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

Youqi Han, Marschall 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 Sar^1 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, Sar^1 Ang II treatment induced IL-6 transcription in a manner completely dependent on the nuclear factor-κB (NF-κB) motif. Sar^1 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 IκBα 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 IκBα proteolysis, TPCK, completely blocked Sar^1 Ang IIAng 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

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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 vasopressors known when bound to its G-protein–coupled receptor, the type 1 angiotensin receptor (AT_1).[1,2] In addition, Ang II promotes long-term changes in vascular smooth muscle cell (VSMC) function by its ability to induce cellular hypertrophy,[3] extracellular matrix production,[4] and early gene expression.[5–7] 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)[5,7] and tyrosine-receptor–coupled transcription factors (signal transducers and activators of transcription)[8] in VSMCs, early growth response-1 in cardiomyocytes,[9] and activator protein 1 in adrenal glomerulsa cells.[10] In addition, we recently reported that Ang II induces the activity of the nuclear factor-κB (NF-κB) transcription factor in hepatocytes.[11] 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.[12–14] 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.[11,13,15] 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.[16,17] 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.[18–20] 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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From the Department of Internal Medicine (Y.H.), Sealy Center for Molecular Cardiology (M.S.R.), and Sealy Center for Molecular Science (A.R.B.), University of Texas Medical Branch, Galveston, Tex.

Correspondence to Allan R. Brasier, Division of Endocrinology, MRB 8.138, University of Texas Medical Branch, 301 University Blvd, Galveston, TX 77555-1060. E-mail arbrasie@utmb.edu

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tors.[16,22] Both human and rat VSMCs inducibly 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 Sar[1] Ang II (100 nmol/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 IκBα and β 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 AT_1 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, Sar[1] Ang II (Sigma) was added at a concentration of 100 nmol/L or recombinant human TNF-α (CalBiochem) was added at a concentration of 1.1 nmol/L. TPCK 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 (TelTex). For Northern blots, 30 μg of total cellular RNA was fractionated on a MOPS/formaldehyde agarose gel as described.[15] The IL-6 cDNA probe was generated by reverse transcription–polymerase chain reaction (RT-PCR).[22,24] The upstream primer for rat IL-6 was 5′-CAAGAGACTTCCAGCCAGTTGC-3′ (hybridizing to nucleotides [nt] 81 to 102 of the rat mRNA) and the downstream primer was 5′-TTGCCGAGTAGACCTCATAGTGACC-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 confirm authenticity. The cDNA probe was produced in the PCR with the rat IL-6 downstream primer and [α^{32}P] dATP. After the blots were hybridized, they were washed and exposed to a Molecular Dynamics PhosphorImager cassette for quantification.

Plasmid Construction and Transfection

The IL-6 promoter/luciferase reporter was produced with the use of the PCR with the IL-6 gene in the pGEM plasmid (a gift from S. Akira, Osaka University, Japan) as a template with the upstream primer 5′-TAATAAGGGATCCAATCAGCCCC ACCCGCTCT-3′ [hybridizing to nt −310 to −279 of the gene promoter,[22] incorporating a *Bam*H1 site (underlined)] and a downstream primer 5′-AAGAAGCTTGCTTCGAGGGCAGAATGAG-3′ [hybridizing nt 25 to −3 of the gene promoter, which incorporates a *Hin*dIII site (underlined)]. The 328-bp *Bam*H1/*Hin*dIII fragment was then subcloned into the same restriction sites of the promoterless luciferase plasmid, poLUC.[25] Site-directed mutagenesis of the NF-κB site in the context of −303/+23 IL-6 promoter was introduced with the technique of PCR "SOEing"[26] with the mutagenic primers (mutations underlined): Δκ sense 5′-ATCAAATGTTGTATTTTACA-ATGAGTCTCAATA-3′, and Δκ antisense 5′-ATTGAGACTCT-ATGTAAAATACAACATTTTGATAAATC-3′. The *Bam*H1/*Hin*dIII fragment that contained the IL-6 promoter that mutated in the NF-κB site was then subcloned into the *Bam*H1/*Hin*dIII-digested poLUC reporter vector. Plasmids were purified by ion exchange chromatography (Qiagen) and sequenced to verify authenticity.

Transient transfections were performed by electroporation. Resuspended VSMCs (1 to 2×10^7) 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 phosphastase (SV2APAP) reporter plasmids on ice in a final volume of 0.5 mL of serum-free medium per cuvette, and subjected to a 35.4- 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 nmol/L Sar[1] 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′-tetracetic 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 described[28] 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.[28]

Gel Mobility Shift and Microaffinity Purification Assays

Nuclear extracts (25 μg) were incubated with 40 000 cpm of ^{32}P-labeled duplex NF-κB binding site, 5′-GATCCACCACAGT-TGGGATTTCCCAACCTGACCA-3′, 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.[28] The complexes were fractionated on 6% native polyacrylamide gels run in TBE buffer (25 mmol/L Tris, 25 mmol/L boric acid, 0.5 mmol/L EDTA), dried, and exposed to Kodak X-AR film at −70°C.

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

WT: GATCCATCAGTTGCAAATCGTGGAATTTCCTCTA
GTAGTCAACGTTTAGCACCTTAAAGGAGATCTAG
ΔκB: GATCCATCAGTTGCAAATCGTTTAATTTAATCTA
GTAGTCAACGTTTAGCAAATTAAATTAGATCTAG
NκM: GATCCATCAGCTACGAGTCGTGGAATTTCCTCTA
GTAGTCGATGCTCAGCACCTTAAAGGAGATCTAG

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 $MgCl_2$, 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

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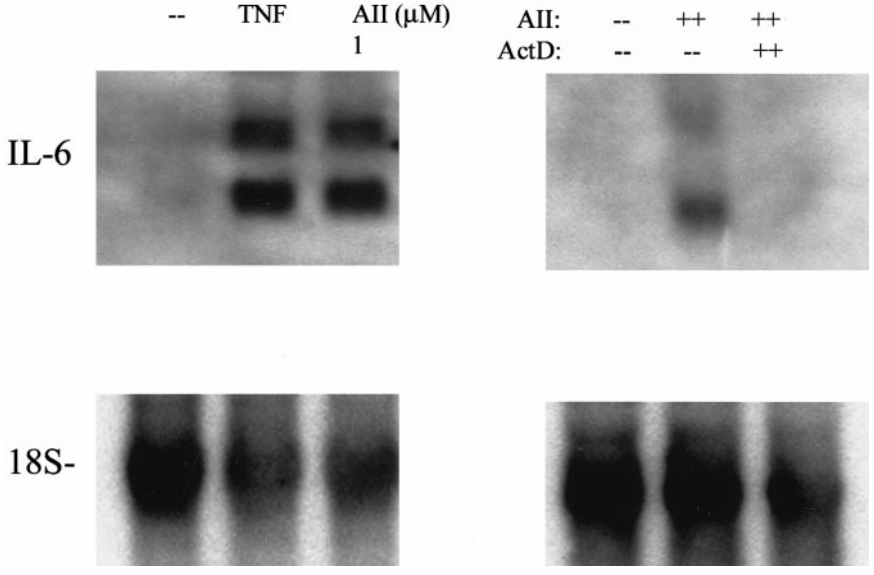

Figure 1. Ang II induces IL-6 gene expression in VSMCs. Northern blot. Left, Serum-starved VSMCs were stimulated with Sar[1] Ang II (100 nmol/L) or recombinant TNF-α (1.1 nmol/L) for 1 hour. Shown is a representative blot performed in 3 separate experiments in which total cellular RNA was fractionated. 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 Sar[1] 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.

streptavidin-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 mmol/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 Sar[1] 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 Sar[1] 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 analyze 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, Sar[1] 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, Sar[1] 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 Sar[1] 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 $\Delta\kappa$/LUC). After stimulation, the −303/+22 IL-6 $\Delta\kappa$/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 Sar[1] 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

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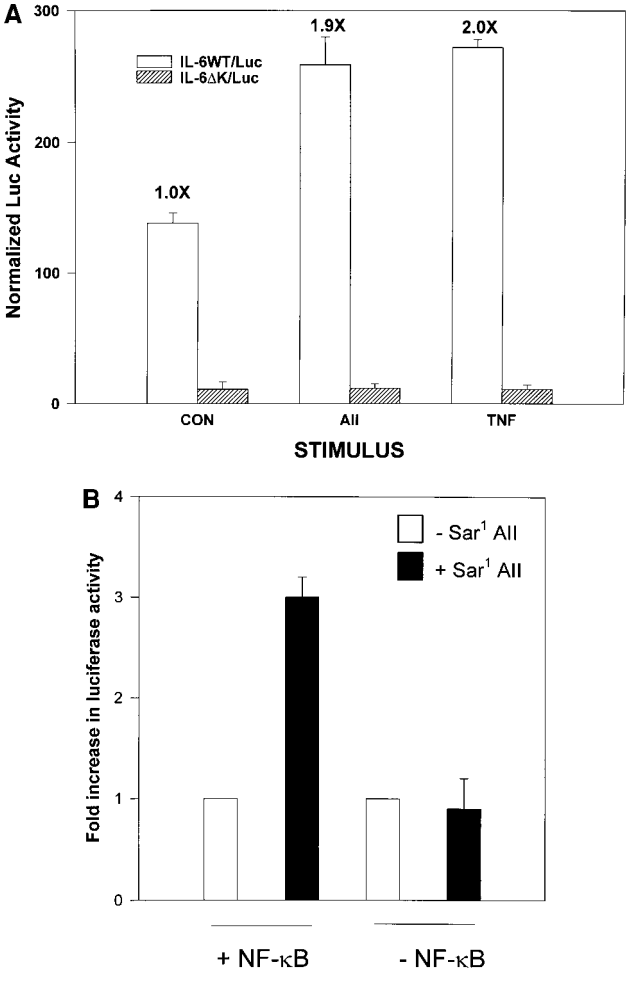

Figure 2. Ang II activates IL-6 gene transcription in VSMCs. A, Transient transfections of IL-6 promoter in VSMCs. −303/+22 IL-6/LUC plasmid and internal control alkaline phosphatase reporter plasmids were transiently transfected into VSMCs and serum starved. After Ang II stimulation, reporter gene activity was extracted and measured. IL-6ΔK/LUC 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[15] reporter was tested for Ang II inducibility. Ang II induction (3±0.2-fold; n=3) occurred only in the presence of NF-κB.

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±1.8-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 Sar[1] 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.

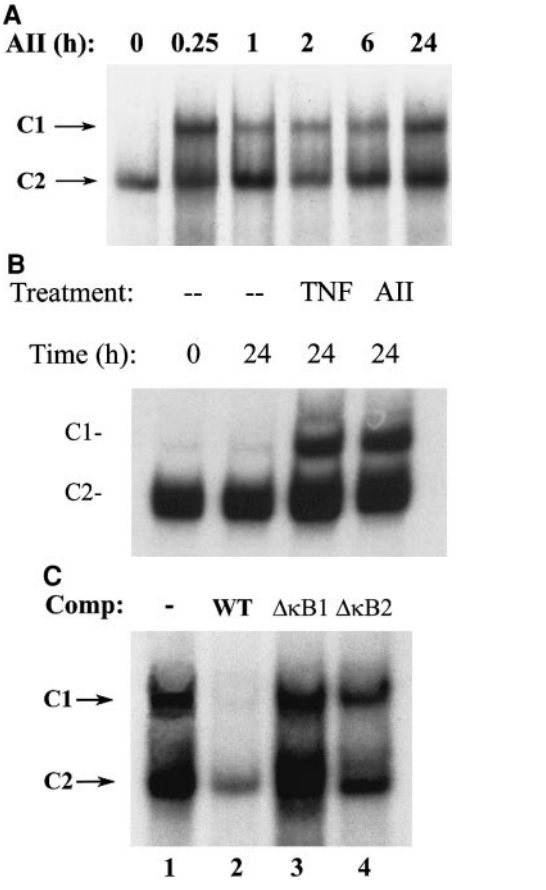

Figure 3. Ang II induces NF-κB binding in VSMCs. A, Ang II induced NF-κB DNA-binding activity in a time-dependent manner in VSMCs. Cultured VSMCs were either untreated or stimulated with 100 nmol/L Ang II for the indicated time (in hours, top) before extraction of nuclear proteins. Shown is an autoradiogram 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 Sar[1] 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′-GTTGGGATTTCCCAACC-3′; ΔκB1 contained the sequence TGTTGGATTTCCGATAC; ΔκB2 contained the sequence GTTGTGATTTCACAAC [mutations in the NF-κB binding site are underlined].[28] 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,[15] were included at a molar excess to

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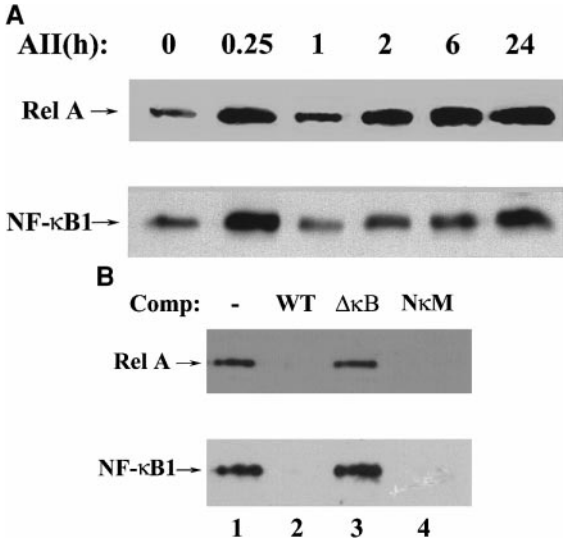

Figure 4. Ang II-inducible NF-κB binding subunits assayed by microaffinity purification. A, Kinetics Rel A and NF-κB1 binding in Sar[1] Ang II–treated VSMCs. Nuclear extracts from either control or Sar[1] Ang II (100 nmol/L)–treated VSMCs for indicated times (in hours, top) were subjected to the microaffinity isolation/Western immunoblot assay (see Materials and Methods) and probed with either anti-Rel A or anti–NF-κB1 antibodies. Specific staining of 65-kDa Rel A and 50-kDa NF-κB1 is shown by arrow at left. Ang II induces nuclear Rel A and NF-κB1 binding in a biphasic fashion, with an early binding peak at 15 minutes and later induction after 2 hours that persists to 24 hours. B, Sequence-specific binding of Rel A and NF-κB1 induced by Sar[1] Ang II. Ang II-treated VSMC nuclear extract was affinity purified for Bt–NF-κB binding in the absence or presence of nonbiotinylated binding sites WT, ΔκB, or NκM competitors and analyzed. Binding of both Rel A and NF-κB1 is specifically completed by WT and NκM but not ΔκB, which indicates sequence-specific NF-κB interaction. Experiments were reproduced 3 times.

labeled probe in the binding reaction (Figure 3C). Only the WT NF-κB competed successfully for the binding of the C1 and C2 complexes. These data indicated that both complexes bind with NF-κB specificity.

To specifically demonstrate the changes in DNA-binding activity of the Ang II-inducible NF-κB subunits, we used a 2-step microaffinity isolation/Western immunoblot DNA-binding assay.[15] In this assay, nuclear proteins from control or Sar[1] Ang II–stimulated VSMCs are incubated in the presence of Bt duplex NF-κB binding site (Methods), captured by the addition of streptavidin-agarose beads, and bound proteins are detected with Western blot. This assay allows the precise detection and quantification of NF-κB proteins whose epitopes may be masked within the NF-κB · DNA complex.[15] As shown in Figure 4A, both the 65-kDa Rel A and 50-kDa NF-κB1 subunits were present and constitutively bound in unstimulated nuclei. However, the binding activity of both proteins was inducible by Ang II. Moreover, the abundance of both Rel A and NF-κB1 was biphasic and was rapidly increased in Ang II-stimulated nuclei at 0.25 hours, decreased at 1 hour, and gradually reaccumulated over the next 24 hours. We note that the relative changes in Rel A and NF-κB1 binding exactly paralleled the relative changes in inducible C1 complex binding in the gel shift assay (Figure 3A).

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 cushion–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 A[28] 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 Sar[1] 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.

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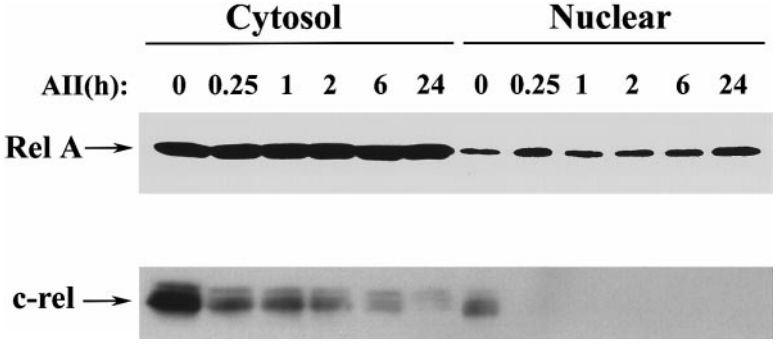

Figure 5. Nuclear translocation of Rel A and c-Rel in response to Ang II. Western immunoblotting from 250 μg of cytoplasmic (left) and 300 μg of nuclear extracts (right) of VSMCs treated with Ang II for indicated times (in hours) with either anti-Rel A (top) or c-Rel (bottom) as a primary antibody. In unstimulated cells, the majority of Rel A is cytoplasmic. However, the nuclear abundance of Rel A rapidly increases after Ang II treatment within 15 minutes. Bottom, c-Rel is also primarily cytoplasmic in control cells. Surprisingly, c-Rel disappears from the cytoplasmic fraction after Ang II treatment and is not translocated into the nucleus, which indicates that it undergoes Ang II-inducible proteolysis.

Compared with our experience of the effect of TNF-α on NF-κB translocation and IκB proteolysis in other cell systems, the effects of Sar[1] 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 Sar[1] Ang II. These data indicate that TNF-α is a more potent activator of NF-κB (and IκBα proteolysis) than Sar[1] 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 Sar[1] Ang II–stimulated VSMCs were incubated with NH_2-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

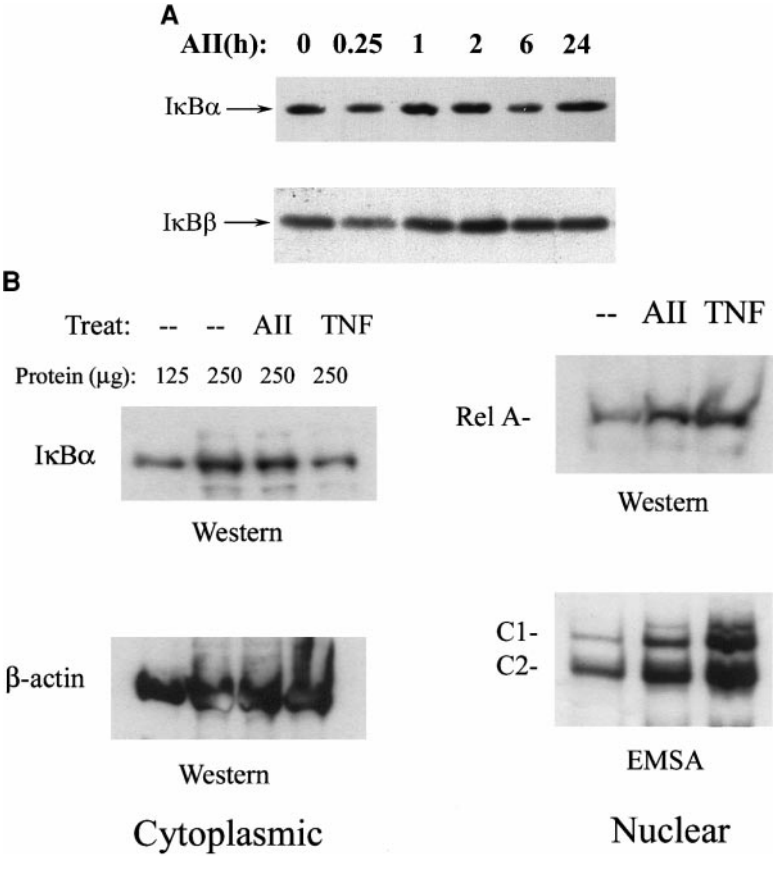

Figure 6. Effects on IκB subunits. A, Changes in cytoplasmic IκBα and IκBβ abundance. Western immunoblotting from 250-μg cytoplasmic extracts of VSMCs treated with Ang II for indicated times (in hours, top) with anti-IκBα or IκBβ primary antibodies. Ang II treatment induces a weak proteolysis of IκBα at 15 minutes and 6 hours and of IκBβ at 15 minutes. Experiments have been repeated 3 times. B, Comparison of effect of Ang II and TNF-α on IκBα proteolysis and NF-κB activation. Quiescent VSMCs were stimulated for 1 hour with Sar[1] Ang II (100 nmol/L) or recombinant TNF-α (1.1 nmol/L) after 72 hours of serum starvation. Left, Cytoplasmic fractions were assayed for changes in IκBα abundance. Control cytoplasmic protein were loaded at 125 and 250 μg protein; 250 μg of protein was assayed for the treated samples. Top, Staining with IκBα antibody; bottom, staining with β-actin antibody. TNF-α induces 80% IκBα proteolysis compared with 40% by Ang II. Right, Nuclear protein was analyzed for changes in Rel A by Western blotting (top) and electrophoretic mobility shift assay (bottom). TNF-α induced a greater change in Rel A abundance (5-fold compared with 3-fold) and stronger NF-κB binding. This experiment is quantitatively different from Figure 3B, probably because of the length of time cells were maintained in the absence of serum and the duration of Sar[1]- Ang II treatment. Experiments were repeated twice.

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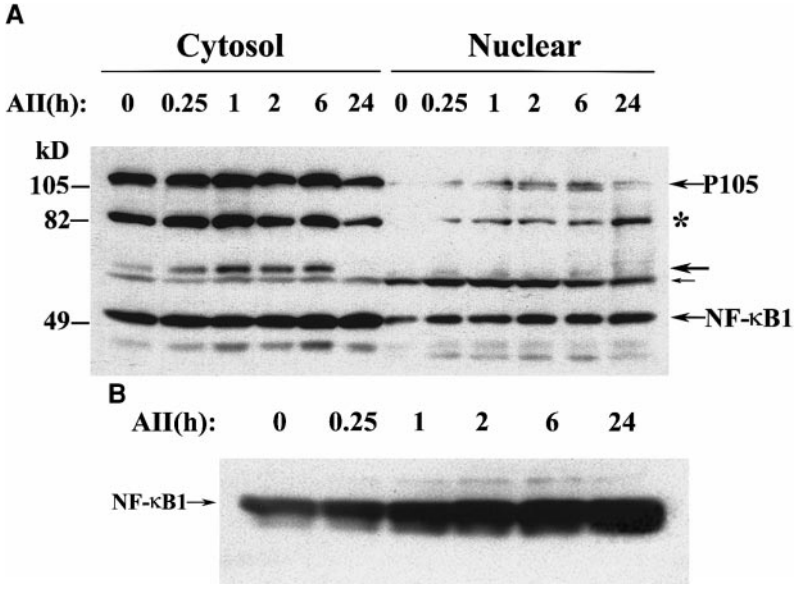

Figure 7. Mechanism of late-phase induction of NF-κB binding. A, NF-κB1/p105 nuclear translocation and p105 processing after Ang II stimulation. Western immunoblotting from 250-μg cytoplasmic (left) and 300-μg nuclear (right) extracts of VSMCs treated with Sar[1] Ang II for indicated times (in hours, top) with anti–NF-κB1 primary antibody. The 105-kDa NF-κB1 precursor (p105), the alternatively spliced 84-kDa NF-kB1 precursor (p84, asterisks), 2 ≈60-kDa NF-κB processing intermediates (large and small arrow), and the mature 50-kDa NF-κB1 (NF-κB1) are indicated. In control cells, the majority of NF-κB1 p105, p84, and p50 are cytoplasmic. After Ang II treatment, there is accumulation of the 60-kDa NF-κB1 intermediate (large arrow). In the nuclear compartment, after Ang II treatment, NF-κB1 p50 is rapidly translocated, whereas the p105 precursor is translocated later (1 to 6 hours) along with the NF-κB1 p84 isoform. B, Enhanced abundance of NF-κB1 in whole lysates of Ang II-treated VSMCs. Western immunoblotting from 200-μg whole-cellular lysates of VSMCs treated with Ang II for indicated times (in hours, top) with anti–NF-κB1 primary antibody.

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[31] 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 AT_1. 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,[32] 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,[12, 33] we report in this study for the first time that Ang II is also a pleiotropic activator of the NF-κB transcription factor family in VSMCs, one of whose function is to induce the secretion of the acute-phase mediator and vascular growth factor IL-6. The relationship of NF-κB activation to IL-6 is based on the observations that (1) 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[31]; 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

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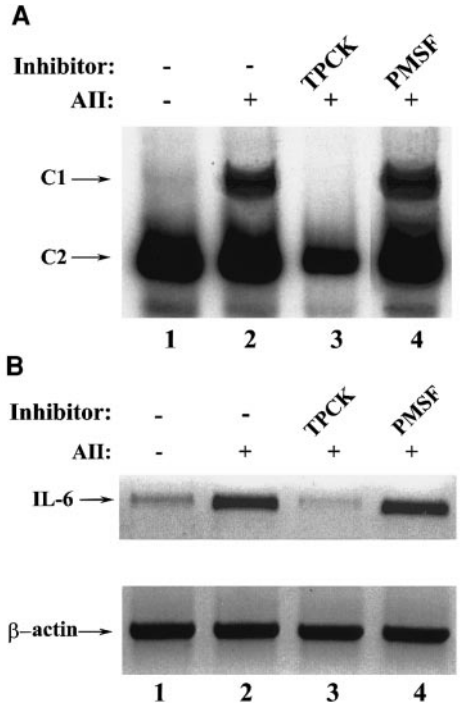

Figure 8. Effect of IκB inhibitor TPCK on Ang II-induced NF-κB translocation and endogenous IL-6 gene expression in VSMCs. A, Gel mobility shift assay. Serum-starved VSMCs were pretreated with nothing, 50 μmol/L TPCK, or 50 μmol/L PMSF 30 minutes before stimulation in the absence or presence of Ang II (100 nmol/L, 15 minutes). Shown is an autoradiogram of NF-κB binding. Pretreatment with TPCK blocks inducible (C1 and C2 complex) NF-κB binding activity. B, Effects on endogenous IL-6 gene expression. RT-PCR for relative changes in IL-6 gene expression was performed in parallel plates of VSMCs treated as in Figure 8A for 1 hour. Shown is an ethidium bromide–stained cDNA product (top with IL-6 primers, bottom with β-actin primers) after agarose gel electrophoresis. TPCK pretreatment completely blocks inducible IL-6 gene expression.

is no longer expressed even though NF-κB binding activity is detectable. Together, these data probably indicate that the IL-6 gene may be actively repressed (after its activation) in a manner dominant to NF-κB binding.

Other investigators have shown that in VSMCs, the effect of NF-κB–inducing hormones on steady-state levels of the IκB inhibitors is not equivalent. For example, the cytokine IL-1β induces a pattern of sequential IκB proteolysis, with a rapid IκBα proteolysis (at 30 minutes) followed by a later IκBβ proteolysis (2 hours), which results in a sustained profile of nuclear Rel A · NF-κB1 binding.[12] The IL-1–like cytokine TNF-α only induces IκBα but not IκBβ proteolysis and only a transient pattern of NF-κB binding.[12] In this study we observe that at maximal agonist doses, Sar[1] Ang II and TNF-α induce IκBα proteolysis to a different extent, with Ang II inducing a combined (weak, but statistically significant) IκBα and IκBβ proteolysis. These data indicate that the IκB molecules are subject to differential hormonal regulation by Ang II and cytokines in VSMCs.

Our data also implicate a delayed effect of Ang II on NF-κB1 processing in VSMCs. NF-κB1 p50 is the NH_2-terminal DNA-binding product of p105 precursor processing.[30] The proteolyzed COOH terminal region of p105 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.[31] 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 cells[15] 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 boundary[39]; we speculate its proteolysis may be required for Ang II-induced activation of quiescent VSMCs.[3]

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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References

1. Murphy TJ, Alexander RW, Griendling KK, Runge MS, Bernstein KE. Isolation of a cDNA encoding the vascular type-1 angiotensin II receptor. *Nature*. 1991;351:233–236.
2. Griendling KK, Ushio-Fukai M, Lassegue B, Alexander RW. Angiotensin II signaling in vascular smooth muscle. *Hypertension*. 1997;29: 366–373.
3. Hershey JC, Hautman M, Thompson MM, Rothblum LI, Haystead TAJ, Owens GK. Angiotensin II-induced hypertrophy of rat vascular smooth muscle is associated with increased 18S rRNA synthesis and phosphorylation of the rRNA transcription factor, upstream binding factor. *J Biol Chem*. 1995;270:25096–25101.
4. Kato H, Suzuki H, Tajima S. Angiotensin II stimulates collagen synthesis in cultured vascular smooth muscle cells. *J Hypertens*. 1991;9:17–22.
5. Naftilan AJ, Pratt RE, Dzau VJ. Induction of platelet-derived growth factor A-chain and c-*myc* gene expressions by angiotensin II in cultured rat vascular smooth muscle cells. *J Clin Invest*. 1989;83:1419–1424.
6. Taubman MB, Berk BC, Izumo S, Tsuda T, Alexander RW, Nadal-Ginard B. Angiotensin II induces c-*fos* mRNA in aortic smooth muscle: role of Ca^{2+} mobilization and protein kinase C activation. *J Biol Chem*. 1989;264:526–530.
7. Naftilan A, Pratt RE, Eldridge CS, Lin HL, Dzau V. Angiotensin II induces c-*fos* expression in smooth muscle via transcriptional control. *Hypertension*. 1989;13:706–711.
8. Marrero MB, Schieffer B, Paxton WG, Heerdt L, Berk BC, Delafontaine P, Bernstein KE. Direct stimulation of Jak/STAT pathway by the angiotensin II AT1 receptor. *Nature*. 1995;375:247–250.
9. Neyses L, Nouskas J, Luyken J, Fronhoffs S, Oberdorf S, Pfeifer U, Williams RS, Sukhatme VP, Vetter H. Induction of immediate-early genes by angiotensin II and endothelin-1 in adult rat cardiomyocytes. *J Hypertens*. 1993;11:927–934.
10. Clark AJ, Balla T, Jones MR, Catt KJ. Stimulation of early gene expression by angiotensin II in bovine adrenal glomerulosa cells: roles of calcium and protein kinase C. *Mol Endocrinol*. 1992;6:1889–1898.
11. Li J, Brasier AR. Angiotensinogen gene activation by AII is mediated by the Rel A (NF-κB p65) transcription factor: one mechanism for the renin angiotensin system (RAS) positive feedback loop in hepatocytes. *Mol Endocrinol*. 1996;10:252–264.
12. Bourcier T, Sukhova G, Libby P. The nuclear factor-κB signaling pathway participates in dysregulation of vascular smooth muscle cells in vitro and in human atherosclerosis. *J Biol Chem*. 1997;272:15817–15824.
13. Siebenlist U, Franzoso G, Brown K. Structure, regulation and function of NF-κB. *Annu Rev Cell Biol*. 1994;10:405–455.
14. Brasier AR, Li J. Mechanisms for inducible control of angiotensinogen gene transcription. *Hypertension*. 1996;27:465–475.
15. Brasier AR, Jamaluddin M, Casola A, Duan W, Shen Q, Garofalo R. A promoter recruitment mechanism for TNFα-induced IL-8 transcription in type II pulmonary epithelial cells: dependence on nuclear abundance of Rel A, NF-κB1 and c-Rel transcription factors. *J Biol Chem*. 1998;273: 3551–3561.
16. Akira S, Kishimoto T. IL-6 and NF-IL6 in acute-phase response and viral infection. *Immunol Rev*. 1992;127:25–50.
17. Loppnow H, Libby P. Proliferating or interleukin-1 activated human vascular smooth muscle cells secrete copious IL-6. *J Clin Invest*. 1990; 85:731–738.
18. Gaumond F, Fortin D, Stankova J, Rola-Pleszczynski M. Differential signaling pathways in platelet-activating factor-induced proliferation and interleukin-6 production by rat vascular smooth muscle cells. *J Cardiovasc Pharmacol*. 1997;30:169–175.
19. Beasley D. Phorbol ester and interleukin-1 induce interleukin-6 gene expression in vascular smooth muscle cells via independent pathways. *J Cardiovasc Pharmacol*. 1997;29:323–330.
20. Ikeda U, Ikeda M, Oohara T, Oguchi A, Kamitani T, Tsuruya Y, Kano S. Interleukin-6 stimulates growth of vascular smooth muscle cells in a PDGF-dependent manner. *Am J Physiol*. 1991;260:H1713–H1717.
21. Loppnow H, Bil R, Hirt S, Schonbeck W, Herzberg M, Werdan K, Rietschel ET, Brandt E, Flad HD. Platelet derived IL-1 induces cytokine production, but not proliferation of human vascular smooth muscle cells. *Blood*. 1998;91:134–141.
22. Yasukawa K, Hirano T, Watanabe Y, Muratani K, Matsuda T, Nakai S, Kishimoto T. Structure and expression of human B-cell stimulatory factor-2 (BSF-2/IL-6) gene. *EMBO J*. 1987;6:2939–2945.
23. Gunther S, Alexander RW, Atkinson WJ, Gimbrone M Jr. Functional angiotensin II receptors in cultured vascular smooth muscle. *J Cell Biol*. 1982;92:289–298.
24. Azuma H. Blockade of T-cell costimulation prevents development of experimental chronic renal allograft rejection. *Proc Natl Acad Sci U S A*. 1996;93:12439–12444.
25. Brasier AR, Tate JE, Habener JF. Optimized use of the firefly luciferase assay as a reporter gene in mammalian cell lines. *Biotechniques*. 1989; 7:1116–1122.
26. Garofalo R, Sabry M, Jamaluddin M, Yu RK, Casola A, Ogra PL, Brasier AR. Transcriptional activation of the interleukin-8 gene by RSV infection in alveolar epithelial cells: nuclear translocation of the Rel A transcription factor as a mechanism producing airway mucosal inflammation. *J Virol*. 1996;70:8773–8781.
27. Brasier AR, Li J, Wimbish KA. Tumor necrosis factor activates angiotensinogen gene expression by the Rel A transactivator. *Hypertension*. 1996;27:1009–1017.
28. Han Y, Brasier AR. Mechanism for biphasic Rel A: NF-κB1 nuclear translocation in tumor necrosis factor α-stimulated hepatocytes. *J Biol Chem*. 1997;272:9823–9830.
29. Grumont RJ, Fecondo J, Gerondakis S. Alternate RNA splicing of murine *nfkb1* generates a nuclear isoform of the p50 precursor NF-κB1 that can function as a transactivator of NF-κB-regulated transcription. *Mol Cell Biol*. 1994;14:8460–8470.
30. Kieran M, Blank V, Logeat F, Vandekerckhove J, Lottspeich F, Le Bail O, Urban MB, Kourilsky P, Baeuerle PA, Israel A. The DNA binding subunit of NF-κB is identical to factor KBF1 and homologous to the *rel* oncogene product. *Cell*. 1990;62:1007–1018.
31. Palombella VJ, Rando OJ, Goldberg AL, Maniatis T. The ubiquitin-proteasome pathway is required for processing the NF-κB1 precursor protein and the activation of NF-kB. *Cell*. 1994;78:773–785.
32. Ross R. The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature*. 1993;362:801–809.
33. Obata H, Biro S, Armia N, Kaieda H, Kihara T, Eto Hi, Miyata M, Tanaka H. NF-κB is induced in the nuclei of cultured rat aortic smooth muscle cells by stimulation of various growth factors. *Biochem Biophys Res Comm*. 1996;224:27–32.
34. Mercurio F, DiDonato JA, Rosette C, Karin M. p105 and p98 precursor proteins play an active role in NF-κB-mediated signal transduction. *Genes Dev*. 1993;7:705–718.
35. Baldassarre F, Mallardo M, Mezza E, Scala G, Quinto I. Regulation of NF-kB through the nuclear processing of p105 (NF-kB1) in Epstein-Barr Virus-immortalized B cell lines. *Mol Cell Biol*. 1995;270:31244–31248.
36. Thompson JE, Phillips RJ, Erdjument-Bromage H, Tempst P, Ghosh S. IκB-β regulates the persistent response in a biphasic activation of NF-κB. *Cell*. 1995;80:573–582.
37. Rice NR, Ernst MK. In vivo control of NF-κB activation by IκB alpha. *EMBO J*. 1993;12:4685–4695.
38. Ernst MK, Dunn LL, Rice NR. The PEST-like sequence of IκB is responsible for inhibition of DNA binding but not for cytoplasmic retention of c-Rel or Rel A homodimers. *Mol Cell Biol*. 1995;15: 872–882.
39. Bash J, Zong WX, Gelinas C. c-Rel arrests the proliferation of HeLa cells and affects critical regulations of the G1/S-phase transition. *Mol Cell Biol*. 1997;17:6526–6536.
40. Brand K, Page S, Rogler G, Bartsch A, Brandl R, Kneuchel R, Page M, Kaltschmidt C, Baeuerle PA, Neumeier D. Activated transcription factor nuclear factor-κB is present in the atherosclerotic lesion. *J Clin Invest*. 1996;97:1715–1722.

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