Angiotensin II Induces Interleukin-6 Transcription in Vascular Smooth Muscle Cells Through Pleiotropic Activation of Nuclear Factor-κB Transcription Factors

Youqi Han, Marshall S. Runge, Allan R. Brasier

Abstract—Interleukin-6 (IL-6) is a multifunctional cytokine expressed by angiotensin II (Ang II)-stimulated vascular smooth muscle cells (VSMCs) that functions as an autocrine growth factor. In this study, we analyze the mechanism for Ang II-inducible IL-6 expression in quiescent rat VSMCs. Stimulation with the Ang II agonist Sar² Ang II (100 nmol/L) induced transcriptional expression of IL-6 mRNA transcripts of 1.8 and 2.4 kb. In transient transfection assays of IL-6 promoter/luciferase reporter plasmids, Sar² Ang II treatment induced IL-6 transcription in a manner completely dependent on the nuclear factor-κB (NF-κB) motif. Sar² Ang II induced cytoplasmic-to-nuclear translocation of the NF-κB subunits Rel A and NF-κB1 with parallel changes in DNA-binding activity in a biphasic manner, which produced an early peak at 15 minutes followed by a nadir 1 to 6 hours later and a later peak at 24 hours. The early phase of NF-κB translocation was dependent on weak simultaneous proteolysis of the Iκβα and β inhibitors, whereas later translocation was associated with enhanced processing of the p105 precursor into the mature 50-kDa NF-κB1 form. Pretreatment with a potent inhibitor of Iκβα proteolysis, TPCK, completely blocked Sar² Ang II-induced NF-κB activation and induction of endogenous IL-6 gene expression, which indicated the essential role of NF-κB in mediating IL-6 expression. We conclude that Ang II is a pleiotropic regulator of the NF-κB transcription factor family and may be responsible for activating the expression of cytokine gene networks in VSMCs. (Circ Res. 1999;84:695-703.)

Key Words: nuclear factor-κB ■ renin-angiotensin system ■ angiotensin II ■ cytokine

Angiotensin II (Ang II), the effector peptide of the renin-angiotensin system (RAS), is formed as a result of sequential proteolysis of the angiotensinogen precursor. Ang II is one of the most potent vasoressors known when bound to its G-protein–coupled receptor, the type 1 angiotensin receptor (AT₁). In addition, Ang II promotes long-term changes in vascular smooth muscle cell (VSMC) function by its ability to induce cellular hypertrophy, extracellular matrix production, and early gene expression. In this manner, Ang II regulates VSMC tone under normal conditions and mediates VSMC hypertrophy in the pathology of chronic hypertension and atherosclerosis.

Ang II activates genetic networks in various cell types by influencing the activity and expression of nuclear regulatory proteins, including immediate–early transcription factors (activator protein 1) and tyrosine-receptor–coupled transcription factors (signal transducers and activators of transcription) in VSMCs, early growth response-1 in cardiomyocytes, and activator protein 1 in adrenal glomerulosa cells. In addition, we recently reported that Ang II induces the activity of the nuclear factor-κB (NF-κB) transcription factor in hepatocytes. NF-κB is a family of cytoplasmic transcription factors composed of homodimeric and heterodimeric complexes of the potent transactivating subunits Rel A and c-Rel and the inert DNA-binding subunit NF-κB1. Rel A and c-Rel complexes are actively sequestered in the cytoplasm by association with the inhibitory proteins (IκB), which inactivate the transcriptional activator subunits by binding and masking their nuclear translocation domains. After activation, NF-κB translocates into the nucleus, where it binds and induces expression of cytokine and acute-phase response genes. Although Ang II increases NF-κB transcriptional activity in hepatocytes, the mechanism (and subunits affected) has not been determined, nor has it been established whether this phenomenon occurs in other cell types.

Interleukin-6 (IL-6) is a multifunctional cytokine that mediates B lymphocyte proliferation/induction of antibody synthesis and mediates the hepatic acute-phase response. In the vessel wall, locally secreted IL-6 also plays an important role as a VSMC growth factor through a paracrine mechanism, which involves production of PDGF. IL-6 is encoded by a highly inducible promoter that is a target for tissue-specific and cytokine-inducible transcription fac-

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tors.16,22 Both human and rat VSMCs inductively secrete IL-6 in response to the inflammatory cytokines IL-1 and tumor necrosis factor (TNF).17 Bacterial cell wall–derived lipopolysaccharide,17 platelet-derived thrombin,21 and Ang II.19 However, the mechanism for transcriptional activation of IL-6 in VSMCs has not been determined.

In this experiment, we examined the mechanisms for Ang II–induced activation of IL-6 gene expression in cultured rat VSMCs. Treatment with Sar1 Ang II (100 mmol/L) rapidly activated expression of IL-6 mRNA transcripts in a transcription-dependent manner and was completely blocked by actinomycin D. In gene transfer studies with plasmids that contained the IL-6 promoter–driven luciferase reporter gene, the NF-κB binding site was essential for this effect. Site mutations of the NF-κB binding site blocked basal and Ang II–activated IL-6 luciferase reporter activity. Ang II induced nuclear translocation of the Rel A·NF-κB1 isoforms in VSMCs concomitant with 1κBα and NF-κB proteolysis. In addition, chronic Ang II stimulation induced processing of the mature form of NF-κB1 (50-kDa subunit) from its 105-kDa precursor, p105. These data implicate Ang II as an activator of NF-κB translocation and cytokine expression in VSMCs.

Materials and Methods

Cell Culture and Treatment

Primary cultures of rat VSMCs that expressed coupled AT1 receptors were isolated by enzymatic dispersion and grown in DMEM as described.23 Experiments were performed with 90% confluent cells grown on culture plates and used before passage 25. Before stimulation, cells were cultured for 72 hours in serum-free medium. In indicated locations, Sar1 Ang II (Sigma) was added at a concentration of 100 mmol/L or recombinant human TNF-α (CalBiochem) was added at a concentration of 1.1 mmol/L. TCPK was from Calbiochem and used at a final concentration of 50 μmol/L.

RNA Extraction and IL-6 Transcript Analysis

VSMC RNA was extracted with acid guanidine HCl/phenol extraction (Trizol). For Northern blots, 30 μg of total cellular RNA was fractionated on a MOPS/formaldehyde agarose gel as described.19 The IL-6 cDNA probe was generated by reverse transcription–polymerase chain reaction (RT-PCR).22,24 The upstream primer for the IL-6 gene in the pGEM plasmid (a gift from S. Akira, Osaka University, Japan) as a template with the upstream primer 5′-CAAGAGACTTTCCAGGCAGTTGC-3′ (hybridizing to nt 81 to 102 of the rat mRNA) and the antisense primer 5′-ATCAAATGTTGTAGATTTCCAATGAAT-3′ (hybridizing to nt 694 to 670 of the rat mRNA) was subcloned into the pCR2.1 plasmid (Invitrogen) giving a probe that hybridized to nucleotides [nt] 81 to 102 of the rat mRNA. The rat IL-6 cDNA probe was generated by reverse transcription–polymerase chain reaction (RT-PCR).22,24 The upstream primer for the IL-6 gene in the pGEM plasmid (a gift from S. Akira, Osaka University, Japan) as a template with the upstream primer 5′-CAAGAGACTTTCCAGGCAGTTGC-3′ (hybridizing to nt 81 to 102 of the rat mRNA) and the antisense primer 5′-ATCAAATGTTGTAGATTTCCAATGAAT-3′ (hybridizing to nt 694 to 670 of the rat mRNA). The 590-bp cDNA was subcloned into the pCR2.1 plasmid (Invitrogen) and sequenced to verify authenticity.

Gel Mobility Shift and Microaffinity Purification Assays

Nuclear extracts (25 μg) were incubated with 40,000 cpm of 32P-labeled duplex NF-κB binding site, 5′-GATCCACACAGTGTTGATTTCCCACGTACAG3′, and 2 μg of poly(dA-dT) in a buffer that contained 8% glycerol, 100 mmol/L NaCl, 5 mmol/L MgCl2, 5 mmol/L DTT, and 0.1 mg/mL PMSF in a final volume of 20 μL for 15 minutes at room temperature.25 The complexes were fractionated on 6% native polyacrylamide gels run in TBE buffer (25 mmol/L Tris, 25 mmol/L boric acid, 0.5 mm/L EDTA), dried, and exposed to Kodak X-AR film at −70°C.

Microaffinity purification of NF-κB binding proteins was performed with chymotactically synthesized oligonucleotides that contained 5′-biotin (Bt) on a flexible linker (Genosys). Sequences of duplex oligonucleotides are

WT: GATCCACATCTGGAATATCCGTTTCTGCAATTTCTCTA GTATGCAATGTTAGGACCTGAATGTTTCTA GTAACCTACTGCTGCTGCTGAAGTACCAATGTTTCTA

ΔκB: GATCCACATCTGGAATATCCGTTTCTGCAATTTCTCTA GTATGCAATGTTAGGACCTGAATGTTTCTA GTAACCTACTGCTGCTGCTGAAGTACCAATGTTTCTA

Nκm: GATCCACATCTGGAATATCCGTTTCTGCAATTTCTCTA GTAACCTACTGCTGCTGCTGAAGTACCAATGTTTCTA

Forty picomoles of Bt oligonucleotide duplex was incubated with 800 μg of nuclear extracts from VSMCs either untreated or stimulated with Ang II for the indicated time in the presence of 10 μg of poly(dA-dT) in a 1-mL volume of binding buffer that contained 12.5 mmol/L HEPES (pH 7.9), 4 mmol/L MgCl2, 60 mmol/L KCl, 1 mmol/L EDTA, 1 mmol/L DTT, 6% glycerol, and protease inhibitor cocktail for 30 minutes at 4°C. The bound proteins were collected by the addition of 40 μL of a 50% (vol/vol) slurry of

Technique of PCR “SOEing”26 with the mutagenic primers (mutations underlined): Δκ α sense 5′-ATCAAATGTTGTAGATTTCCAATGAAT-3′, and Δκ antisense 5′-ATTGAAGACTCTTAGTAACATACGGATTTCATTGATAAAAATC-3′. The BamH1/HindIII fragment that contained the IL-6 promoter that mutated in the NF-κB site was then subcloned into the BamH1/HindIII-digested pUC reporter vector. Plasmids were purified by ion exchange chromatography (Qiagen) and sequenced to verify authenticity.

Transient transfections were performed by electroporation. Resuspended VSMCs (1 × 106) were incubated for 15 minutes with 60 μg of the indicated IL-6/LUC and 20 μg of internal control SV40 early region promoter/alkaline phosphatase (SV2APAP) reporter plasmids on ice in a final volume of 0.5 mL of serum-free medium per cuvette, and subjected to a 35- to 42.1-ms pulse of 300 V and 950 mF with a Gene Pulser transfection apparatus (Bio-RAD). The transfection mixtures were incubated for another 15 minutes at room temperature before being plated onto 60-mm culture dishes. Cells were then serum-starved for 72 hours before stimulation with 100 mmol/L Sar1 Ang II for 5 hours. In the luciferase assays, transfected cells were washed with PBS 3 times and lysed on the plate by the addition of 200 μL of lysis buffer (25 mmol/L Tris-phosphate, pH 7.8; 2 mmol/L DTT; 2 mmol/L trans-1,2-diaminocyclohexane-N,N,N′,N′-tetraacetic acid; 10% glycerol; 1% Triton X-100), and cytoplasmic lysates (100 μL) were assayed for luciferase and alkaline phosphatase reporter activities.27 Luciferase activity was determined by subtraction of machine background and normalization of each plate to alkaline phosphatase activity.

Western Immunoblots and Immunoprecipitation

Two to three hundred micrograms of cytoplasmic or nuclear extracts were prepared as described28 and fractionated on SDS-PAGE, transferred to polyvinylidene difluoride (PVDF) membrane, and subjected to Western immunoblots. Primary antibodies were rabbit polyclonal anti-IκB, IκBβ, Rel A, c-Rel, and NF-κB1 antibodies. Immune complexes were detected by binding donkey anti–rabbit IgG conjugated to horseradish peroxidase followed by reaction in the enhanced chemiluminescence assay (ECL, Amersham International). The specificity of each antibody has been documented by the ability to identify the correct molecular weight product and whether it is blocked after preadsorption with the appropriate peptide.29
strepavidin-agarose beads (Pierce) and incubation for 2 more hours at 4°C. After the binding proteins were washed 4 times with binding buffer and transferred to fresh microtubes, they were eluted in a 100-μL volume a of mixture of Laemmli buffer and 500 nmol/L KCl and fractionated in SDS-PAGE for the Western immunoblotting probed with anti-Rel A or anti-NF-κB1 antibodies. For competition, a 10-fold excess of nonbiotinylated oligonucleotides was included in the initial binding reaction.

**Results**

**Ang II Activates Endogenous IL-6 Gene Expression in Quiescent VSMCs**

Both human saphenous vein and rat aortic VSMCs have been previously shown to inducibly express IL-6 mRNA and protein.17,19,21 To confirm that Ang II induces expression of IL-6, cell culture supernatants of quiescent VSMCs stimulated for 3 hours with Sar1 Ang II were assayed by ELISA. During this period, untreated control cultures produced 100±80 ng/mL IL-6, whereas cells stimulated with 100 nmol/L Sar1 Ang II accumulated 850±120 ng/mL, and those stimulated with 1 μmol/L accumulated 970±120 ng/mL (mean±SD, n=4 experiments, P<0.05 for each treatment compared with control, Student’s t test). To examine whether IL-6 secretion occurred as a consequence of increased transcript abundance, a preliminary time course with RT-PCR was performed that revealed a maximal IL-6 expression at 1 hour (not shown). To examine the magnitude of induction and determine the transcript sizes produced, Northern blot analysis was performed on VSMCs stimulated for 1 hour (Figure 1). Relative to control, Sar1 Ang II rapidly induced a 3±1-fold change in steady state IL-6 abundance (mean±SD, n=3, P<0.05). To determine whether IL-6 induction was transcriptional, actinomycin D–pretreated cells were stimulated with Ang II (Figure 1, right panel). Actinomycin D completely blocked IL-6 transcript accumulation. These data indicate that Ang II induces a rapid but transient transcriptional induction of IL-6 gene expression in rat VSMCs.17

To isolate the component of IL-6 induction that was due to changes in IL-6 transcription, −303/+22-bp of the IL-6 promoter/luciferase reporter (−303/+22 IL-6/LUC) gene was transfected into rat VSMCs and subsequently stimulated for 5 hours with either vehicle, Sar1 Ang II (100 nmol/L), or TNF-α (1.1 nmol/L). As shown in Figure 2, normalized luciferase activity (IL-6/luciferase to internal control alkaline phosphatase) after Sar1 Ang II stimulation increased 1.7±0.3-fold relative to control (mean±SD, n=3, P<0.05, Student t test), whereas after stimulation with TNF-α increased 2.3±0.5-fold (mean±range, n=2). The role of NF-κB in Ang II–induced activation of −303/+22 IL-6/LUC reporter activity was assayed by introduction of a site mutation of the NF-κB binding site into the −303/+22 IL-6/LUC reporter (−303/+22 IL-6 Δκ/LUC). After stimulation, the −303/+22 IL-6 Δκ/LUC was inert to stimulation by both agents (Ang II and TNF-α). To determine whether the NF-κB binding site is sufficient to confer Ang II inducibility, we analyzed an NF-κB response element in a promoter context where basal and inducible activity could be measured. In VSMCs, the IL-8 promoter is also Ang II inducible; deletion of the NF-κB binding site completely blocked Ang II induction (Figure 2B). We have previously demonstrated that the NF-κB binding site is an Ang II–inducible enhancer that can confer Ang II induction onto a heterologous promoter.11 Although the unlikely possibility of cryptic Ang II–inducible elements in the IL-6 promoter cannot be completely excluded, we interpret these data to indicate that the NF-κB binding site is necessary and sufficient for Ang II–mediated transcription.

**Ang II Stimulates NF-κB DNA-Binding Activity in a Biphasic Manner**

To determine whether Ang II stimulates changes in NF-κB DNA binding activity in VSMCs, NF-κB binding was assayed in nuclear extracts from cells stimulated for various times with Sar1 Ang II (Figure 3A). In unstimulated nuclei, a single binding activity was seen (complex C2), and on longer exposures, a faint binding of a slower migrating complex, C1, could be seen (not shown). In contrast, within 0.25 hours after Ang II treatment, the second complex, C1, and to a lesser extent C2 were both induced. Although C1 binding was detectable at all times after Ang II treatment (0.25 to 24 hours, data not shown).

**Figure 1.** Ang II induces IL-6 gene expression in VSMCs. Northern blot. Left, Serum-starved VSMCs were stimulated with Sar1 Ang II (100 nmol/L) or recombinant TNF-α (1.1 nmol/L) for 1 hour. Shown is a representative blot performed in 3 separate experiments in which total cellular RNA was fractionated. Top, Autoradiographic signal after hybridization for IL-6. Bottom, After hybridization with 18S probe. Ang II and TNF induce 2 IL-6 transcripts of 1.8 and 2.4 kb in size. Right, Control or actinomycin D–pretreated (5 μg/mL; 5 minutes) VSMCs were stimulated with vehicle or Sar1 Ang II for 1 hour. Shown is a representative Northern blot. Top, Autoradiographic signal after IL-6 hybridization; bottom, after 18S hybridization. Experiment was repeated twice with identical results.
hours), its abundance was maximal at 0.25 hours, fell at 1 hour, and reaccumulated at 24 hours. Compared with control, aggregate (C1+C2) NF-κB binding activity was increased 3.2±1.6-fold at 0.25 hours and 2.8±1.4-fold at 24 hours (mean±SD, n=3, P<0.05 for each time point, Student t test). To exclude potential nonspecific effects for the NF-κB induction at 24 hours, control experiments were performed. Cells starved for 72 hours were harvested, and NF-κB binding was compared with cells starved an additional 24 hours in the absence or presence of Sar1 Ang II (Figure 3B). As a positive control, cells maintained in serum-free medium for 24 hours were subsequently stimulated with TNF-α. A strong induction of NF-κB binding was observed only in the 24-hour Ang II- and 15-minute TNF-α–treated plates, which indicates NF-κB binding requires the presence of hormone.

Figure 2. Ang II activates IL-6 gene transcription in VSMCs. A, Transient transfections of IL-6 promoter in VSMCs. –303/+22 IL-6/LUC plasmid and internal control alkaline phosphatase reporter plasmids were transiently transfected into VSMCs and serum starved. After Ang II stimulation, reporter gene activity was extracted and measured. IL-6/KLUC contains a site mutation of the NF-κB binding site (see Materials and Methods). Shown is the mean±SD of normalized (luciferase/alkaline phosphatase) reporter activity independently transfected triplicate plates from a representative experiment. B, NF-κB binding site confers Ang II inducibility. The NF-κB site upstream of the inert IL-8 TATA box in the luciferase reporter was tested for Ang II inducibility. Ang II induction (3±0.2-fold; n=3) occurred only in the presence of NF-κB.

Figure 3. Ang II induces NF-κB binding in VSMCs. A, Ang II induced NF-κB DNA-binding activity in a time-dependent manner in VSMCs. Cultured VSMCs were either untreated or stimulated with 100 nmol/L Ang II for the indicated time (in hours, top) before extraction of nuclear proteins. Shown is an autoradiogram of gel mobility shift assay with 25 μg of nuclear proteins binding to NF-κB oligonucleotide. Migration of various binding complexes (C1, C2) is shown on the left. C1 and to a lesser extent C2 exhibited a biphasic induction pattern, with an early binding peak at 15 minutes and later induction after 2 hours of Ang II treatment. B, Late induction of NF-κB binding is hormone dependent. VSMCs were serum starved for 72 hours. A control plate was harvested at that time (“0” hours). The remainder were maintained an additional 24 hours in serum-free medium in the absence (−) or presence (+) of Sar1 Ang II. For the plate that received TNF-α, stimulation was performed for only 15 minutes before harvest. The C1 plus C2 binding induction occurs only in the presence of hormone. C, Competition in gel shift assay. Autoradiogram of mobility shift assay. Nuclear proteins from cultured VSMCs (25 μg) were stimulated for 15 minutes with 100 nmol/L Ang II bound to radiolabeled NF-κB oligonucleotide in the absence or presence of 100-fold molar excess of cold oligonucleotide competitor. The WT NF-κB binding site contained the core sequence 5′-GGTTGGATTTCCCA-CC-3′; ΔκB1 contained the sequence GGTGTGATTTCCGATAC; ΔκB2 contained the sequence GGTGTGATTTCAAC [mutations in the NF-κB binding site are underlined]. The constitutive C2 complex and the Ang II–inducible C1 complex both compete with the wild type but not the NF-κB site mutations, which indicates they are sequence-specific NF-κB binding complexes.

Binding specificity for the NF-κB complexes was demonstrated with competition in gel shift assay in which unlabeled oligonucleotides that represented the wild-type NF-κB site (WT) and 2 unrelated mutations (ΔκB1, ΔκB2), neither of which bind to NF-κB, were included at a molar excess to
Sequence specificity of Rel A and NF-κB1 binding activities was determined in the experiment shown in Figure 4B. In this competition assay, the Bt-duplex NF-κB binding step was done in the presence of 10-fold excess of nonbiotinylated duplex WT, NF-κB (ΔκB), or a non-NF-κB mutation (NκM). Both Rel A and NF-κB1 binding were competed for by the addition of the WT or the NκM mutations but not the NF-κB (ΔκB), which indicates NF-κB binding specificity.

Ang II Increases Nuclear Translocation of the 65-kDa Rel A Isoform
To confirm that the nuclear abundance of Rel A is influenced by Ang II treatment, which accounts in part for its changes in DNA-binding activity, Western immunoblots to measure Rel A abundance in cytoplasmic and sucrose–purified nuclei were performed (Figure 5, top panel). Sixty-five kilodaltons of Rel A was predominately cytoplasmic in unstimulated cells, although a small fraction was nuclear. After Ang II treatment, cytoplasmic Rel A did not change significantly. However, nuclear Rel A rapidly increased within 0.25 hours; compared with control levels, Rel A abundance increased 3.0±1-fold (mean±SD, n=3, P<0.05). This indicated that although a statistically significant change in nuclear Rel A was detected, the fraction of Rel A translocated into the nucleus was only a minor fraction of the total cytoplasmic reservoir.12,28 More importantly, the relative steady-state changes in Rel A observed in the nuclear compartment after Ang II stimulation exactly paralleled the changes in NF-κB binding (Figures 3A and 4A).

Ang II Increases Cytoplasmic Proteolysis of the c-Rel Isoform
In epithelial and liver cells,15 c-Rel is a ∼78-kDa cytoplasmic protein that is regulated by a nuclear translocation mechanism in parallel with Rel A28 and inducibly binds with high affinity to NF-κB regulatory elements.15,28 Western immunoblots of cytoplasmic and nuclear fractions of Ang II-stimulated VSMCs were also analyzed for changes in c-Rel abundance (Figure 5). We were surprised to observe that c-Rel disappears rapidly from both the cytoplasmic and nuclear compartments. These data indicate that Ang II induces a paradoxical disappearance of c-Rel in VSMCs.

Ang II-Induced Transient Proteolysis of the IκBα and β Isoforms
The IκB isoforms α and β are the predominant inhibitors responsible for Rel A inactivation expressed in VSMCs.12 To determine whether Ang II stimulation influences steady-state abundance of IκBα and/or β, Western immunoblots were performed on Sar1 Ang II–stimulated cytoplasmic extracts. Thirty-seven kilodaltons of IκBα and 46-kDa of IκBβ were detected in control cytosolic extracts (Figure 6A). For IκBα, 0.25 hours after Ang II treatment, proteolysis was consistently but weakly discernible. In 3 separate experiments, IκBα abundance was 60±25% of control values at this time point (P<0.05, n=3, Student t test). IκBβ was also proteolyzed at 0.25 hours to 70% (range 50% to 90%, n=2) but not thereafter.
Compared with our experience of the effect of TNF-α on NF-κB translocation and IκB proteolysis in other cell systems, the effects of Sar1 Ang II were small. To determine whether there are intrinsic differences between NF-κB inducibility and that of the hormone, the effects of both hormones were analyzed (Figure 6B). At maximal doses of each ligand, TNF-α produced a greater degree of NF-κB binding, Rel A translocation, and IκBα proteolysis than produced by Sar1 Ang II. These data indicate that TNF-α is a more potent activator of NF-κB (and IκBα proteolysis) than Sar1 Ang II.

Late-Phase NF-κB Induction: Ang II Increases 50-kDa NF-κB1 Translocation and Processing From the p105 Precursor

NF-κB1 p50 is the primary DNA binding subunit of NF-κB and is a processed form of the cytoplasmic 105-kDa precursor.30 In Figure 7A, immunoblots of cytoplasmic and sucrose cushion–purified nuclear fractions from Sar1 Ang II–stimulated VSMCs were incubated with NH2-terminal anti–NF-κB1 antibody to detect the precursor and mature forms. In control cells, the majority of NF-κB1 was cytoplasmic, which includes discrete 105-kDa, 84-kDa, and the mature 50-kDa forms. Similar to Rel A and c-Rel, the majority of the protein was cytoplasmic, with little of the 50-kDa form being nuclear. After Ang II treatment, the abundance of 50-kDa NF-κB1 increased monotonically over 24 hours. In addition, we note that the NF-κB1 p105 and p84 precursors also accumulated in the nucleus.

The later increase in nuclear NF-κB1 p50 may be the result of enhanced processing from the p105 precursor as suggested by the cytoplasmic accumulation of 60-kDa intermediate NF-κB1 isoforms (large arrow, Figure 7A). To help establish the latter phenomenon, immunoblots of whole-cell lysates from the same Ang II treatment times were prepared and
stained with NF-κB1 antibody (Figure 7B). The whole-cell abundance of NF-κB1 p50 increased from 1 to 24 hours in parallel with the latter phase of nuclear NF-κB1. These data indicate Ang II perhaps has a biphasic effect on NF-κB1, initially by promoting its translocation and secondarily by increasing its processing from the p105 precursor.

**Protease Inhibitors That Block NF-κB Activation Also Block Ang II-Induced Endogenous IL-6 Gene Expression**

Taken together, our data indicated that proteolysis of IκB is required for the initial phase of Rel A NF-κB1 translocation after Ang II stimulation in VSMCs. To provide additional information for this mechanism and determine the relationship of Rel A · NF-κB1 translocation to endogenous IL-6 gene expression, we pretreated VSMCs with the IκB proteolysis inhibitor TPCK before Ang II stimulation. A gel mobility shift assay of control and Ang II-stimulated VSMCs in the absence or presence of the protease inhibitor TPCK (50 μmol/L) and PMSF as a control is shown in Figure 8A. Pretreatment with TPCK but not PMSF completely blocked the appearance of inducible NF-κB binding. Parallel-treated plates of VSMCs were subjected to RT-PCR to detect changes in endogenous IL-6 gene transcription (Figure 8B). IL-6 mRNA was induced in the Ang II-treated cells but not in Ang II-treated cells pretreated with TPCK. These data indicate that Rel A · NF-κB1 translocation is required for endogenous IL-6 gene expression after Ang II stimulation.

**Discussion**

Ang II regulates VSMC growth and gene expression through signal transduction cascades coupled to the AT1. Several reports indicate that the pathological changes in the vessel wall observed as a result of atherosclerosis and hypertensive vascular injury are inflammatory in nature. Injured VSMCs phenotypically switch from a contractile to a synthetic phenotype, which produces cytokines and growth factors. In this way, VSMCs assume an endocrine function in the vessel wall. Although VSMCs are highly responsive to other NF-κB activators such as IL-1, TNF, and unidentified serum components, we report in this study for the first time that Ang II induces NF-κB binding in parallel with IL-6 gene transcription, (2) site mutations of the NF-κB binding site block both basal and inducible IL-6 expression, (3) inhibition of NF-κB translocation by the proteasome inhibitor blocks endogenous IL-6 expression, and (4) NF-κB is an Ang II-inducible cis regulatory element (Figure 2 and Reference 11). These data provide an important link among the activated RAS, inflammatory response, and VSMC hypertrophy in the vessel wall.

NF-κB in both human and rat VSMCs is an inducible complex composed of Rel A · NF-κB1 (p50) heterodimers whose increased nuclear binding is mediated through a process that involves cytoplasmic-nuclear translocation (this study and 12). After Ang II treatment of VSMCs, we observe a biphasic pattern of activation of Rel A · NF-κB1 heterodimers, with a rapid early activation (0.25 hours), followed by a variable nadir (1 to 2 hours), and a late activation (6 to 24 hours). The early activation is simultaneous with a weak degree of IκBα and β proteolysis in the cytoplasm. The late activation phase primarily occurs as a result of enhanced processing of the NF-κB1 p105 precursor into the mature 50-kDa nuclear DNA-binding subunit. Both IκB proteolysis and p105 processing are mediated by the ubiquitin-proteasome pathway; together these observations indicate that Ang II induces intracellular proteolysis of various NF-κB isoforms through the 26S proteasome. Taken together, these data implicate a pleiotropic effect of Ang II on members of the NF-κB–IκB family. The early phase of Rel A · NF-κB1 translocation is associated with IL-6 gene expression (measured by Northern blot) and transcriptional activity (measured by transfection assay). However, after 24 hours, IL-6 mRNA...
contains repeated domains homologous to erythrocyte ankyrin and functions as an IκB-like molecule retaining Rel A and/or c-Rel in the cytoplasm.34 In various cell types, p105 is inducibly phosphorylated and proteolyzed through a mid-molecule glycine-rich region.35 After p105 processing, sequestered Rel A and NF-κB1 DNA-binding subunits are released for subsequent translocation into the cell nucleus. Our data indicate that NF-κB1p105 is not only inducibly processed but also undergoes nuclear translocation 6 hours after Ang II stimulation. We do not believe that this is due to cytoplasmic contamination of our nuclear preparations because our highly purified nuclei (prepared by sucrose cushion centrifugation) are devoid of cytoplasmic markers.28 Cytoplasmic-nuclear translocation of p105 has not been described in VSMCs. However, in Epstein barr virus-infected B lymphocytes, NF-κB1 p105 was observed to be nuclear and processed within the nucleus.39 One well-described activator of NF-κB processing is the potent protein kinase C agonist phorbol 12-myristate 13-acetate.35 It is noteworthy that Ang II is a potent activator of protein kinase C; we speculate that this may be a common pathway used in Ang II induction of NF-κB1 processing.

c-Rel is a transactivating protein found in cytokine-inducible NF-κB complexes in epithelial cells15 whose nuclear-cytoplasmic translocation is also controlled by complex with IκBα and IκBβ.36–38 A surprising observation in this study is that cytoplasmic c-Rel is proteolyzed after Ang II treatment and not translocated into the nucleus. c-Rel has been implicated in growth arrest at the G1/S-phase boundary39; we speculate its proteolysis may be required for Ang II-induced activation of quiescent VSMCs.

Within the context of the atheromatous arterial wall, a variety of NF-κB activators have been identified, including cytokines, IL-1, TNF-α, thrombin, and oxidized LDL,12,32,40 that act on endothelial, macrophage, T-lymphocyte, and smooth muscle cells to express genes important in the fibroproliferative response, including vascular cell adhesion molecule-1 tissue factor, colony stimulating factors, and chemotactic cytokines. These factors function in a paracrine way on resident cells to facilitate intercellular interactions and amplify the inflammatory response. We propose that Ang II, the central effector molecule of the activated RAS, also participates in the phenotypic switch of VSMCs into cells with endocrine functions, thereby producing IL-6 and perhaps other NF-κB-regulated genes. Within the vessel wall, IL-6 is likely to play several important roles. Although IL-6 is an activator of acute-phase reactant expression in liver cells and antibody production in B lymphocytes, IL-6 functions as an autocrine growth factor in VSMCs through its ability to influence PDGF expression.20 Additionally, IL-6 may also play an important chemotactic role in stimulating progenitor cells that have transmigrated through the activated endothelium.16

In summary, Ang II the octapeptide effector of the activated RAS, mediates IL-6 expression in VSMCs through pleiotropic effects on the NF-κB family of transcriptional regulators. These observations further underscore the link between the activated RAS and inflammatory cytokine expression or activation in the vascular wall.
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