Original Article

Angiotensin II Increases Expression of IP-10 and the Renin-Angiotensin System in Endothelial Cells

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Angiotensin II promotes vascular inflammation, which plays important roles in vascular injury. In this study, we found that angiotensin II–stimulated human endothelial cells increased the release of a CXC chemokine, IP-10, according to an antibody array. IP-10 expression was higher in the endothelium of coronary blood vessels in mice infused with angiotensin II than in control. Quantitative real-time PCR analysis revealed that angiotensin II significantly increased IP-10 mRNA expression compared to control. Pretreatment with valsartan, but not with PD123319, blocked angiotensin II–induced IP-10 mRNA expression. IP-10 levels in conditioned media detected by ELISA increased in response to angiotensin II compared to control, which was blocked by the pretreatment with valsartan. These data indicate that angiotensin II stimulates IP-10 production from endothelial cells *via* angiotensin II type 1 receptors. In endothelial cells, IP-10 significantly increased mRNA expression of renin, angiotensin-converting enzyme, and angiotensinogen. IP-10 also increased angiotensin II levels in conditioned media compared to control. Angiotensin II significantly increased mRNA expression of renin, angiotensin converting enzyme and angiotensinogen, which was blocked by neutralization of IP-10 with antibody in endothelial cells. IP-10 neutralization with antibody blocked angiotensin II–induced apoptosis and cell senescence in endothelial cells. These data indicate that IP-10 is involved not only in leukocyte-endothelial interaction but also in the circuit of endothelial renin-angiotensin system activation that potentially promotes atherosclerosis. (*Hypertens Res* 2008; 31: 1257–1267)

***Key Words*: angiotensin II, angiotensin II type 1 receptor, chemokine, IP-10, renin-angiotensin system**

Introduction

Angiotensin II is a multifunctional peptide that is the principal effector of the systemic and local renin-angiotensin system (*1–5*). The effects of angiotensin II are mediated by two types of plasma membrane receptors: type 1 (AT_1) and type 2 (AT_2). A number of studies have shown the involvement of angiotensin II in the development of atherosclerosis and vascular remodeling, as well as in the regulation of blood pressure and neurohumoral homeostasis (*1–5*).

It has been shown that leukocyte chemotaxis plays important roles in the progression of atherosclerosis (*6–9*). Chemokines produced by vascular cells, including endothelial and vascular smooth muscle cells, stimulate leukocytes to migrate into the vessel wall by activating signaling pathways through membrane receptors (*6–10*). In monocytes, macrophages, vascular smooth muscle cells, and endothelial cells, angiotensin II activates nuclear factor κB (NF-κB), which induces the production of chemokines such as monocyte chemoattractant protein-1 (MCP-1), interleukin 6, and interleukin 8 (*1*, *11–17*).

In this study, we used an antibody array to identify chemokines of which release by endothelial cells changes in response to angiotensin II. We found that levels of a CXC chemokine, IP-10, in conditioned media is increased by

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angiotensin II in human umbilical vein endothelial cells (HUVECs) and a human endothelial cell line, EA hy.926 cells (*18*). We also demonstrate that IP-10 expression was increased in the endothelium of coronary blood vessels in mice *in vivo* that were infused with angiotensin II. It has been shown that IP-10 expression is induced by interferons in leukocytes as well as in vascular cells such as endothelial and vascular smooth muscle cells (*19*, *20*). IP-10 is also expressed in human atherosclerotic lesions (*19*).

IP-10 exerts biological actions on leukocytes through a membrane receptor, CXCR3, that is reportedly expressed in T lymphocytes, natural killer cells, and endothelial cells (*6*, *19*, *21–24*). In the present study, we investigated the effects of IP-10 independent of leukocyte-endothelial interactions on endothelial cells. We demonstrate that IP-10 increases the expression of the components of the renin-angiotensin system, such as renin, angiotensin-converting enzyme (ACE), and angiotensinogen, suggesting that IP-10 in endothelial cells upregulates the local renin-angiotensin system, which may play a role in the promotion of atherosclerosis and vascular remodeling.

Methods

Materials

Human recombinant IP-10 was purchased from PeproTech (Rocky Hill, USA). Angiotensin II and PD123319 were purchased from Sigma Chemical (St. Louis, USA). CGP42112 was from Peptide Institute (Osaka, Japan). Valsartan was supplied by Novartis Pharmaceutical (Basel, Switzerland). Mouse IgG 1 and monoclonal anti-human CXCL10/IP-10 antibody were purchased from R&D Systems (Minneapolis, USA). Anti-SAPA/JNK polyclonal antibody, anti-phospho-SAPA/JNK, and HRP-conjugated anti-rabbit IgG were purchased from Cell Signaling Technology (Danvers, USA).

Cell Culture

HUVECs were cultured in RPMI medium (Sigma Chemical) containing 20% FBS, 25 U/mL heparin, 20 mg/mL endothelial cell growth supplement, 100 U/mL penicillin, and 100 μg/mL streptomycin. EA hy.926 cells, a gift from Dr. Cora-Jean S. Edgell (University of North Carolina at Chapel Hill, Chapel Hill, USA), were maintained at 37°C under an atmosphere of 5% CO_2 in DMEM containing 5% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin (*18*).

Antibody Array

HUVECs cultured in normal media and EA hy.926 cells cultured in serum-starved media were stimulated with control vehicle and angiotensin II (1 μmol/L) for 24 h, and then the conditioned media were collected. The conditioned media were analyzed using RayBio Human Inflammation Antibody Array III and 3.1 according to the manufacturer's instructions (RayBiotech, Norcross, USA). Briefly, inflammation array membranes were blocked in 2 mL of a blocking buffer for 30 min and then incubated with 1 mL of conditioned medium at room temperature for 2 h. The membranes were then rinsed three times with 2 mL of wash buffer I, followed by two washes with 2 mL of wash buffer II at room temperature. Membranes were then incubated in 1:200-diluted biotin-conjugated anti-immunoglobulin antibody at room temperature for 2 h and washed as described above before incubation in 1:1,000-diluted horseradish peroxidase-conjugated streptavidin for 2 h. Membranes were rinsed, incubated with a peroxidase substrate, and exposed to X-ray films.

Animals

Thirty-two-week-old male mice (C57BL/6J strain, CLEA Japan, Tokyo, Japan) with an average weight of 29–35 g were used in the study. The mice were implanted subcutaneously with an osmotic mini-pump (Alzet 2004; Charles River Laboratories, Wilmington, USA) containing angiotensin II (Sigma Chemical) dissolved in saline, which was delivered at the rate of 1.44 mg/kg/d for 4 weeks. After the mice were anesthetized with pentobarbital sodium (80 mg/kg i.p.; Abbot Laboratories, Abbott Park, USA), the heart was perfused with saline containing 10 U/mL heparin. The heart sample was then dissected and fixed in 4% paraformaldehyde overnight. All experiments were approved by the institutional animal research committee of Saga University and conformed to the animal care guidelines of the American Physiological Society.

Immunohistochemistry

IP-10 expression was determined immunohistochemically in paraffin-embedded heart specimens fixed in 4% paraformaldehyde. A Histofine Simple Stain PO (M) kit (Nichirei, Tokyo, Japan) was used according to the manufacturer's instructions. After the endogenous peroxidase was quenched with 3% hydrogen peroxidase in absolute methanol, samples were incubated with a blocking solution and then with monoclonal anti-IP10 antibody (Santa Cruz Biotechnology, Santa Cruz, USA) for 60 min at room temperature. After rinsing in PBS, the samples were incubated with Histofine Simple Stain PO (M) for 30 min. After rinsing, the samples were incubated with chromogen/substrate reagent. Antibody binding was then visualized with 3-amino-9-ethyl carbazole (Nichirei). Samples were observed microscopically (Axio Imager; Carl Zeiss, Oberkochen, Germany).

Gel Electrophoresis and Immunoblotting

The protein contents of the samples were determined using a Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, USA). The samples (40 μg protein) were resolved by one-

dimensional SDS-PAGE and then electrophoretically transferred to polyvinylidene difluoride membranes (ATTO, Tokyo, Japan). The membranes were subjected to immunoblotting as described previously (*25*).

Real-Time PCR

Total RNA was isolated from cells with Isogen (Nippon Gene, Tokyo, Japan) following the manufacturer's protocol. cDNA synthesis was performed using 2 μg total RNA and the RETROscript Kit (Ambion, Austin, USA). Primers were designed using Primer Express software (Applied Biosystems, Foster City, USA), and real-time PCR was conducted using the SYBR Green PCR Master Mix (Applied Biosystems). Real-time PCR was performed with an ABI 7300 Sequence Detection System (Applied Biosystems) using a standard temperature profile (2 min at 50°C, 10 min at 95°C, and 40–45 cycles of 15 s at 95°C, 1 min at 60°C). Cycle threshold values were determined by automated threshold analysis with ABI Prism version 1.3.1 software (Applied Biosystems). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as an internal control. The specific primer sets for the target gene were as follows: IP-10, (forward) 5′-GTCCACGTGTTGAGATCATTGC-3′ and (reverse) 5′-TCTTGATGGCCTTCGATTCTG-3′ (GenBank Accession No. NM001565); GAPDH (forward), 5′-GGTGGTCTC CTCTGACTTCAAC-3′ and (reverse) 5′-TCTCTCTTC CTCTTGTGTTCTTG-3′ (GenBank Accession No. NM002046); renin, (forward) 5′-CAGGGACAGTCAGTG GCTTTC-3′ and (reverse) 5′-ACATCTGTGTCACCG TGATTCC-3′ (GenBank Accession No. NM000537); ACE, (forward) 5′-ATGAAGACCTGTTATGGGCATGG-3′ and (reverse) 5′-ATTTCGGGTAAAACTGGAGGATGG-3′ (GenBank Accession No. NM152831); angiotensinogen, (forward) 5′-GACAACTTCTCGGTGACTCAAGTG-3′ and (reverse) 5′-GGCATAGTGAGGCTGGATCAG-3′ (GenBank Accession No. NM000029). These primers were synthesized by JBioS (Saitama, Japan). The mRNA expression levels were normalized by GAPDH expression and presented relative to control levels.

Enzyme-Linked Immunosorbent Assay (ELISA) for IP-10

IP-10 was measured using a commercially available kit (R&D Systems) according to the manufacturer's protocol. Each sample was assayed in duplicate along with a standard provided in the kit to generate a standard curve used to determine the unknown amount of targets.

Enzyme Immunoassay (EIA) for Angiotensin II

Angiotensin II levels were determined in conditioned media using commercial ELISA kits (Peninsula Laboratories, Belmont, USA) following the manufacturer's protocol.

Measurement of Endothelial Apoptosis

For the quantitive determination of apoptosis, we measured DNA fragmentation by measuring histone-associated DNA fragments using a photometric enzyme immunoassay (Cell Death Detection ELISA; Roche, Mannheim, Germany) according to the manufacturer's instructions.

Senescence-Associated β-Galactosidase Activity

Cells were fixed and stained for senesence-associated β-galactosidase (SA-β-gal) activity as described (*26*). In brief, the cells were fixed for 10 min in 2% formaldehyde and 0.2% glutaraldehyde in PBS, and incubated for 12 h at 37°C with fresh β-gal staining solution containing 1 mg/mL, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, 5 mmol/L potassium ferrocyanide, 5 mmol/L potassium ferricyanide, and 2 mmol/L $MgCl_2$, pH 6.0. Senescent cells were identified by blue staining under a phase-contrast microscope (Axiovert 40CFL, Carl Zeiss), and the number SA-β-gal–positive cells among 100 cells were counted in each of 20 random fields from six independent experiments.

Statistical Analysis

Data were expressed as the mean±SEM based on five independent experiments. Statistical analysis was performed by Student's *t*-test and the Tukey-Kramer post hoc test. Values of $p<0.05$ were considered statistically significant.

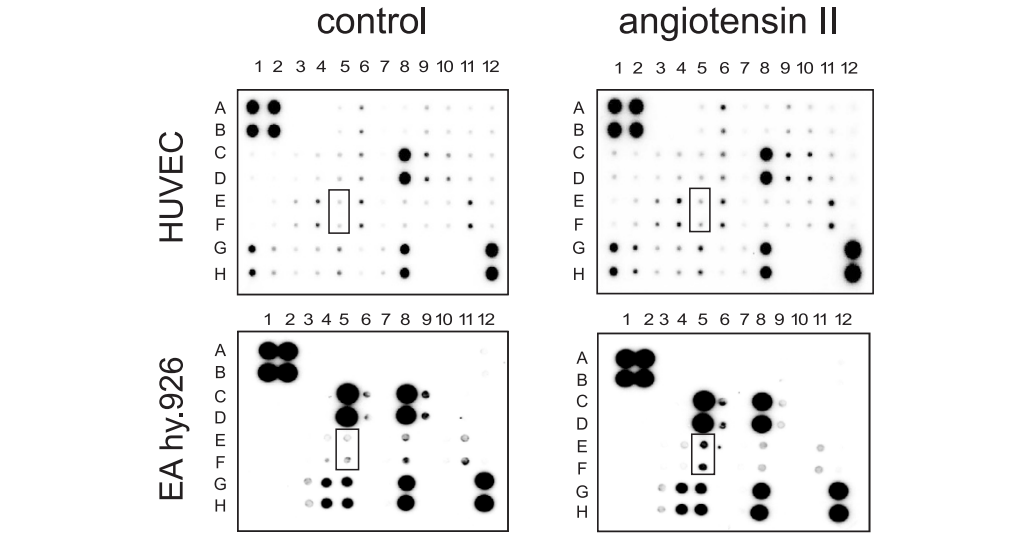
Results

Angiotensin II–Induced IP-10 Expression *via* AT_1 Receptors in Endothelial Cells

To study the effects of angiotensin II on the release of chemokines from endothelial cells, we used an antibody array to analyze conditioned media of HUVECs and a human endothelial cell line, EA hy.926 cells, stimulated with control vehicle and angiotensin II for 24 h. As shown in Fig. 1A, we identified an increased release of a CXC chemokine IP-10 from angiotensin II–stimulated HUVECs and EA hy.926 cells compared to unstimulated control cells. To study the effects of angiotensin II on IP-10 expression in endothelial cells *in vivo*, immunohistochemical analysis was performed in heart tissue from mice infused with control vehicle and angiotensin II. As shown in Fig. 1B, IP-10 expression in the endothelium of coronary blood vessels was higher in mice infused with angiotensin II than in those infused with control vehicle. These data suggest that angiotensin II increases IP-10 protein expression in endothelial cells.

In EA hy.926 cells, we examined the effects of angiotensin II on IP-10 mRNA expression. Total RNA extracted from cells stimulated with control vehicle and angiotensin II after

A



	1	2	3	4	5	6	7	8	9	10	11	12
A	POS	POS	NEG	NEG	Eotaxin	Eotaxin-2	GCSF	GM-CSF	ICAM-1	IFN-γ	I-309	IL-1α
B	POS	POS	NEG	NEG	Eotaxin	Eotaxin-2	GCSF	GM-CSF	ICAM-1	IFN-γ	I-309	IL-1α
C	IL-1β	IL-2	IL-3	IL-4	IL-6	IL-6sR	IL-7	IL-8	IL-10	IL-11	IL-12 p40	IL-12 p70
D	IL-1β	IL-2	IL-3	IL-4	IL-6	IL-6sR	IL-7	IL-8	IL-10	IL-11	IL-12 p40	IL-12 p70
E	IL-13	IL-15	IL-16	IL-17	IP-10	MCP-1	MCP-2	M-CSF	MIG	MIP-1α	MIP-1β	MIP-1δ
F	IL-13	IL-15	IL-16	IL-17	IP-10	MCP-1	MCP-2	M-CSF	MIG	MIP-1α	MIP-1β	MIP-1δ
G	RANTES	TGF-β1	TNF-α	TNF-β	sTNF RI	sTNF RII	PDGF-BB	TIMP-2	BLANK	BLANK	NEG	POS
H	RANTES	TGF-β1	TNF-α	TNF-β	sTNF RI	sTNF RII	PDGF-BB	TIMP-2	BLANK	BLANK	NEG	POS

B

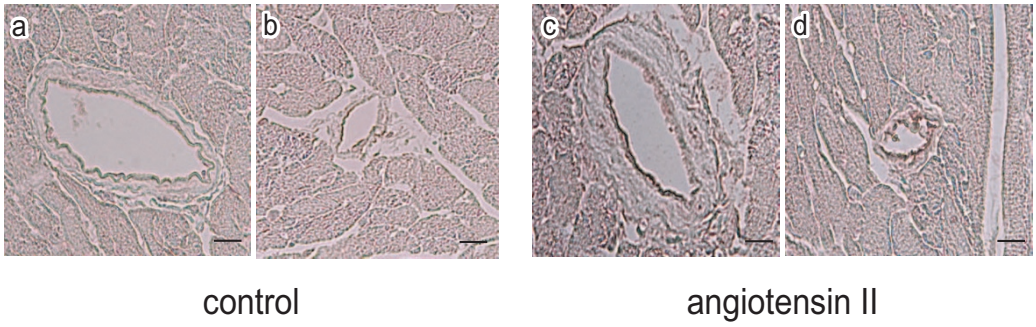


Fig. 1. *A: An antibody array to detect chemokines in conditioned media from HUVECs and EA hy.926 cells. Conditioned media prepared from unstimulated and angiotensin II–treated HUVECs and EA hy.926 cells were incubated with an antibody array membrane. Protein-antibody complexes were detected with horseradish peroxidase–conjugated anti-immunoglobulin, as described in Methods. Each inflammation-related factor is depicted by duplicate spots in its location. An antibody spotting map on the antibody array membrane is shown in the lower panel. POS, positive control; NEG, negative control; GCSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte macrophage colony-stimulating factor; ICAM-1, intercellular adhesion molecule-1; IFN-γ, interferon-γ; IL, interleukin; IL-6sR, interleukin-6–soluble receptor; IP-10, interferon-γ–inducible protein-10; MCP, monocyte chemoattractant protein; M-CSF, macrophage colony-stimulating factor; MIG, monokine-induced by γ-interferon; MIP, macrophage inflammatory protein; RANTES, regulated upon activation, normal T-cell expressed and secreted; TGF, transforming growth factor; TNF, tumor necrosis factor; sTNFR, soluble tumor necrosis factor receptor; PDGF-BB, platelet-derived growth factor-BB; TIMP-2, tissue inhibitor of metalloproteinases-2. B: Angiotensin II–induced expression of IP-10 in coronary blood vessels of mice. Cross sections of heart tissue from mice infused with saline as a control vehicle (a and b) or angiotensin II (c and d) were immunohistochemically stained with an anti–IP-10 antibody, as described in Methods. Representative images are shown. Bars: 10 μm.*

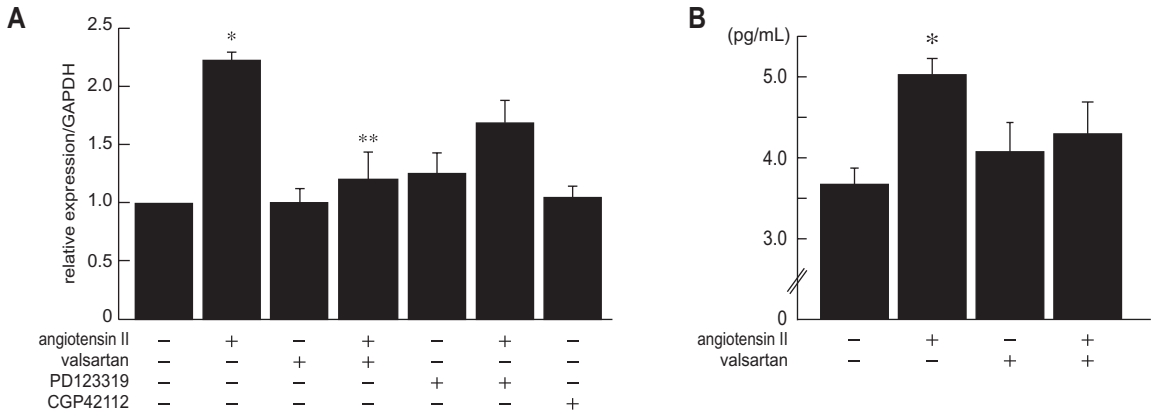


Fig. 2. *A: Angiotensin II–induced IP-10 mRNA expression via AT_1 receptors. EA hy.926 cells pretreated with control vehicle, valsartan (1 μmol/L, 30 min), or PD123319 (1 μmol/L, 30 min) were stimulated with control vehicle or angiotensin II (100 nmol/L, 8 h). EA hy.926 cells were also treated with CGP42112 (100 nmol/L, 8 h). Total RNA was extracted from the cells and IP-10 mRNA expression was examined by quantitative real-time PCR. Expression levels were based on the amount of the target RNA relative to the endogenous control gene GAPDH in order to normalize the amount and quality of total RNA. Data shown are means±SEM of three samples from each of five independent experiments, and are expressed as fold increases compared with the value for control cells. * and **$p < 0.05$ vs. untreated control and angiotensin II–stimulated cells, respectively. B: IP-10 levels measured by ELISA in conditioned media from control and angiotensin II–stimulated cells. Conditioned media prepared from cells treated with control vehicle or angiotensin II (1 μmol/L, 24 h) in the presense or absence of valsartan (1 μmol/L) were subjected to ELISA assay for IP-10. Data shown are means±SEM of three samples from each of five independent experiments. *$p < 0.05$ vs. untreated control cells.*

starvation for 24 h was subjected to analysis by quantitative real-time PCR. As shown in Fig. 2A, angiotensin II (100 nmol/L) significantly increased IP-10 mRNA expression, up to 2.2-fold that of control. Angiotensin II–induced IP-10 mRNA expression was inhibited by pretreatment with an AT_1 receptor antagonist, valsartan, but not with an AT_2 receptor antagonist, PD123319. An AT_2 receptor agonist, CGP42112, had no significant effect on IP-10 mRNA expression. To determine the release of IP-10 protein from endothelial cells, we measured IP-10 levels in conditioned media from EA hy.926 cells stimulated with control vehicle and angiotensin II for 24 h by ELISA. IP-10 detectable in conditioned media showed an increase in response to angiotensin II to 1.3-fold compared to control, which was partially blocked by the pretreatment with valsartan (Fig. 2B). These data suggest that angiotensin II stimulates IP-10 production *via* AT_1 receptors in endothelial cells.

IP-10 Upregulates the Local Renin-Angiotensin System in Endothelial Cells

We examined the effects of IP-10 on the expression of the components of the local renin-angiotensin system in endothelial cells. Several papers have shown that IP-10, in the range of 0.1 ng/mL to 100 ng/mL, activates cell signaling and modulates cell function in various types of cells, including T lymphocytes and both epithelial and non-epithelial cells (*27–29*). Therefore, we performed the following experiments using 10 ng/mL of IP-10. Total RNA extracted from EA hy.926 cells stimulated with control vehicle and human recombinant IP-10 after starvation for 24 h was subjected to analysis by quantitative real-time PCR. As shown in Fig. 3A, treatment with recombinant human IP-10 significantly increased mRNA expression of renin, ACE, and angiotensinogen, up to 1.4-, 1.2- and 1.9-fold that of control, respectively, in EA hy.926 cells. Although we studied the effects of 0.1, 1, and 10 ng/mL IP-10 on the expression of renin, ACE, and angiotensinogen, no dose-dependency was found in IP-10–induced expression (data not shown). These results indicate that IP-10 upregulates the mRNA expression of renin-angiotensin system components in endothelial cells.

To examine the effects of IP-10 on angiotensin II release from endothelial cells, we measured angiotensin II levels in conditioned media from EA hy.926 cells stimulated with control vehicle or human recombinant IP-10 for 24 h by EIA. The angiotensin II concentration in conditioned media was 1.1-fold of control in the presence of IP-10 (Fig. 3B). It was previously shown that angiotensin II activates the signaling pathways mediated by the phosphorylation of mitogen-activated protein kinases such as c-Jun N-terminal kinase (JNK), p38, and extracellular signal–regulated kinase (*30*, *31*). Here we examined whether conditioned media from EA hy.926 cells treated with control vehicle or with recombinant human IP-10 stimulates JNK phosphorylation in EA hy.926 cells. As shown in Fig. 3C, the conditioned media from cells treated with IP-10 increased JNK phosphorylation compared to con-

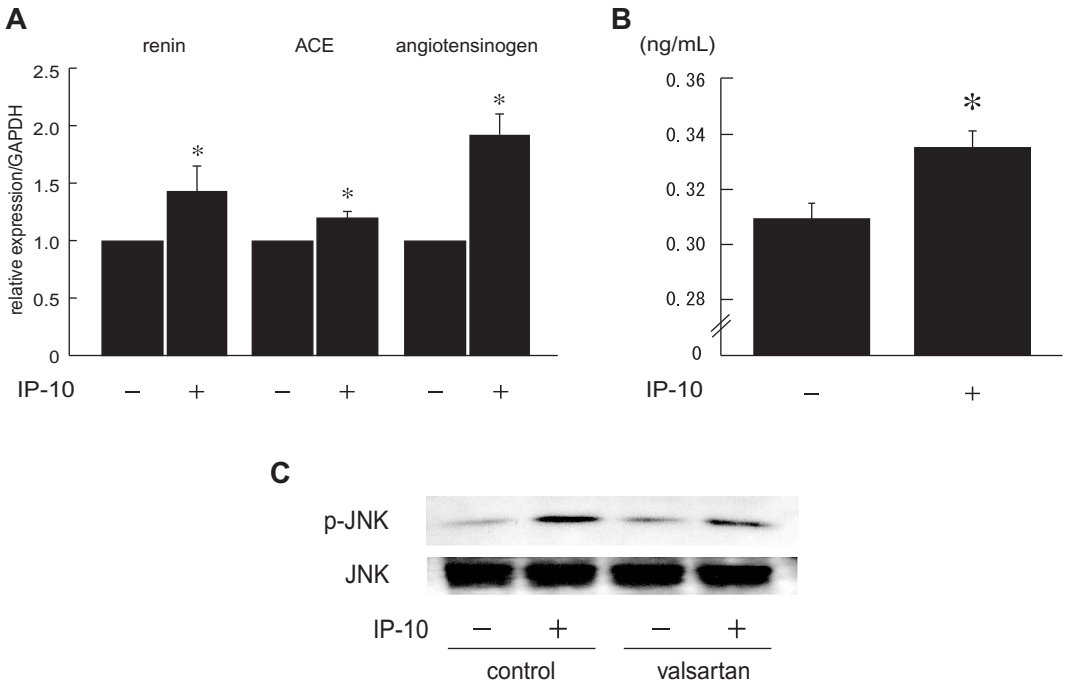


Fig. 3. *A: IP-10 increases mRNA expression of renin, ACE, and angiotensinogen in endothelial cells. EA hy.926 cells were stimulated with control vehicle or human recombinant IP-10 (10 ng/mL, 16 h). Total RNA was extracted from the cells, and the mRNA expression of renin, ACE, and angiotensinogen was examined by quantitative real-time PCR. Expression levels were based on the amount of the target RNA relative to the endogenous control gene GAPDH in order to normalize the amount and quality of total RNA. Data shown are means±SEM of three samples from each of five independent experiments, and are expressed as fold increases compared with the value for control cells. *$p < 0.05$ vs. untreated control cells. B: Angiotensin II levels in conditioned media from control and IP-10–stimulated cells. Angiotensin II levels in conditioned media that were prepared from EA hy.926 cells treated with vehicle or human recombinant IP-10 (50 ng/mL) for 24 h were determined by EIA. Data shown are means±SEM of three samples from each of five independent experiments. *$p < 0.05$ vs. untreated control cells. C: Conditioned media from IP-10–treated cells evoke the AT_1 receptor–mediated phosphorylation of JNK. Whole cell lysates (40 μg protein) from EA hy.926 cells, which were stimulated for 30 min with the conditioned media from EA hy.926 cells treated with vehicle or human recombinant IP-10 (50 ng/mL) for 24 h after pretreatment with control vehicle or valsartan (1 μmol/L) for 30 min, were subjected to immunoblotting with antibodies against JNK and phosphorylated JNK (p-JNK). Representative immunoblot images are shown.*

ditioned media from cells treated with the control vehicle. Valsartan pretreatment inhibited this increase. Treatment with conditioned media did not change total JNK levels in whole cell lysates. These results suggest that IP-10 promotes angiotensin II release, which is functionally relevant to the activation of AT_1 receptor–mediated cell signaling in endothelial cells.

We studied the effects of angiotensin II on mRNA expression of renin, ACE, and angiotensinogen in EA hy.926 cells. Total RNA extracted from cells stimulated with control vehicle or angiotensin II after starvation was subjected to analysis by quantitative real-time PCR. As shown in Fig. 4A, mRNA expression of renin, ACE, and angiotensinogen significantly increased, to 1.3-, 1.4-, and 2.1-fold that of control, respectively. Pretreatment with valsartan significantly inhibited angiotensin II–induced mRNA expression of renin and angiotensinogen and tended to decrease angiotensin II–induced expression of ACE. These results indicate that angiotensin II increases the expression of the components of the renin-angiotensin system, such as renin, ACE, and angiotensinogen, *via* AT_1 receptors in endothelial cells. Next, we examined the involvement of angiotensin II–induced IP-10 in the expression of renin, ACE, and angiotensinogen. In the presence of a neutralizing antibody against IP-10 in culture media, angiotensin II–induced mRNA expression of ACE and angiotensinogen was significantly inhibited and angiotensin II–induced mRNA expression of renin tended to decrease (Fig. 4B). Mouse IgG 1, an isotype-matched control of the neutralizing antibody, showed no significant effect on angiotensin II–induced mRNA expression of renin, ACE, and angiotensinogen. These data suggest that angiotensin II–induced IP-10 plays a critical role in the mRNA expression of the compo-

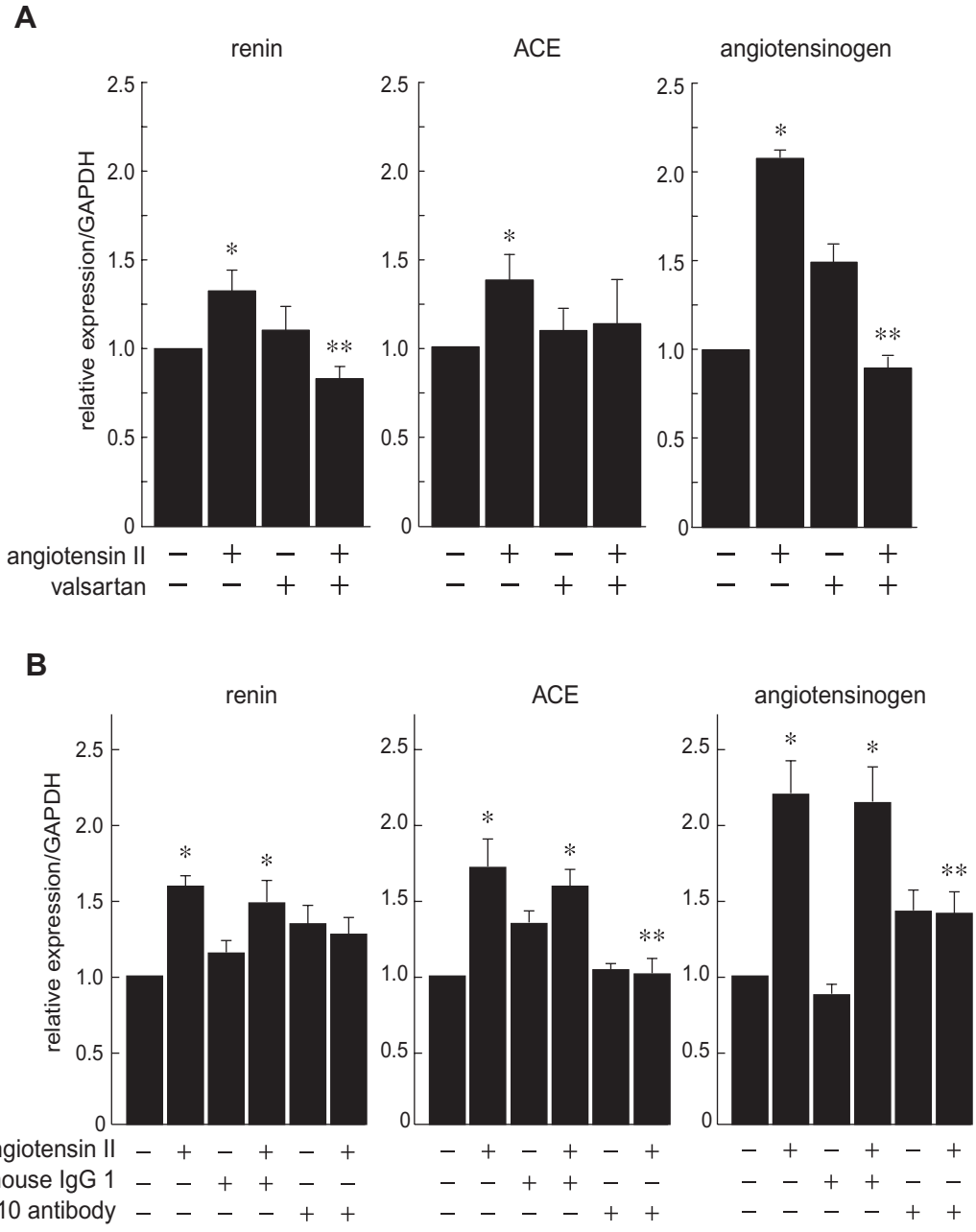


Fig. 4. *A: Angiotensin II increases mRNA expression of renin, ACE, and angiotensinogen via AT_1 receptors in endothelial cells. EA hy.926 cells pretreated with control vehicle and valsartan (1 μmol/L, 60 min) were stimulated with control vehicle or angiotensin II (1 μmol/L, 24 h). Total RNA was extracted from the cells, and the mRNA expression of renin, ACE, and angiotensinogen was examined by quantitative real-time PCR. The levels were based on the amount of the target RNA relative to the endogenous control gene GAPDH in order to normalize the amount and quality of total RNA. Data shown are means±SEM of three samples from each of five independent experiments, and are expressed as fold increases compared with the value for control cells. * and **$p < 0.05$ vs. untreated control and angiotensin II–stimulated cells, respectively. B: IP-10 neutralization with an antibody blocks angiotensin II–induced mRNA expression of renin, ACE, and angiotensinogen in endothelial cells. EA hy.926 cells were stimulated with control vehicle or angiotensin II (1 μmol/L, 24 h) in the presence of an anti–IP-10 neutralizing antibody or an isotype-matched control mouse IgG 1 (1 μg/mL). Total RNA was extracted from the cells, and the mRNA expression of renin, ACE, and angiotensinogen was examined by quantitative real-time PCR. Expression levels were based on the amount of the target RNA relative to the endogenous control gene GAPDH in order to normalize the amount and quality of total RNA. Data shown are means±SEM of three samples from each of five independent experiments, and are expressed as fold increases compared with the value for control cells. * and **$p < 0.05$ vs. untreated control and angiotensin II–stimulated cells, respectively.*

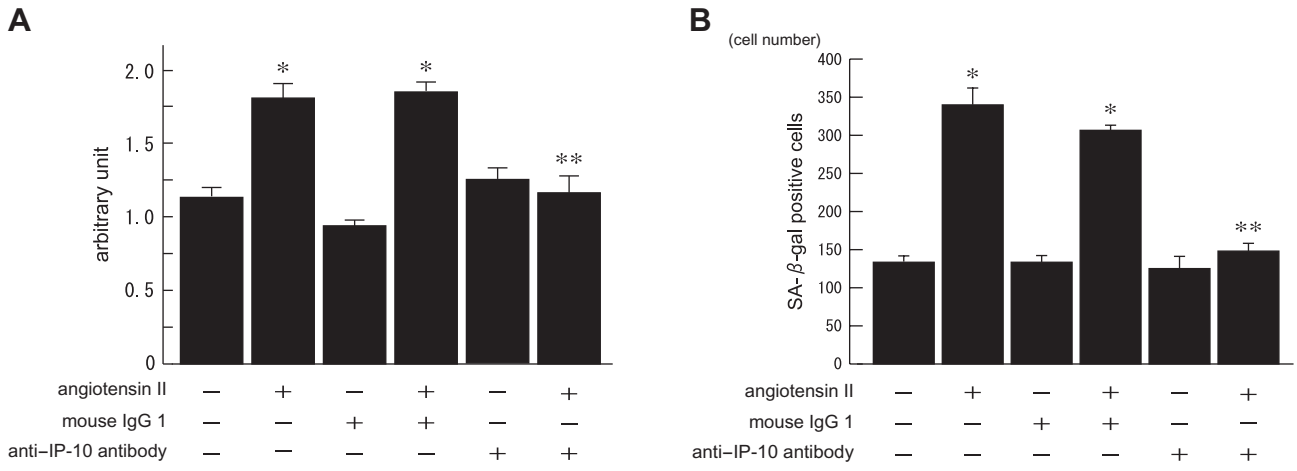


Fig. 5. *A: IP-10 neutralization inhibits angiotensin II–induced DNA fragmentation in endothelial cells. HUVECs were stimulated with angiotensin II (1 μmol/L, 24 h) in the presence of an anti–IP-10-neutralizing antibody or isotype-matched control mouse IgG 1 (1 μg/mL). DNA fragmentation was estimated by measuring histone-associated DNA fragments using a photometric enzyme immunoassay, as described in Methods. * and **$p < 0.05$ vs. untreated control and angiotensin II–stimulated cells, respectively. B: Angiotensin II–induced endothelial senescence is prevented by IP-10 neutralization. HUVECs were stimulated with angiotensin II (1 μmol/L, 24 h) in the presence of an anti–IP-10 neutralizing antibody or an isotype-matched control mouse IgG 1 (1 μg/mL). Cells were stained for SA-β-gal as described in Methods. Data presented are means±SEM of senescent SA-β-gal–positive cell numbers among 100 cells in 20 random fields from each of six independent experiments. * and **$p < 0.05$ vs. untreated control and angiotensin II–stimulated cells, respectively.*

nents of the renin-angiotensin system such as renin, ACE, and angiotensinogen, in endothelial cells. Taken together with the results that suggest angiotensin II stimulated the release of IP-10 and IP-10 induced upregulation of the renin-angiotensin system, it is indicated that angiotensin II and IP-10 form a circuit that stimulates the local renin-angiotensin system in endothelial cells.

We studied the involvement of IP-10 in the pathological roles of angiotensin II in endothelial cells. Akishita *et al*. reported that angiotensin II induces endothelial apoptosis and senescence (*32*). We evaluated apoptosis by measuring DNA fragmentation in HUVECs. As shown in Fig. 5A, angiotensin II caused an increase in DNA fragmentation in HUVECs, and coincubation with an anti–IP-10 neutralizing antibody significantly blocked this increase. Coincubation with an isotype-matched control mouse IgG 1 had no obvious effect. These data suggest that IP-10 is involved in endothelial cell apoptosis induced by angiotensin II. We also examined endothelial cell senescence by SA-β-gal staining. Angiotensin II significantly increased the number of cells stained with SA-β-gal in HUVECs (Fig. 5B). Coincubation with an anti–IP-10 neutralizing antibody blocked the increase induced by angiotensin II, whereas coincubation with an isotype-matched control mouse IgG 1 had no obvious effect. These findings indicate that IP-10 mediates angiotensin II–induced endothelial cell senescence.

Discussion

It was previously demonstrated that pharmacological AT_1 receptor blockade inhibited diet-induced atherosclerosis in apolipoprotein E (ApoE) knockout mice (*33*, *34*). Also, genetic disruption of AT_1 receptor ameliorates vascular oxidative stress, endothelial dysfunction, and atherosclerotic lesion formation in ApoE knockout mice (*33*, *34*). Thus, it is suggested that AT_1 receptor–mediated angiotensin II signaling promotes atherosclerosis.

Chemokine-induced leukocyte migration to the vessel wall plays important roles in atherogenesis. Previous studies have shown that angiotensin II increases chemokine release from vascular cells. Mateo *et al*. demonstrated that angiotensin II induces mononuclear leukocyte-endothelial interactions by stimulating the release of CC chemokines such as MCP-1, RANTES, and MIP-1α from endothelial cells (*35*). They also showed the differential release of CC chemokines depending on the source of endothelial cells. Our present study shows the increased release of a CXC chemokine, IP-10, from endothelial cells by angiotensin II, and that valsartan, an AT_1 receptor antagonist, blocks this increase. STAT1 and NF-κB were previously shown to be involved in the induction of IP-10 gene expression in leukocytes (*36*, *37*). Further studies are necessary to elucidate downstream signaling pathways in AT_1 receptor–mediated IP-10 expression in endothelial cells. The cultured endothelial cells used in our study originated from

HUVECs (*18*). IP-10 expression in human atherosclerotic lesions has been reported (*19*). It was also demonstrated that a deficiency of CXCR3, an IP-10 receptor, reduces atherosclerotic lesion formation, upregulates anti-inflammatory molecules, and increases the number of regulatory T cells within atherosclerotic lesions in ApoE knockout mice, suggesting that IP-10 signaling promotes atherosclerosis (*38*). Akishita *et al*. demonstrated that angiotensin II augments apoptosis and cell senescence of endothelial cells implicated in atherogenesis (*32*). In the present study, IP-10 neutralization inhibited angiotensin II–induced apoptosis and cell senescence of endothelial cells, suggesting that angiotensin II-AT_1 receptor–induced IP-10 release from endothelial cells contributes to atherogenesis.

Effects of IP-10 on endothelial cells that are independent of leukocyte-endothelial interactions are not fully understood. The local renin-angiotensin system is reportedly expressed in cardiomyocytes and vascular cells (*39–42*). Our present study demonstrates that IP-10 upregulates gene expression of the components of the renin-angiotensin system such as renin, ACE, and angiotensinogen, and increases the release of angiotensin II in endothelial cells. IP-10 activates signaling pathways in which MAP kinases such as ERK, p38 and JNK, phosphatidylinositol 3-kinase and JAK/STAT participate, by binding to CXCR3, a membrane receptor (*27*, *28*, *43–45*). The expression of functional CXCR3 has been reported in endothelial cells (*23*, *46*, *47*). The signaling pathways responsible for the expression of renin, ACE, and angiotensinogen have not been fully understood. The involvement of STAT in the regulation of renin and angiotensinogen expression has been reported (*48–50*). Also, NF-κB upregulates angiotensinogen (*50*). Thus, multiple signaling pathways that potentially crosstalk with IP-10 signaling seem to be involved in the regulation of renin, ACE, and angiotensinogen expression. In the present study, renin, ACE and angiotensinogen show different expression changes in response to IP-10 and angiotensin II, suggesting that divergent signaling pathways are involved in the expression of each gene. Further studies are necessary to elucidate the mechanisms responsible for the expression of the renin-angiotensin system components in endothelial cells.

It is likely that angiotensin II released from endothelial cells exerts biological actions in both paracrine and autocrine fashions, and that angiotensin II-AT_1 receptor–induced IP-10 links angiotensin II and upregulation of the local renin-angiotensin system in endothelial cells. Therefore, it is suggested that the postulated angiotensin II-renin-angiotensin system circuit in endothelial cells sustains proinflammatory signals. Pharmacological blockade of AT_1 receptors with AT_1 receptor antagonist may prevent not only chemokine-induced leukocyte-endothelial interaction but also upregulation of the endothelial renin-angiotensin system, which potentially promotes vascular injury including atherosclerosis.

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