Mechanism of Angiotensin II-mediated Regulation of Fibronectin Gene in Rat Vascular Smooth Muscle Cells*

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This study was performed to investigate a mechanism of angiotensin II (Ang II)-mediated activation of the fibronectin (FN) gene in rat vascular smooth muscle cells. Actinomycin D and CV11974 completely inhibited Ang II-mediated increase in FN mRNA levels. Inhibitors of protein kinase C (PKC), protein-tyrosine kinase (PTK), phosphatidylinositol-specific phospholipase C, Ras, phosphatidylinositol 3-kinase, p70 S6 kinase, and Ca²⁺/calmodulin kinase also decreased Ang II-induced activation of FN mRNA. In contrast, cycloheximide; PD123319; or inhibitors of Gα, protein kinase A, or mitogen-activated protein kinase kinase did not affect the induction. FN promoter contained a putative AP-1 binding site (rFN/AP-1; −463 to −437), and the results of a transient transfection and electrophoretic mobility shift assay showed that Ang II enhanced rFN/AP-1 activity. CV11974 and inhibitors of PCK or PTK suppressed increases in rFN/AP-1 activity, although neither PD123319 nor a protein kinase A inhibitor affected the induction. Furthermore, mutation of rFN/AP-1 that disrupted nuclear binding suppressed Ang II-induced transcription in the native FN promoter (−1908 to +136) context. Thus, Ang II activates transcription of the FN gene through the Ang II type 1 receptor in vascular smooth muscle cells, at least in part, via the activation of AP-1 by a signaling mechanism dependent on PCK and PTK.

Hemodynamic and endocrine factors are among the most important factors implicated in the physiology and pathophysiology of the vascular wall. Arterial hypertension evokes structural and functional changes of the vascular wall (1, 2). Modifications of the extracellular matrix, including fibronectin (FN) and collagen, have been previously reported in vessel walls of hypertensive animals (3–5). Activation and qualitative changes in the extracellular matrix participate in vascular wall remodeling and in the pathogenesis of atherosclerosis. Vascular remodeling in hypertension may be an adaptive response to increased transmural pressure (6–9). Mechanical stress seems to play a direct role in vascular remodeling, since mechanical stretch is able to increase protein synthesis by vascular smooth muscle cells (VSMCs) (10). However, neuronal and humoral factors may be critical in hypertension-induced remodeling of vascular wall. Especially, several in vivo studies have reported that hypertension activates the vascular renin-angiotensin system (RAS) including angiotensin-converting enzyme (ACE) (11), and infusion of pressor and subpressor doses of angiotensin II (Ang II) increases aortic FN mRNA in both hypertensive and normotensive animals (12, 13). Ang II evokes diverse physiological response including arterial vasoconstriction to elevate blood pressure in vivo (14) and increases production of collagen with a growth-promoting effect on VSMCs in vitro (15). Pharmacological evidence has defined at least two subtypes of Ang II receptors, Ang II type 1 (AT1) receptor and Ang II type 2 (AT2) receptor. Previous results of molecular cloning have revealed that both receptor subtypes belong to the superfamily of G protein-coupled receptors with seven transmembrane helices (16–19). According to the recent results of in vitro studies, Ang II initially activates a phosphatidylinositol-specific phospholipase C (PI-PLC) via its binding to AT1 receptor on the surface of VSMCs, leading to the generation of inositol trisphosphate and diacylglycerol (20), which are involved in intracellular Ca²⁺ mobilization (21) and protein kinase C (PKC) activation (22), respectively. In VSMCs, Ang II also induces a rapid increase in expression of the growth-associated nuclear proto-oncogenes and stimulates tyrosine phosphorylation of multiple substrates (23, 24). These findings, taken together with relatively abundant expression of AT1 receptor in vascular wall and VSMCs, indicate that Ang II plays an important role in vascular remodeling via an AT1 receptor pathway. Thus, investigation of the mechanism of Ang II-induced regulation of extracellular matrix and tissue RAS in VSMCs is essential in elucidating the mechanism of vascular remodeling and the pathogenesis of atherosclerosis.

In the present study, we examined the effects of Ang II on gene expression of extracellular matrix components (FN and collagen) and investigated a mechanism of Ang II-induced activation of FN gene transcription in VSMCs.

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**The abbreviations used are: FN, fibronectin; VSMC, vascular smooth muscle cell; ACE, angiotensin-converting enzyme; Ang II, angiotensin II; AT1, angiotensin II type 1; AT2, angiotensin II type 2; PI-PLC, phosphatidylinositol-specific phospholipase C; PKC, protein kinase C; PTX, pertussis toxin; CAT, chloramphenicol acetyltransferase; FN/AP-1, rat fibronectin promoter AP-1 binding motif; EMSA, electrophoretic mobility shift assay; PTK, protein-tyrosine kinase; PI3K, phosphatidylinositol 3-kinase; MAP, mitogen-activated protein; S6K, p70 S6 kinase; TK, thymidine kinase; kb, kilobase pair; RAS, renin-angiotensin system.
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collagen) and RAS components (angiotensinogen, ACE, and AT1 receptor) in VSMCs. We obtained evidence for induction of expression of FN and collagen but not angiotensinogen, ACE, or AT1 receptor. Furthermore, interactions of several signal transduction pathways were involved in Ang II-induced expression of FN in VSMCs, and a specific promoter region, the AP-1 element, of the FN gene might play an important role in the Ang II-mediated increase in FN mRNA.

**Experimental Procedures**

**Materials**—RPMI 1640 medium, fetal calf serum, penicillin, and streptomycin were obtained from Life Technologies, Inc. Ang II, phosphor-12-myristate-13-acetate, A23187, 8-bromo-cAMP, HA-1004, H-7, genistein, pertussis toxin (PTX), manumycin, rapamycin, actinomycin D, and cycloheximide were purchased from Sigma. Calphostin C, herbinycin A, H-89, U-73122, PD98059, BAPTA-AM, and calmidazolium chloride, were obtained from Calbiochem. Wortmannin was purchased from BioMol.

**AT1 receptor-specific antagonist CV11974 and AT2 receptor-specific antagonist PD123329 were supplied from Takeda Chemical and Perk Davis, respectively.**

**Cell Culture of VSMCs—**VSMCs were aseptically isolated from the rat aortic explants of 5-week-old Sprague-Dawley rats. Subconfluent cells were passaged by trypsinization at approximately 80% confluence in dishes were made quiescent by incubation with serum-free RPMI 1640 medium for 24 h, unless otherwise stated.

**RNA Isolation and Northern Blot Hybridization—**Total cellular RNA was isolated from VSMCs using the single-step procedure by acid guanidium thiocyanate-phenol chloroform extraction (26). Twenty micrograms of each total RNA sample were denatured with 1 M glyoxal and 50% dimethyl sulfoxide, electrophoresed on a 1.2% agarose gel, and transferred to GeneScreen Plus (Nylon) membrane (DuPont). Filters were prehybridized for 1 h at 65 °C in a solution consisting of 1% SDS, 1 M NaCl, and 10% dextran sulfate. Hybridization proceeded for 24 h in the same solution containing 100 ng/ml of denatured salmon sperm DNA and 3 × 10^6 cpm/ml of the 32P-labeled probes. Filters were washed twice with 2× SSC (1× SSC: 0.15 M NaCl, 0.01 M sodium citrate) for 5 min at room temperature, twice with 2× SSC and 1× SDS for 30 min at 60 °C, and twice with 0.1× SSC for 30 min at room temperature. Dried filters were exposed to x-ray film or to the imaging plate of FUJIX Bio-Image Analyzer BAS2000 (Fuji Photo Film).

**cDNA Probes—**Rat FN cDNA probes kindly provided by Dr. Hynes were utilized for Northern blot analysis. One probe, which encodes the 10th and 11th type I repeat (a 0.51-kb HindIII/NheI fragment) shared by all isoforms of FN, was used to detect total FN mRNA (27). Other rat FN cDNA probes for EIIA (a 0.55-kb PstI/NheI fragment), EIIIB (a 0.59-kb PstI/NheI fragment), and V region (a 0.60-kb PstI/NheI fragment) were used to detect only FN mRNA with EIIIA, EIIIB, and V domain, respectively (28). Rat α1 chain of type I collagen cDNA (a 1.3-kb PstI/BamHI fragment) was supplied by Dr. Rowe (29). Rat angiotensinogen cDNA probe (a 1.1-kb Accl/Acc1 fragment) was generously provided by Drs. Ohkubo and Nakanishi (30). ACE (a 2.3-kb EcoRl/BglII fragment) cDNA was provided by Dr. Corvol (31). AT1 receptor cDNA (a 0.5-kb SacII/SmaI fragment) was provided by Dr. Sugaya (32). 18 S ribosomal RNA cDNA probe (a 0.3-kb SmaI/SmaI fragment) was provided by Dr. Raynal (33).

**Plasmid Construction, DNA Transfection, and Chloramphenicol Acetyltransferase (CAT) Assay—**To examine whether Ang II was a major activator of FN production in VSMCs, we first assessed effects of serum and several vasoactive substances including Ang II on FN gene expression by Northern blot analysis. After VSMCs were made quiescent by incubation with serum-free medium for 24 h, VSMCs were incubated with the indicated medium for 36 h. As shown in Fig. 1, a low level of FN mRNA could be detected in untreated control VSMCs. The mRNA level was markedly increased after exposure of the cells to FBS (10%), phosphor-12-myristate-13-acetate (10⁻⁷ M), or Ang II (10⁻⁷ M), whereas treatment with A23187 (10⁻⁵ M) or 8-bromo-cAMP (10⁻⁴ M) did not affect the FN mRNA levels. Then we examined the concentration dependence of Ang II–induced expression of FN mRNA in VSMCs. As shown in Fig. 2, the activation of FN mRNA expression was dependent on the concentration of Ang II. The concentration of Ang II that induced the maximal expression of FN mRNA was determined to be 10⁻⁷ M.
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Angiotensin II; increased expression was initially detectable at $10^{-11}$ M, half-maximal at approximately $5 \times 10^{-9}$ M, and maximal at $10^{-7}$ to $10^{-6}$ M.

We next examined the time course of extracellular matrix and RAS components mRNA expression induced by Ang II ($10^{-7}$ M). Expression of FN mRNA started to increase 3 h after Ang II treatment and peaked at 36 h, and at 48 h FN mRNA was still elevated compared with control (Fig. 3). We also found an increase in the level of type I collagen mRNA as early as 1 h after treatment, with its peak at 24 h. In contrast, the ACE mRNA level did not show any significant change after exposure to Ang II, and mRNA expression of angiotensinogen and AT1 receptor was decreased after exposure to Ang II. These results indicate that Ang II treatment specifically increases extracellular matrix mRNA expression but not RAS components mRNA expression in VSMCs. Several studies using antagonists of Ang II receptor subtypes and angiotensinogen-deficient mice suggest that Ang II exerts various effects on the expression of major component genes of the RAS by positive or negative feedback mechanisms (42–45). Although we and others showed that angiotensinogen gene expression was up-regulated in response to Ang II in hepatocytes, cardiac myocytes, and renal fibroblasts (46–48), the present results indicate that Ang II decreases expression of angiotensinogen mRNA in VSMCs. In addition, previous studies showed that Ang II down-regulated the vascular smooth muscle AT1 receptor by transcriptional and post-transcriptional mechanisms (49, 50), and the results in this study were consistent with these results. FN exists in several forms as a result of the alternative splicing of a single gene. The different isoforms are distinguished by the presence or absence of exon products and are designated in the rat as FN-EIIIA, FN-EIIIB, and FN-V (29). Changes in FN biosynthesis by VSMCs may have a causative role in hypertension and atherosclerosis (51), and a selective induction of the isoform containing the EIIIA insert, which is a fetal gene transcript, has been shown to occur during the development of vascular hypertrophy in response to hypertension (52, 53). In the present study, Ang II appeared to increase the FN-EIIIA mRNA to a greater extent than the other isoforms (Fig. 4), thereby indicating that Ang II actually changed the phenotype of VSMCs.

Effects of Ang II Receptor Antagonists, Transcriptional Inhibitor, and Protein Synthesis Inhibitor on Ang II-mediated Increase in FN mRNA Expression—To determine the type of Ang II receptor(s) involved in mediating the enhanced expression of FN mRNA in response to Ang II, the effects of Ang II receptor antagonists were investigated. VSMCs were incubated for 30 min with CV11974 ($10^{-6}$ and $10^{-5}$ M), followed by treatment with Ang II ($10^{-7}$ M) for 24 h. Treatment of VSMCs with CV11974 abolished the stimulatory effect of Ang II (Fig. 5, lanes 3 and 4). In contrast, incubation of cells with PD123319 ($10^{-6}$ and $10^{-5}$ M) did not affect the response to Ang II at all (Fig. 5, lanes 14 and 15). None of these Ang II receptor antag-
onists alone increased the expression of FN mRNA (Fig. 5, lanes 5 and 16). These results indicate that Ang II activates FN mRNA expression through an AT1 receptor pathway in VSMCs.

Next, to determine whether de novo RNA or protein synthesis was required for Ang II-induced increase in FN mRNA, VSMCs were pretreated with actinomycin D or cycloheximide for 30 min and incubated for 24 h in the presence or absence of Ang II (10^{-7} M) (Fig. 5). The RNA synthesis inhibitor actinomycin D not only abolished Ang II-mediated increase in FN mRNA but also lowered the basal FN mRNA level (Fig. 5, lanes 6–8), whereas the induction of FN mRNA by Ang II and the basal FN mRNA expression were not significantly altered by cycloheximide in VSMCs (Fig. 5, lanes 9–11). These results indicate that de novo mRNA transcription is required for the induction of FN mRNA expression by Ang II but that the effects of Ang II do not require de novo protein synthesis to stimulate the expression of FN mRNA.

FN mRNA turnover was examined by inhibiting new mRNA transcription with actinomycin D (5 mg/ml; Fig. 6). Half-lives for FN mRNA in control VSMCs were ~6.9 h. Treatment of VSMCs with Ang II for 12 h caused a slight increase (1.1-fold) in the half-life of FN mRNA (~7.3 h). Taken together, these results suggest that up-regulation of FN mRNA is mainly induced by enhancement of gene transcription.

Putative Role of Signal Transduction Pathways in Ang II-mediated Expression of FN mRNA—The interaction of Ang II with Ang II receptors on VSMCs activates several intracellular signal transduction pathways, which result in increases in [Ca^{2+}], and activation of PKC and protein-tyrosine kinases (PTKs) (20–24). Therefore, the role of these signal transduction pathways in modulating expression of the FN mRNA was examined (Fig. 7). VSMCs were incubated with signaling inhibitors for 30 min, followed by treatment with Ang II (10^{-7} M) for 24 h. First, we examined a possible role of protein kinase A in Ang II-mediated up-regulation of FN mRNA. Incubation of VSMCs with protein kinase A inhibitors, HA-1004 (10 mg/ml and 100 mg/ml) or H-89 (10^{-5} and 10^{-4} M) had no effect on Ang II-induced FN mRNA levels (Fig. 7, lanes 3–6). Next, involvement of PKC in Ang II-stimulated expression of FN mRNA was assessed by use of two different PKC inhibitors, H-7 (10 and 100 mg/ml) and calphostin C (10^{-7} and 10^{-6} M). Both PKC inhibitors significantly decreased the response to Ang II (Fig. 7, lanes 7–10). Third, the role of PTKs in mediating the cellular...
effects of Ang II was assessed with genistein (10^{-5} and 10^{-4} M) and herbimycin A (10^{-6} and 10^{-5} M), two different inhibitors of PTKs. Both treatments significantly inhibited the Ang II-mediated increase in FN mRNA (Fig. 8). These results indicate that PKC and PTK are involved in the activation of FN mRNA by Ang II.

AT1 receptor has been reported to be coupled to either G_{s} or G_{i}, which activates PI-PLC or inhibits adenylate cyclase, respectively (20–24). To determine which G protein-mediated signaling is involved in the FN activation, the effects of a specific PI-PLC inhibitor U-73122 and a G_{i} inhibitor PTX on Ang II-induced FN activation were studied in VSMCs (Fig. 8). Treatment with a U-73122 (10^{-6} and 10^{-5} M) for 24 h significantly inhibited FN activation induced by Ang II, whereas PTX

1(1 and 10 \mu M) did not affect Ang II-induced increase in FN mRNA (Fig. 8, lanes 3–6). These data suggest that PI-PLC activation through a PTX-insensitive G protein (probably G_{i}) plays a critical role in Ang II-induced FN activation in VSMCs. In cultured VSMCs, Ang II also stimulates Ras, phosphatidylinositol 3-kinase (PI3K), mitogen-activated protein (MAP) kinase, and p70 S6 kinase (S6K) (54–56) and has been shown to cause an elevation of cytosolic Ca^{2+} released from the inositol triphosphate-sensitive intracellular stores by the activation of PI-PLC. Treatment with the Ras farnesyltransferase inhibitor manumycin (10^{-7} and 10^{-6} M), the PI3K inhibitor wortmannin (10^{-7} and 10^{-6} M), the S6K inhibitor rapamycin (10^{-6} and 10^{-5} M), the intracellular Ca^{2+} chelator BAPTA-AM (10^{-6} and 10^{-5} M), or the calmodulin inhibitor calmidazolium chloride for 24 h resulted in significant loss of FN mRNA activation induced by Ang II (Fig. 8, lanes 7, 8, and 11–20). In contrast, blockade of MAP kinase kinase with PD98059 failed to inhibit Ang II-induced increase in FN mRNA (Fig. 8, lanes 9 and 10). These results indicate that Ang II-induced FN activation was dependent on Ras, PI3K, and S6K, whereas it was independent of MAP kinase in VSMCs. In addition, although the Ca^{2+} ionophore A23187 was not able to up-regulate FN mRNA expression, the results suggest that Ang II stimulates FN expression through a Ca^{2+}/calmodulin-dependent mechanism.

Ang II-mediated Activation of AP-1-Binding Site-containing Promoter—Selective gene expression is mostly controlled at transcriptional level (57), and the above results suggest that the AT1 receptor-mediated activation of transcription via signaling pathways may play an important role in Ang II-induced expression of FN mRNA in VSMCs. The regulation of transcriptional activity is achieved through the binding of a series of transcriptional factors to sequence-specific DNA elements that usually locate in the 5’-flanking region of the gene (58–60). The identification of both cis-acting elements and nuclear factors in the specialized cells would give a clue to understanding the molecular mechanisms that underlie expression of genes induced by various stimuli. Previous studies showed that the 5’-flanking region of the rat FN gene contained the motifs for transcription factors E4TF1, AP-1, AP-2, PEA2, and cAMP response element (61, 62). Among them, the AP-1-binding motif

![Fig. 4. Effect of Ang II on expression of alternative spliced forms of FN mRNA in VSMCs. VSMCs were stimulated with Ang II (10^{-7} M) for the indicated durations, and FN mRNA/18S ribosomal RNA (18S) levels were estimated. Representative Northern blots are shown of FN, FN-EIIIA, FN-EIIIB, and FN-V mRNAs and 18S ribosomal RNA from VSMCs (total RNA, 20 µg) treated with Ang II. Northern blot hybridization was performed as described under "Experimental Procedures."](image)

![Fig. 5. Effect of Ang II receptor antagonists, transcriptional inhibitor, and protein synthesis inhibitor on Ang II-induced activation of FN mRNA expression. VSMCs incubated with serum-free medium were preincubated for 30 min with CV11974 (lanes 3 and 4), actinomycin D (lanes 6 and 7), cycloheximide (lanes 9 and 10), or PD123319 (lanes 14 and 15), followed by treatment with Ang II (lanes 2–4, 6, 7, 9, 10, and 13–15, 10^{-7} M) for 24 h, and FN mRNA/18S ribosomal RNA (18S) levels were estimated. A, representative Northern blots of FN mRNA and 18S ribosomal RNA from VSMCs (total RNA, 20 µg) treated with Ang II and inhibitors. Northern blot hybridization was performed as described under "Experimental Procedures." B, relative FN mRNA levels. The levels of mRNA expression were measured as described in the legend to Fig. 1. Bars represent means of four independent experiments.](image)
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is known to be one of the targets of Ang II-mediated transcriptional activation via a PKC-dependent pathway (63).

Thus, we examined whether Ang II activates transcription through the AP-1 binding motif of the FN promoter. We transfected a CAT reporter gene containing the rat FN AP-1 binding motif linked upstream of the thymidine kinase minimum promoter (rFN/AP-1/TK-CAT) into VSMCs and stimulated VSMCs with Ang II (Fig. 9). Thirty-six hours after the DNA transfection, transfected VSMCs were incubated with Ang II receptor antagonists or signaling inhibitors for 30 min, followed by treatment with Ang II (10^{-7} M) for 18 h. Ang II increased CAT activity of rFN/AP-1/TK-CAT by 4.6-fold (Fig. 9, lanes 3 and 4). On the other hand, CAT activity of a CAT reporter gene containing only the thymidine kinase minimum promoter (TK-CAT) was not enhanced by Ang II (data not shown). We also examined what type of Ang II receptor(s) was involved in mediating the enhanced CAT activity through rFN/AP-1 in response to Ang II. Incubation of VSMCs with CV11974 (10^{-5} M) abolished the stimulatory effect of Ang II (10^{-7} M), whereas incubation of cells with PD123319 (10^{-5} M) did not affect the response to Ang II at all (Fig. 9, lanes 5 and 6). In addition, we examined a possible role of several signaling pathways in Ang II-mediated increase in CAT activity of rFN/AP-1/TK-CAT. Although incubation of VSMCs with a protein kinase A inhibitor HA-1004 (100 μg/ml) had no effect on Ang II-induced CAT activity, treatment with the PKC inhibitor calphostin C (10^{-6} M) or the PTK inhibitor genistein (10^{-4} M) significantly inhibited the Ang II-mediated increase in CAT activity of rFN/AP-1/TK-CAT (Fig. 9, lanes 7–9). These results indicate that activation of AP-1-binding site-containing promoter through PKC- and PTK-dependent pathways may play an important role in Ang II-induced transcription of the FN gene in VSMCs.

**Ang II-mediated Increase in AP-1 Binding Activity of FN Promoter**—Since the results of the DNA transfection study showed that Ang II stimulated transcription directed by rFN/AP-1 in VSMCs, we carried out EMSA using rFN/AP-1 as the probe to examine the effect of Ang II on nuclear binding activity to rFN/AP-1 (Fig. 10A). VSMCs were treated with Ang II (10^{-7} M) for 18 h, and nuclear extracts were prepared. Incubation of VSMC nuclear extracts with the 32P-labeled rFN/AP-1 produced a single shifted band, and Ang II significantly increased the intensity of this band (Fig. 10A, lanes 2 and 3). The Ang II-induced nuclear binding activity to rFN/AP-1 was specifically competed by the unlabeled rFN/AP-1 or double-stranded oligonucleotides containing the consensus binding site for AP-1 but not by those for AP-2 (Fig. 10A, lanes 4–7). Then VSMCs were incubated with Ang II receptor antagonists or signaling inhibitors for 30 min, followed by treatment with Ang II (10^{-7} M) for 18 h (Fig. 10B). Incubation of VSMCs with CV11974 (10^{-5} M) significantly decreased the stimulatory effect of Ang II (10^{-7} M), while incubation of cells with PD123319 (10^{-5} M) did not affect the response to Ang II (Fig. 10B, lanes 3 and 4). Furthermore, incubation of VSMCs with a PKC inhibitor calphostin C (10^{-6} M) or a PTK inhibitor genistein (10^{-4} M) significantly decreased the Ang II-mediated enhancement of nuclear binding activity to rFN/AP-1, although treatment with the protein kinase A inhibitor HA-1004 (100 μg/ml) had no effect on the Ang II-induced increase (Fig. 10B, lanes 5–7).

From the above results, rFN/AP-1 seems to exert a critical influence on Ang II-mediated promoter activity of the FN gene in VSMCs. Thus, to evaluate a functional significance of rFN/AP-1 in Ang II-mediated FN promoter activity, we assayed the effects of a mutation in the native FN promoter sequences (from −1908 to +136 of the transcriptional start site) that disrupted binding of nuclear factors to this element. Although the DNA-protein complex formed by the rFN/AP-1 element binding activity could be competed out by a nonlabeled rFN/AP-1 element, the rFN[mAP-1]-CAT element, which contained substitution mutations interrupting the AP-1 binding motif, did not compete out this binding (Fig. 11A, lanes 2–4). In transiently transfected VSMCs, the FN promoter (−1908 to +136)-CAT hybrid gene with this mutated rFN/AP-1 element (rFN/m[AP-1]-CAT) showed a significant decrease in Ang II-mediated promoter activity (Fig. 11B). These data suggest that an AT1 receptor-mediated stimulation of AP-1 binding activity may be involved in Ang II-induced transcription of the FN gene through PKC- and PTK-dependent mechanism in VSMCs.

**DISCUSSION**

Preceding studies have shown that the expression of aortic FN was increased relatively early not only in acute experimental hypertension models induced by Ang II infusion, deoxycorticosterone-salt treatment, or clipping of a renal artery (two-kidney, one-clip hypertension) but also in a chronic hypertension model such as spontaneously hypertensive rats (5, 51–53). These previous results raised a possibility that elevation of blood pressure and activation of the vascular RAS may be synergistically perceived by vascular cells as a signal that is transduced to increase the expression of aortic FN (64).

Ang II, which is a main effector of the RAS, regulates various physiological responses, including electrolyte and water bal-
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Ang II is produced both systemically and locally in the vessel wall by the actions of renin, which converts angiotensinogen into angiotensin I (Ang I), and ACE, which cleaves Ang I to form Ang II. Ang II has been shown to act directly, stimulate VSMC growth, and enhance the production of FN and collagen in VSMCs, mesangial cells, and renal fibroblasts (15, 47, 65). Thus, contribution of Ang II in regulating aortic FN expression in vivo was examined by several investigators using a hypertension model induced by continuous infusion of Ang II in Wistar and Sprague-Dawley rats (3, 12, 13). They found that the expression of aortic FN in this model was induced not by blood pressure elevation but mainly by Ang II through an AT1 receptor-pathway. In addition, a recent study examined the effects of AT1 receptor blockade on the gene expression of immediate early genes, including c-jun and c-fos, and FN after endothelial denudation of the left common carotid artery by balloon catheter in Sprague-Dawley rats (66). The results showed that blockade of AT1 receptor inhibited the induction of AP-1 and FN in rat injured artery. These results suggest that Ang II may play a pivotal role through an AT1 receptor pathway in the induction of FN during the process of vascular remodeling in vivo. Previous studies showed that Ang II acted directly and enhanced the production of FN in mesangial cells and renal fibroblasts and demonstrated that Ang II increased synthesis of FN proteins in mesangial cells and renal fibroblasts through induction of TGF-β expression by Ang II (47, 65). With respect to regulation of the FN gene in VSMCs, a recent study showed that Ang II increased expression of the FN gene in VSMCs (67), and FN converts VSMCs from a contractile to a synthetic phenotype and plays an important role in the proliferative response of VSMCs. However, Ang II-induced mitogenesis and cellular pro-
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In this study, we showed that Ang II enhanced the expression of FN mRNA in VSMCs in a concentration- and time-dependent manner, while Ang II did not increase mRNA expression of the RAS components. Treatment of hypertensive animals with ACE inhibitors appears to be more effective in causing regression of vascular hypertrophy than other treatments that lead to an equivalent decrease in blood pressure (69). In addition, the steady-state mRNA levels for aortic angiotensinogen, ACE, and AT1 receptor were significantly elevated in experimental hypertensive rats (70, 71), and vascular injury induced angiotensinogen and ACE gene expression in injured vessels (72, 73), thereby suggesting that vascular RAS plays an important role in the vascular hypertrophy in hypertension and in the myointimal proliferation in response to injury. Although the results of this study do not support one possible mechanism for the RAS positive feedback loop in VSMCs in the pathogenesis of vascular hypertrophy, the results may provide a rationale for the use of AT1 receptor antagonists, which is effective in inhibition of extracellular matrix production, in the management of vascular hypertrophy associated with hypertension.

The effects of Ang II on growth, gene expression, and FN production are mediated primarily by AT1 receptor. In this study, CV11974 completely blocked Ang II-mediated expression of the FN mRNA, whereas PD123319 did not interfere with the Ang II response. These results indicated that the AT1 receptor essentially mediates activation of the FN gene by Ang II in VSMCs. We further examined some mechanisms in response to Ang II stimulation in VSMCs. Binding of Ang II to AT1 receptor stimulates PI-PLC, increases protein tyrosine phosphorylation, and activates Ras and several protein kinases, such as PI3K, PKC, MAP kinase, Ca2+/calmodulin kinase, and S6K (24, 74). Previous studies propose an important role for PKC as an intracellular mediator of the effects of several hypertrophic growth stimuli (22), including mesangial cell FN production (75). In this study, we showed that Ang II-mediated expression of FN mRNA was inhibited by PKC inhibitors, suggesting that activation of PKC is involved in Ang II responses. In addition, recent studies in VSMCs suggest that many Ang II effects, such as MAP kinase activity, protein synthesis, and vascular contraction, require tyrosine phosphorylation (76), and we showed that Ang II-induced FN mRNA expression was blocked by PTK inhibitors in this study. Although AT1 receptor is a typical G protein-coupled receptor that lacks tyrosine kinase activity, these results suggest that a receptor-associated tyrosine kinase may be involved in Ang II signaling and propose a potential cross-talk between PKC and PTK in AT1 receptor-mediated activation of FN mRNA expression by Ang II in VSMCs. Furthermore, these results also suggest that Ang II-induced expression of FN mRNA is initiated by stimulation of PI-PLC through a PTX-insensitive G protein (probably G1) and is dependent on Ras, PI3K, S6K, and Ca2+/calmodulin kinase.

Comparison of the kinetics of FN mRNA expression by treatment with actinomycin D in the presence or absence of Ang II revealed that a Ang II-induced mRNA stabilization process played only a partial role in FN mRNA regulation. Since Ang II-induced increases in the mRNA levels of FN were blocked by pretreatment with actinomycin D but not by cycloheximide, it is unlikely that Ang II treatment induces de novo protein synthesis, whose products function to stabilize FN mRNA. Given that FN expression is mainly regulated at the transcriptional level in VSMCs, it is conceivable that constitutively expressed factors are activated by Ang II treatment through signaling pathways, which in turn induces the expression of FN mRNA transcriptionally.

Another finding of this study was that Ang II treatment increased transcriptional activity through the AP-1 binding site of the FN promoter (rFN/AP-1) and enhanced the binding
activity of nuclear factors to rFN/AP-1. In addition, the results of functional analysis by site-directed mutation of rFN/AP-1 site demonstrated that the rFN/AP-1 site plays a critical role in Ang II-induced transcriptional activity in the native FN promoter context. CV11974 significantly inhibited Ang II-mediated stimulation of rFN/AP-1 cis-element activity, whereas PD123319 did not interfere with the Ang II response, thereby confirming that AT1 receptor mainly mediates activation of AP-1 binding activity of the FN gene by Ang II in VSMCs. AP-1 was originally described as a transcriptional activator that is induced as part of the immediate early response to phorbol ester stimulation (77, 78) and was showed to regulate tran-

**Fig. 10.** Effect of Ang II on AP-1 binding activity of FN promoter and effect of Ang II receptor antagonists and signaling inhibitors on Ang II-induced activation of AP-1 binding activity. A, representative results of EMSA using nuclear extracts from VSMCs treated with Ang II. VSMCs were treated with Ang II (10^{-7} M) for 18 h, and EMSA of rFN/AP-1 was performed as described under “Experimental Procedures.” Nuclear extracts from VSMCs (lanes 2–7, 15 μg) were incubated with the probe (rFN/AP-1). In competition assay, 50- or 100-fold molar excess of the competitor DNA was added to the reaction mixture. Lane 1 contained no nuclear extract. The solid and open arrowheads indicate specific DNA-protein complex and free probe, respectively. B, representative results of EMSA using nuclear extracts from VSMCs treated with Ang II and inhibitors. VSMCs were preincubated for 30 min with CV11974 (lane 3, 10^{-5} M), PD123319 (lane 4, 10^{-5} M), HA-1004 (lane 5, 100 μg/ml), calphostin C (lane 6, 10^{-6} M), or genistein (lane 7, 10^{-4} M) and treated with Ang II (10^{-7} M) for 18 h. EMSA of rFN/AP-1 was performed as described under “Experimental Procedures.” Nuclear extracts from VSMCs (lanes 1–7, 15 μg) were incubated with the probe (rFN/AP-1). The solid and open arrowheads indicate specific DNA-protein complex and free probe, respectively.

**Fig. 11.** Effect of rFN/AP-1 mutation on nuclear factor binding and on promoter activity of the native FN gene. A, representative results of EMSA using nuclear extracts (15 μg) from VSMCs treated with Ang II (10^{-7} M) for 18 h. EMSA was performed as described under “Experimental Procedures.” Nuclear extracts from VSMCs (lanes 1–4, 15 μg) were incubated with the probe (rFN/AP-1). In competition assay, 100-fold molar excess of the competitor DNA was added to the reaction mixture. Lanes 5 and 6 show the effects of the native rFN/AP-1 and the mutated rFN/AP-1 (rFN/m[AP-1]) as cold competitors of the DNA-protein interaction, respectively. The solid and open arrowheads indicate specific DNA-protein complex and free probe, respectively. B, representative results of the CAT assay using cell extracts (80 μg) from VSMCs subjected to cyclic stretch. VSMCs were transfected with a plasmid rFN-CAT (3 μg) or rFN/m[AP-1]-CAT (3 μg). Thirty-six hours after the transfection, VSMCs were treated with Ang II (lanes 4–9, 10^{-7} M) for an additional 18 h, and promoter activity was estimated by CAT assay. CAT assay was performed as described under “Experimental Procedures.” Relative CAT activities were measured as described in the legend of Fig. 9. Bars represent means of four independent transfection experiments.
Angiotensin II Activates Vascular Cell Fibronectin

The data presented here demonstrate that Ang II activates expression of FN mRNA in VSMCs and that this effect is mediated mainly via a G_{i}-coupled AT1 receptor pathway, which involves a signaling mechanism dependent on PI-PLC, Ras, PKC, PTK, P38, S6K, and Ca^2+/calmodulin kinase in the Ang II-induced activation of the rFN/AP-1 site in VSMCs.

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Angiogenesis

Schniederich activity of genes through binding to AP-1 consensus motif in the promoter region in a PKC-dependent manner in VSMCs. In the present study, stimulation of rFN/AP-1 cis-element activity by Ang II was blocked by PTK inhibitors. A previous study using renal mesangial cells also showed that a cross-talk between PKC and PTK contributed to mitogenic signaling by endothelin-1 (80). Although it is suggested that a PTK-sensitive mechanism for recruiting new AP-1 complexes to DNA (e.g. PTK-sensitive c-fos induction by Ang II to form higher affinity AP-1 complexes), further experiments are necessary to determine the mechanism underlying the effects of PTK inhibitors on AP-1 DNA binding and to delineate a role of other protein kinases including P38, S6K, and Ca^2+/calmodulin kinase in the Ang II-induced activation of the rFN/AP-1 site in VSMCs.

The data presented here demonstrate that Ang II activates expression of FN mRNA in VSMCs and that this effect is mediated mainly via a G_{i}-coupled AT1 receptor pathway, which involves a signaling mechanism dependent on PI-PLC, Ras, PKC, PTK, P38, S6K, and Ca^2+/calmodulin kinase. In particular, Ang II appears to increase transcription of the FN gene through AP-1 binding motif of the FN promoter (rFN/AP-1), and Ang II may regulate the activity of rFN/AP-1 element by an AT1 receptor-mediated signaling pathway involving PKC and PTK. Ang II-induced activation of FN gene expression in VSMCs may play a pathophysiological role in the abnormal growth of VSMCs observed in cardiovascular diseases, thereby providing a rationale for the use of AT1 receptor antagonists in the management of vascular hypertrophy in response to hypertension.