Induction of Interleukin-6 Expression by Angiotensin II in Rat Vascular Smooth Muscle Cells

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Abstract—Recent studies suggest that atherosclerosis is a kind of inflammatory process and that cytokine plays important roles in this process. Although it is generally accepted that angiotensin II (Ang II) plays an important role in atherogenesis, the role of Ang II in cytokine production has not been explored. In this report, we investigated the effect of Ang II on the production of interleukin-6 (IL-6), which is a multifunctional proinflammatory cytokine in rat vascular smooth muscle cells. Ang II significantly increased the expression of IL-6 mRNA and protein in a dose-dependent manner (10^{-10} to 10^{-6} mol/L). The expression of IL-6 mRNA induced by Ang II showed 2 peaks at 30 minutes and 12 to 24 hours after stimulation. The effect of Ang II on IL-6 release and mRNA expression was completely blocked by an Ang II type 1 receptor antagonist, CV11974; however, an Ang II type 2 receptor antagonist, PD123319, showed no effect. Chelating of intracellular Ca^{2+} with BAPTA-AM, inhibition of tyrosine kinase with genistein, and inhibition of mitogen-activated protein kinase kinase with PD98059 completely abolished the effect of Ang II. However, downregulation of protein kinase C by pretreatment with a phorbol ester for 24 hours or a specific protein kinase C inhibitor, calphostin C, did not affect the Ang II–induced expression of IL-6 mRNA. Deletion and mutational analysis of IL-6 gene promoter showed that cAMP-responsive element was important for Ang II–induced IL-6 gene expression. Gel mobility shift assay showed an increase of cAMP-responsive element binding protein by Ang II. These results provide new insights into Ang II signaling and the role of Ang II in the progression of inflammatory changes of blood vessels. (Hypertension. 1999;34:118-125.)

Key Words: angiotensin II ■ interleukins ■ protein kinases ■ cAMP-responsive element

Atherosclerotic lesions contain elements of inflammatory reaction with fibroproliferative changes. An early atherosclerotic lesion consists largely of macrophages and T lymphocytes. With the advancement of the lesion, smooth muscle cells migrate, proliferate, and produce extracellular matrix components, presumably as part of a repair process of metabolic or physical injury and subsequent inflammation of blood vessels.¹ The repair process after injury is associated with the induction of immediate early genes such as c-fos and c-jun, followed by the production of various kinds of growth factors and cytokines.

Angiotensin II (Ang II) is a multifunctional octapeptide that increases contraction of blood vessels and induces hypertrophy and hyperplasia of vascular smooth muscle cells (VSMC). There are 2 isoforms for Ang II receptor, which are designated as type 1 receptor (AT1R)²,³ and type 2 receptor (AT2R).⁴,⁵ Most of the cardiovascular effects of Ang II are ascribed to the AT1R. Ang II stimulates production of growth factors such as platelet-derived growth factor (PDGF) and transforming growth factor-β (TGF-β) and matrix components such as collagen and fibronectin through the AT1R in VSMC. Several lines of evidence have suggested the important roles of Ang II in vascular cell growth and tissue remodeling following hypertension, vascular injury, and atherosclerosis.⁶–¹⁰

Recent findings showed that the AT1R couples to many intracellular signal transduction pathways. In common with many other growth factors, Ang II causes a rapid induction of the growth-associated immediate early gene expression. Stimulation of AT1R by Ang II elicits tyrosine phosphorylation and activates the mitogen-activated protein kinase (MAPK) pathway.¹¹–¹⁵ Understanding of the mechanism of these Ang II–stimulated signaling pathways is important because these signals eventually activate gene transcription of immediate early genes, growth factors, and extracellular matrix, resulting in hypertrophy or hyperplasia of VSMC.

Interleukin-6 (IL-6) has multiple biological activities, such as induction of B-cell differentiation, T-cell activation, induction of acute-phase protein such as C-reactive protein (CRP) and fibrinogen in the liver, and production of platelets.¹⁶,¹⁷ Ikeda et al¹⁸ showed that IL-6 increased the proliferation of VSMC in a PDGF-dependent manner. IL-6 is secreted from macrophages, T cells, endothelial cells, mesangial cells, and VSMC.¹⁸–²² In terms of VSMC, IL-6 is secreted by stimulation of interleukin-1²³ and

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PDGF. IL-6 mRNA was expressed in the atherosclerotic lesion of Watanabe heritable hyperlipidemic (WHHL) rabbits. The plasma level of IL-6 is elevated in patients with unstable angina. Although these studies suggest an important role of IL-6 in atherosclerosis, the mechanism and the source of IL-6 production are not understood. In addition, the effect of Ang II on IL-6 production has not been elucidated.

In this report we demonstrated that Ang II induced IL-6 expression in cultured rat VSMC through the AT1R. Deletion and mutational analysis of the promoter region of the IL-6 gene showed that cAMP-responsive element (CRE) plays an important role in Ang II–induced upregulation of IL-6 gene expression.

Methods

Reagents

DMEM and fetal bovine serum were purchased from GIBCO BRL. Ang II was purchased from Peptide Institute. PD98059, a MAPK kinase inhibitor, was obtained from Research Biochemicals International. Unless indicated otherwise, other chemical reagents were purchased from Wako Chemicals. [α-32P]dCTP was obtained from Dupont NEN. Bovine serum albumin was purchased from Sigma. CV11974, a specific AT1R antagonist, was obtained from Takeda Chemical Industries, Ltd, and PD123319, a specific AT2R antagonist, was obtained from Warner-Lambert, Park Davis Co. Antibody against Stat 3 binding protein (CREB) was obtained from New England Biolabs Inc. Antibody against Stat 3 binding protein was obtained from Santa Cruz Inc.

Cell Culture

VSMC were isolated from the thoracic aorta of Sprague-Dawley rats as described previously and maintained in DMEM supplemented with 10% fetal bovine serum at 37°C in a humidified atmosphere of 95% air/5% CO2. Passages between 5 and 15 were used for the experiments. Cells were seeded in 6-cm tissue culture dishes or 24 well plates and cultured in DMEM with 10% fetal bovine serum for 4 days. Then the medium was changed to DMEM supplemented with 0.1% BSA. The cells were cultured for an additional 2 days and stimulated with Ang II for the indicated period.

Northern Blot Analysis

Total RNA was prepared by an acid guanidinium-phenol-chloroform extraction method. Northern blot analysis was performed as described previously except that 20 μg of total RNA was analyzed. The hybridized membrane was stripped by boiling in 0.5% SDS extraction method. Northern blot analysis was performed as described previously and maintained in DMEM supplemented with 10% fetal bovine serum at 37°C in a humidified atmosphere of 95% air/5% CO2. Passages between 5 and 15 were used for the experiments. Cells were seeded in 6-cm tissue culture dishes or 24 well plates and cultured in DMEM with 10% fetal bovine serum for 4 days. Then the medium was changed to DMEM supplemented with 0.1% BSA. The cells were cultured for an additional 2 days and stimulated with Ang II for the indicated period.

Deletion Mutants of Rat IL-6 Gene Promoter Region and Construction of Luciferase Fusion DNA Construct

A genomic DNA clone encoding the rat IL-6 promoter region obtained by the polymerase chain reaction technique with 5′ primer (5′ GTGGACAGAAACCCAGGGAC 3′) and 3′ primer (5′ CTGTGTCCTGAAGGCGAGTG 3′). The product of polymerase chain reaction, the IL-6 promoter region, was sequenced by the dyeoxy chain termination method (Thermo Sequenase cycle sequencing kit, Amershams) after cloning into pBluescript (Stratagene). The promoter region of IL-6 gene was digested with restriction endonucleases. The 5′-end of the digested DNA fragments was blunted by Klenow enzyme or T4 DNA polymerase, and the 3′-end was digested with XhoI, which is located in the first exon of the rat IL-6 gene. Five deletion mutants (from No. 1 to No. 5) were cloned into the Smal-XhoI site of pGL3 basic vector (Promega Corporation). Site-directed mutagenesis was performed according to the method of Higuchi et al. In the deletion mutant No. 1, the wild-type sequence of CRE, ATGACGTTCA, was altered to ATCGATTCGA. Nucleotide sequences of the mutation were confirmed by DNA sequencing.

Transfection of IL-6 Promoter-Luciferase Fusion DNA Construct to VSMC

Confluent VSMC were split by trypsin/EDTA solution, and 5×104 cells were prepared in a 6-cm tissue culture dish. After 48 hours, 5 μg of IL-6 promoter-luciferase fusion DNA and 2 μg of β-galactosidase gene driven by an SV40 promoter-enhancer sequence were introduced to VSMC by the DEAE dextran method according to the manufacturer’s instructions (Promega Corporation). After transfection, the cells were cultured in DMEM with 10% FCS for 24 hours, washed twice with phosphate-buffered saline, and stimulated with 10−6 mol/L of Ang II for 24 hours in DMEM with 0.1% BSA. The luciferase activity was measured as described previously. The β-galactosidase activity in the same sample was measured spectrophotometrically according to Sambrook et al3 and used to normalize the luciferase activity.

Quantification of Rat IL-6 by Sandwich ELISA

The medium of unstimulated or Ang II–stimulated VSMC was collected and centrifuged at 12 000 rpm for 1 minute. The supernatant was stored at −70°C until assay. ELISA for rat IL-6 was performed with a Cytoscreen ELISA kit (BioSource International) according to the manufacturer’s instructions. Briefly, serial dilution of recombinant rat IL-6 and test samples were applied to a microtiter plate coated with an anti-rat IL-6 monoclonal antibody, and the incubation was done at 37°C for 3 hours. The solution was discarded, and wells were washed 4 times. Then biotinylated anti-rat IL-6 antibody solution was added. After the incubation at room temperature for 30 minutes and washing 4 times, streptavidin–horseradish peroxidase was added. After the incubation at room temperature for 30 minutes and washing 4 times, a stabilized chromogen, tetramethylbenzidine, was added. The plate was incubated at room temperature for 30 minutes in the dark, and the color was read spectrophotometrically at 450 nm.

Preparation of Nuclear Extracts and Gel Mobility Shift Assay

Nuclear extracts were prepared according to the methods described by Dignam et al and Osborn et al. Gel mobility shift assay was performed as described previously. DNA probe (wild-type CRE: 5′-GCTAAATGACGTTCACTATT-3′) was labeled with 32P. Ten micromolars of nuclear extracts was incubated with 1×106 cpm of labeled DNA probes and 2 μg of deoxycytidine-heteropolymer in a buffer containing 20 mmol/L HEPES, pH 7.9, 50 mmol/L KCl, 1 mmol/L MgCl2, 5 mmol/L dithiothreitol, 1 mmol/L EDTA, 4% glycerol, and 50 mg/L BSA for 30 minutes at room temperature and electrophoresed on 5% acrylamide gel. Wild-type CRE or mutant CRE (5′-GCTAAATGACGTTCACTATT-3′) was added as a competitor. After electrophoresis, gels were dried and exposed to an x-ray film at −80°C.

Statistical Analysis

Statistical analyses were performed by 1-way or 2-way ANOVA and multiple comparison (Fisher) test if appropriate. A P value <0.05 was considered significant. Data were expressed as mean±SE.

Results

Ang II–Increased IL-6 Production

VSMC were stimulated with Ang II, and the protein level of IL-6 in the supernatant was measured by sandwich ELISA. As shown in Figure 1A, the protein level of IL-6 in the tissue culture medium of Ang II (10−7 mol/L)–stimulated VSMC was increased in a time-dependent manner. A small amount
of IL-6 was detected in the supernatant of unstimulated VSMC after 24 hours. A significant increase of IL-6 in the supernatant of Ang II–stimulated VSMC was observed after 6 hours. Next we examined the effect of isoform-specific antagonist for Ang II receptor on the enhancement of IL-6 expression. CV11974 (10^{-5} mol/L), an AT1R-specific antagonist, inhibited the enhancement of IL-6 production by Ang II, but PD123319 (10^{-5} mol/L), an AT2R-specific antagonist, had no significant effect. These results suggest that Ang II stimulates IL-6 production through the AT1R in VSMC. At 24 hours of stimulation, Ang II dose dependently increased IL-6 production (Figure 1B).

Enhancement of IL-6 mRNA Expression by Ang II
We examined the time course of Ang II–induced IL-6 mRNA expression (Figure 2). Two species of IL-6 gene transcript were detected (Figure 2A). The most abundant transcript was \( \sim 1.3 \) kb in length, and the less abundant transcript was \( \sim 2.4 \) kb. The 2 IL-6 mRNA species are generated by an alternative polyadenylation. For the quantification of IL-6 mRNA, the radioactivities of both mRNA species were taken into account. As seen in Figure 2, a biphasic increase of IL-6 mRNA by Ang II was observed. The first peak was at 30 minutes after Ang II stimulation. Then the expression level of IL-6 mRNA was decreased, and the second peak was at 12 to 24 hours after Ang II stimulation.

VSMC were incubated with various concentrations of Ang II for 30 minutes. The expression of IL-6 mRNA by Ang II was increased dose dependently (Figure 3). We also examined the Ang II receptor isoform responsible for IL-6 mRNA expression by using an isoform-specific antagonist. CV11974 completely abolished the IL-6 mRNA induction by Ang II; however, PD 123319 showed no effect (Figure 3). This result suggests that Ang II induces IL-6 mRNA expression through the AT1R and is consistent with the results of IL-6 protein production.
Intracellular Signals in Ang II–Induced IL-6 Expression

We examined the signaling pathway of Ang II important for the induction of IL-6 gene expression (Figure 4). Protein kinase C (PKC) activation\textsuperscript{35} and intracellular Ca\textsuperscript{2+} mobilization\textsuperscript{36} have been reported to be important signals through the AT\(_R\). VSMC were treated for 24 hours with phorbol 12-myristate 13-acetate (PMA) (10\textsuperscript{-6} mol/L) for downregulation of PKC activity or pretreated with a PKC inhibitor, calphostin C (10\textsuperscript{-6} mol/L, 30 minutes), and stimulated with 10\textsuperscript{-6} mol/L of Ang II for 30 minutes. Although prolonged exposure to PMA or calphostin C increased basal IL-6 mRNA expression, response to Ang II was not inhibited by these treatments. Preincubation of VSMC with BAPTA-AM (10\textsuperscript{-5} mol/L), an intracellular Ca\textsuperscript{2+} chelator, for 30 minutes before Ang II stimulation inhibited Ang II–induced IL-6 mRNA expression. EGTA (10\textsuperscript{-2} mol/L, 30 minutes), an extracellular Ca\textsuperscript{2+} chelator, did not inhibit Ang II–induced IL-6 mRNA expression. Basal and Ang II–induced IL-6 mRNA expression was rather enhanced by preincubation with EGTA. These results suggest that Ang II activates IL-6 gene expression through an intracellular Ca\textsuperscript{2+}-dependent pathway. Tyrosine phosphorylation and activation of MAPK are also important signals of AT\(_R\).\textsuperscript{37} Pretreatment with genistein (10\textsuperscript{-4} mol/L), which inhibits tyrosine kinase, and PD98059 (10\textsuperscript{-5} mol/L), which inhibits activation of MAPK kinase, abolished the effect of Ang II on the induction of IL-6 mRNA. These results suggest that Ang II–induced tyrosine phosphorylation and MAPK activation also play important roles in the activation of IL-6 gene expression.

Promoter Activity of IL-6 Gene

To determine the promoter region responsible for the induction of IL-6 gene expression by Ang II, deletion expression. EGTA (10\textsuperscript{-2} mol/L, 30 minutes), an extracellular Ca\textsuperscript{2+} chelator, did not inhibit Ang II–induced IL-6 mRNA expression. Basal and Ang II–induced IL-6 mRNA expression was rather enhanced by preincubation with EGTA. These results suggest that Ang II activates IL-6 gene expression through an intracellular Ca\textsuperscript{2+}-dependent pathway. Tyrosine phosphorylation and activation of MAPK are also important signals of AT\(_R\).\textsuperscript{37} Pretreatment with genistein (10\textsuperscript{-4} mol/L), which inhibits tyrosine kinase, and PD98059 (10\textsuperscript{-5} mol/L), which inhibits activation of MAPK kinase, abolished the effect of Ang II on the induction of IL-6 mRNA. These results suggest that Ang II–induced tyrosine phosphorylation and MAPK activation also play important roles in the activation of IL-6 gene expression.

Promoter Activity of IL-6 Gene

To determine the promoter region responsible for the induction of IL-6 gene expression by Ang II, deletion
analysis of the promoter region was performed. We constructed luciferase expression vectors with various lengths of the promoter region of the IL-6 gene (Figure 5A). Luciferase activity was measured after 24 hours of Ang II (10^{-6} mol/L) stimulation (Figure 5B). Ang II stimulated luciferase activity by \approx 2-fold in constructs of No. 1, No. 2, and No. 3. The response to Ang II was not observed in the construct that lacked the DNA segment between –581 and –150 bp (No. 4). In this DNA segment, several consensus cis DNA elements such as glucocorticoid response element, AP-1, and CRE are present. Among these elements, the AP-1 and the CRE (also known as a multiple response element) were reported to be important for basal and lipopolysaccharide-stimulated IL-6 gene expression. 38 Because downregulation of PKC by prolonged exposure to PMA or a PKC inhibitor showed no effect on Ang II–induced enhancement of IL-6 gene expression, we assumed that the AP-1 site is not critical. Therefore, we introduced a mutation into the CRE site. The luciferase activity of luciferase expression plasmid driven by IL-6 gene promoter with mutation in the CRE site was not enhanced by Ang II stimulation (Figure 6). This suggests that the CRE site in the IL-6 gene promoter is essential for Ang II–induced upregulation. We also examined the effect of PD98059 on IL-6 promoter activity. Pretreatment with PD98059 strongly inhibited Ang II–induced upregulation.

### Induction of CREB by Ang II

Next we examined the DNA binding protein bound to the CRE site using gel mobility shift assay (Figure 7). When nuclear extracts from Ang II–stimulated VSMC were used, DNA binding protein (arrow 2) was increased compared with unstimulated VSMC (lanes 1 to 3). Specificity of the binding was confirmed by adding 100 times molar excess of nonlabeled probe that eliminates this band (lane 4). When oligonucleotide with mutation in the CRE site was added as a competitor, this band did not fade (lane 5). When the antibody against CREB was added (lane 6), the band was supershifted (the shifted band is indicated by arrow 1). However, when an irrelevant antibody (antibody against Stat 3) was added (lane 7), the band was not supershifted. These results suggested that Ang II induces CREB binding to the CRE site of the IL-6 gene promoter.

### Discussion

We showed the following: (1) Ang II stimulated the expression of IL-6 through the AT_{1}R in VSMC; (2) the induction of IL-6 expression by Ang II was dependent on intracellular Ca^{2+}, tyrosine phosphorylation, and MAPK kinase and independent of PKC or extracellular Ca^{2+}; (3) CRE in the promoter region of the IL-6 gene plays an important role in Ang II–induced upregulation.

Previous studies suggested that a Ca^{2+}-dependent signaling mechanism plays an important role in Ang II–induced activation of MAPK through the AT_{1}R. 37 Chelation of intracellular Ca^{2+} by BAPTA-AM suppressed Ang II–induced IL-6 mRNA expression. In contrast, chelation of extracellular Ca^{2+} by EGTA itself enhanced IL-6 mRNA expression and did not inhibit Ang II–induced upregulation. Several reports showed that various Ca^{2+} channel blockers increased the expression of IL-6 mRNA in human VSMC, 39 human mesangial cells, 40 and peripheral blood mononuclear cells. 41 EGTA may exert an effect similar to that of the Ca^{2+} channel blocker. However, the mechanism by which EGTA and Ca^{2+} channel blocker stimulate IL-6 gene expression remains unclear. Pretreatment with PD98059 or genistein completely inhibited Ang II–induced IL-6 expression. Recently, Eguchi et al 37 showed that Ang II–induced MAPK activation is dependent on Ca^{2+}/calmodulin-sensitive tyrosine phosphorylation. Our results were consistent with their results and suggest that the downstream signal(s) of MAPK is important for IL-6 gene activation by Ang II.

There are several important regulatory cis DNA elements such as AP-1, CRE, nuclear factor–IL-6 (NF-IL6), and nuclear factor-κB (NF-κB) in the promoter region of the IL-6 gene. These promoter elements are conserved among species such as mouse, 42 rat, 30 and human 43 and regulate IL-6 gene expression in a cell type–specific manner. AP-1, NF-κB, and NF-IL6 are required for IL-6
The gene expression in monocytes

NF-κB is sufficient for expression in the T-cell line. However, regulatory elements important for expression in VSMC have not been investigated. Although the basal expression of IL-6 mRNA in quiescent VSMC is very weak, deletion analysis showed that the DNA segment between −150 and −27 bp is essential for basal expression because luciferase activity was greatly reduced by deletion of this DNA segment (Figure 5; compare the luciferase activity of No. 5 construct with that of No. 4). In this DNA segment, NF-κB, NF-IL6, and GATA are present and probably are important for the basal expression of IL-6 gene in VSMC. In terms of the response to Ang II, the DNA segment between −581 and −150 bp is essential. Mutational analysis showed that the CRE site in this DNA segment is essential for the response to Ang II. However, transcription factors are known to activate gene transcription in a cooperative manner. Therefore, the cooperation of CREB with other transcription factors such as AP-1, NF-IL6, or NF-κB may play a role, and this possibility is not excluded in this study. Recently, Xing et al reported that MAPK-activated p90 phosphorylates and activates CREB. Because Ang II–induced activation of IL-6 gene transcription is inhibited by a MAPK kinase inhibitor, it is possible that Ang II activates the MAPK-p90-CREB pathway and upregulates IL-6 gene transcription. It is known that protein kinase A, the calmodulin-dependent protein kinases, p90, and MAPK-activated protein kinase-2 phosphorylate CREB at serine-133, which is a key regulatory site controlling transcriptional activity in the response to a variety of extracellular signals, including growth factors and neurotropins. However, Chawla et al reported a dissociation between CREB phosphorylation on serine-133 and CRE-dependent transcription. Therefore, at this point it is not clear that increased binding of CREB by gel mobility shift assay correlates with activation of CRE-dependent transcription of IL-6 gene promoter.

We observed a biphasic increase of IL-6 mRNA by Ang II, as shown in Figure 2. Because Ang II induces production of many growth factors in VSMC, we assumed that the second peak was induced by Ang II–induced growth factors. To test this possibility, VSMC were incubated with cycloheximide (10−8 mol/L, 30 minutes), which is a protein synthesis inhibitor, and incubated with or without 10−6 mol/L of Ang II for 30 minutes or 24 hours. Cycloheximide increased basal mRNA expression at 30 minutes or 24 hours. The induction of IL-6 mRNA by cycloheximide has been reported. The response to Ang II in the presence of cycloheximide was preserved at both incubation periods, suggesting that the IL-6 mRNA expression by Ang II dose not require de novo protein synthesis (data not shown).

Previous studies have shown that IL-6 increases the number of platelets in the circulation and IL-6 activates platelets through arachidonic acid metabolism in vitro. Burstein reported that IL-6 increased plasma fibrinogen and decreased free protein S concentration. These IL-6–induced modifications of platelet and the coagulant phase of the clotting mechanism may lead to pathological thrombosis and instability of plaque. In an experimental model, Ikeda et al reported that IL-6 gene transcript was expressed in the atherosclerotic plaque of WHHL rabbit. However, the role of Ang II in the expression of IL-6 in WHHL rabbit was not defined. Recently, Nickenig et al reported that LDL increased AT,R mRNA in VSMC by increasing its stability and resulted in the elevation of functional response to Ang II. Thus, it is possible that AT,R in the WHHL rabbit plays a pivotal role in the induction of IL-6 transcript in atheromatous plaque.

Although it is not clear from this study that Ang II regulates IL-6 production in vivo, inhibition of Ang II–induced IL-6 production may be one of the mechanism by which an angiotensin-converting enzyme inhibitor or an AT,R antagonist prevents neointimal formation in the balloon injury model of atherosclerosis. Our data clearly showed the role of Ang II in IL-6 production in VSMC, suggesting that Ang II may mediate the inflammatory process in blood vessels.

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