10 expression in the VSMCs of the SHR. In the SHR VSMCs transfected with DDAH-1 siRNA, CCL5-induced IL-10 expression was not detected. This result indicates that CCL5-induced IL-10 expression is mediated by DDAH-1 activity in the VSMCs of SHR.

To understand the effects exerted by chemokines on cells, the interaction between chemokines and the chemokine receptors on the cell wall must be considered first. In this study, Met-RANTES inhibited CCL5-induced IL-10 expression, an inhibition that was more effective than the inhibition mediated by either AT₂ R or the DDAH-1 inhibitor alone. Additionally, the AT₂ R and the DDAH-1 inhibitor and Met-RANTES enhanced the inhibition of CCL5-induced IL-10 expression. These results indicate that the interaction between CCL5 and the CCL5 receptor must occur prior to the effects exerted by CCL5-induced IL-10 expression. Apart from what has been discussed previously, it is also true that AT₂ R and DDAH-1 may exert partial effects on CCL5-induced IL-10 expression in the VSMCs of SHR.

Both 12-LO and ET-1 have been linked to the development of hypertension.^{32–34} Ang II is a potent inducer of 12-LO activity, which is elevated in the setting of SHR. ET-1 is a potent vasoconstrictor secreted by the endothelium and participates in the regulation of vascular tone.³³ In addition to CCL5, IL-10 also inhibits Ang II-induced 12-LO and ET-1 mRNA expression in the VSMCs of SHR.¹⁷ Additionally, IL-10 increases CCL5 expression, and IL-10 and CCL5 synergistically inhibit Ang II-induced 12-LO and ET-1 expression in SHR VSMCs.¹⁷ Therefore, we determined whether IL-10-induced CCL5 activity mediates the inhibitory effects exerted by IL-10 on Ang II-induced 12-LO and ET-1 expression in the VSMCs of SHR. CCL5 partially mediated the inhibitory effects exerted by IL-10 on Ang II-induced 12-LO and ET-1 expression. These results suggest that CCL5 and IL-10 most likely play synergistic antihypertensive roles via interactions with the Ang II-induced hypertensive vasculature.

In conclusion, CCL5 increases IL-10 expression, and the CCL5-induced elevation of IL-10 expression is mediated primarily via DDAH-1 activation via the AT₂ R pathway in the VSMCs of SHR. Moreover, CCL5 partially mediates the inhibitory effects exerted by IL-10 on Ang II-induced 12-LO and ET-1 expression in the VSMCs of SHR. To the best of our knowledge, no direct evidence linking CCL5 to IL-10 activity in the setting of Ang II-induced vascular hypertension had previously been uncovered. Therefore, this study is the first to provide evidence that CCL5 exerts upregulatory effects on IL-10 activity in the VSMCs of SHR. Although additional *in vivo* studies should be performed, it is highly probable that CCL5 plays a cooperative role in the antihypertensive activity of IL-10 in SHR.

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