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Involvement of a Novel Rac/RhoA Guanosine Triphosphatase-Nuclear Factor-κB Inducing Kinase Signaling Pathway Mediating Angiotensin II-Induced RelA Transactivation

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Angiotensin II (Ang II) is the major effector peptide of the renin angiotensin system that induces inflammatory gene expression through the nuclear factor-κB (NF-κB) transcription factor. Activation of latent cytoplasmic NF-κB is controlled by distinct pathways, the best known being the canonical pathway controlling IκB kinase activation. Interestingly, Ang II only weakly activates the canonical pathway. Although basal nucleocytoplasmic RelA shuttling is required for Ang II stimulation, changes in RelA translocation do not account for its transcriptional effect. Instead, Ang II rapidly induced RelA phosphorylation at Ser residue 536, and complex formation with the Ser^{536} kinase known as the NF-κB-inducing kinase (NIK)/MEKK14. The requirement of NIK in Ang II-inducible transcription was shown by expressing a dominant-negative NIK or small interfering RNA (siRNA)-mediated knockdown; both inhibited Ang II-induced transcription. Conversely, constitutively active NIK potently induced RelA transactivation activity. Consistent with its actions independent of the canonical pathway, NIK induces the activity of the RelA transactivation domains -1 and -2 in constitutively nuclear GAL4-RelA fusion proteins that do not bind IκBα. Ang II induces NIK activity, phosphorylation of its endogenous IκB kinase α substrate, and induction of nuclear NF-κB2 (p52) processing. NIK downregulation prevents Ang II-induced phospho-Ser^{536} RelA formation, indicating that it is essential for RelA activation. The Ang II pathway further involves the RhoA small GTP-binding protein because RhoA inhibition blocks Ang II-induced transcriptional activity and formation of phospho-Ser^{536} RelA formation. Finally, we demonstrate that Ang II infusion *in vivo* rapidly induces phospho-Ser^{536} RelA formation and activation of the NF-κB-dependent IL-6 gene. These data indicate that Ang II induces NF-κB-dependent transcription through an alternative pathway, being largely independent of IκB proteolysis, but mediated by the small GTPases Rac/RhoA, required for NIK·RelA complex formation and inducible Ser^{536} RelA phosphorylation. (*Molecular Endocrinology* 21: 2203–2217, 2007)

THE RENIN ANGIOTENSIN system (RAS) is an endocrine loop important in long-term blood pressure control and intravascular fluid homeostasis (1, 2). Responding to physiological regulators, the RAS controls sequential NH2-terminal proteolytic processing of the angiotensinogen precursor into angiotensin II (Ang II), the major RAS effector octapeptide. Newly formed Ang II, in turn, binds the high-affinity type 1 aniotensin receptor (AT_1) expressed by cells in adult liver, brain, adrenal cortex, and vascular tissues (3). Although most known for its potent vasoconstrictive effects, Ang II is now recognized to induce hepatic or vascular gene expression programs controlling cellular fibrosis (4) and inflammatory responses (5). These latter actions are implicated in a broader pathophysiological role of Ang II for enhancing diabetic nephropathy (6), mediating hypertensive cardiomyopathy (7), accelerating atherosclerosis (5, 8), and producing hepatic fibrosis (9). For this reason, the signaling pathways activated by AT_1 are being intensively investigated.

The AT_1 receptor is a seven transmembrane-spanning protein, coupled to heterotrimeric G protein-dependent (10) and -independent signaling pathways (11). G protein-dependent signaling results in activation of phos-

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Abbreviations: Ab, Antibody; Ang II, angiotensin II; AT_1, type 1 angiotensin receptor; Co-IP, coimmunoprecipitation; CT, cycle threshold; DN, dominant negative; DTT, dithiothreitol; ECFP, enhanced cyan fluorescent protein; GST, glutathione-*S*-transferase; IP, immunoprecipitation; KA, kinase activity; MEK, mitogen-erk related kinase kinase; NF-κB, nuclear factor-κB; NIK, NF-κB-inducing kinase; NIK-CA, constitutionally active NIK; PAK1, p21-activated kinase; Q-RT-PCR, quantitative RT-PCR; RAS, renin angiotensin system; RSK, ribosomal kinase; siRNA, short interfering RNA; TAD, transactivation domain; UAS, upstream activating sequence; VSMCs, vascular smooth muscle cells.

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pholipase Cβ to generate inositol trisphosphate and diacylglycerol, which increase intracellular calcium and activate protein kinase C (10). The G protein-independent pathways involve activation of Src and phospho-ERK (11). Downstream, these signaling pathways activate immediate early transcription factors, including activator protein-1 (12), signal transducer and activators of transcription (13), and nuclear factor-κB (NF-κB) (14, 15). These factors are responsible for the fibrotic and inflammatory gene expression programs (5, 16); in fact, studies from our laboratory have specifically identified NF-κB as a major regulator of Ang II-induced inflammation (5, 16).

NF-κB is a family of seven structurally related isoforms that function in various combinations as an inducible transcription factor. Specifically, the NF-κB family includes the proteolytically processed DNA binding subunits termed NF-κB1 (50 and 105 kDa), NF-κB2 (52 and 100 kDa), and the transcriptional activators termed RelA, C-Rel, and Rel B (17, 18). Frequently referred to as a "central regulator of inflammation," NF-κB controls the expression of inducible cytokines, chemokines, cell adhesion molecules, vasoactive and antiapoptotic proteins important in the cellular stress response (19–21).

Currently, NF-κB activation is thought to be controlled by several distinct pathways, termed the "canonical" (22) and the "noncanonical" pathways (23). The canonical pathway largely controls the nuclear levels of the RelA·NF-κB1 complex. Under normal conditions, RelA·NF-κB1 is sequestered and inactivated in the cytoplasm by binding the IκBα-inhibitory protein (24). The hallmark of NF-κB activation via the canonical pathway involves the proteolysis of the IκB inhibitor (22). Signal-induced IκB proteolysis is mediated by activation of the multiprotein cytoplasmic IκB kinase (IKK) (25), a kinase that phosphorylates IκB specifically in its NH_2-regulatory domain, making it a target for ubiquitination and proteolysis (26, 27). As a result, liberated RelA·NF-κB1 enters the nucleus to activate target gene expression by inducing assembly of active promoters and coactivator recruitment (28). By contrast, the noncanonical pathway, primarily operative in B lymphocytes, controls processing of the 100-kDa NF-κB2 precursor (p100) into its mature 52-kDa DNA binding form (NF-κB2) and nuclear translocation of the liberated NF-κB2·Rel B complex (29). Activation of the canonical or noncanonical pathway is stimulus type specific, with some cytokines preferentially activating the canonical pathway (*e.g*. TNF), and others activating the noncanonical pathway (BAFF, LIGHT). The pathways used by Ang II are not completely understood.

The liver represents an important physiological target for Ang II. Here, Ang II signaling induces expression of the angiotensinogen precursor, known as the RAS "positive feedback loop" (30) and mediates hepatic fibrosis (4) and triglyceride accumulation characteristic of insulin resistance syndromes (31). In previous work, we observed that Ang II stimulation activated NF-κB-dependent transcription without producing detectable IκBα proteolysis or changes in RelA nuclear abundance (15). Here we further investigate this apparently novel pathway. Short interfering RNA (siRNA)-mediated "knockdown" of RelA completely inhibited Ang II-induced NF-κB reporter activity, proving that RelA is necessary for transcriptional activation. Instead, Ang II induced phosphorylation at Ser^{536} in the COOH-terminal transactivation domain (TAD). The role of NIK in Ang II-inducible signaling was implicated by overexpression and genetic knockdown experiments. Experiments using constitutive nuclear GAL4-RelA fusion proteins further demonstrated that the RelA TADs-1 and -2 are the functional targets of NIK-dependent transactivation. These data indicate that Ang II induces NF-κB-dependent transcription through a novel pathway, distinct from the canonical pathway, mediated upstream by Rho GTPases leading to NIK-dependent Ser^{536} RelA phosphorylation.

RESULTS

Ang II-Induced Activation of NF-κB Is Dependent on Constitutive Nuclear RelA

In hepatocytes, Ang II rapidly induces a dose-dependent activation of NF-κB-dependent transcription over physiological concentrations of 10–1000 nM, requiring the high-affinity type I AT1 receptor in a manner indistinguishable from that induced by TNFα (15). To determine whether this activation was mediated via the canonical NF-κB activation pathway, HepG2 cells were stimulated and fractionated into cytoplasmic and sucrose cushion-purified nuclear extracts using our validated protocols (32). Using a quantitative near-infrared Western immunoblot (supplemental Figs. 1 and 2, published as supplemental data on The Endocrine Society's Journals Online web site at http://mend.endojournals.org), we found that the 37-kDa IκBα isoform in unstimulated cells was nearly completely proteolyzed at 15 and 30 min in response to TNF stimulation (Fig. 1A, *upper left*). After 60 min of TNF stimulation, a 2-fold increase in IκBα was produced due to NF-κB-dependent overshoot resynthesis (33). By contrast, Ang II did not induce a comparable degree of IκBα proteolysis, producing, at most, a 30% reduction of basal activity (Fig. 1A, *upper right*). We next examined the effect of Ang II on steady-state abundance of nuclear RelA by Western immunoblot. In HepG2 cells, nuclear RelA abundance is approximately 10% of that of the cytoplasmic pool and represents a population undergoing constitutive nucleocytoplasmic shuttling (27, 33, 34). Consistent with the lack of a strong effect on IκBα proteolysis, Ang II did not detectably induce changes in nuclear 65-kDa RelA isoform (Fig. 1B), whereas a 4-fold induction of RelA was induced by TNF (Fig. 1B, *right*).

To further examine whether the canonical NF-κB activation pathway was activated by Ang II, we performed immunoprecipitation (IP)-kinase assays on the IKKβ kinase, the rate-limiting step in the canonical NF-κB pathway. In this experiment, HepG2 cells were stimulated for various times with nothing, TNF, or Ang II, and whole-cell

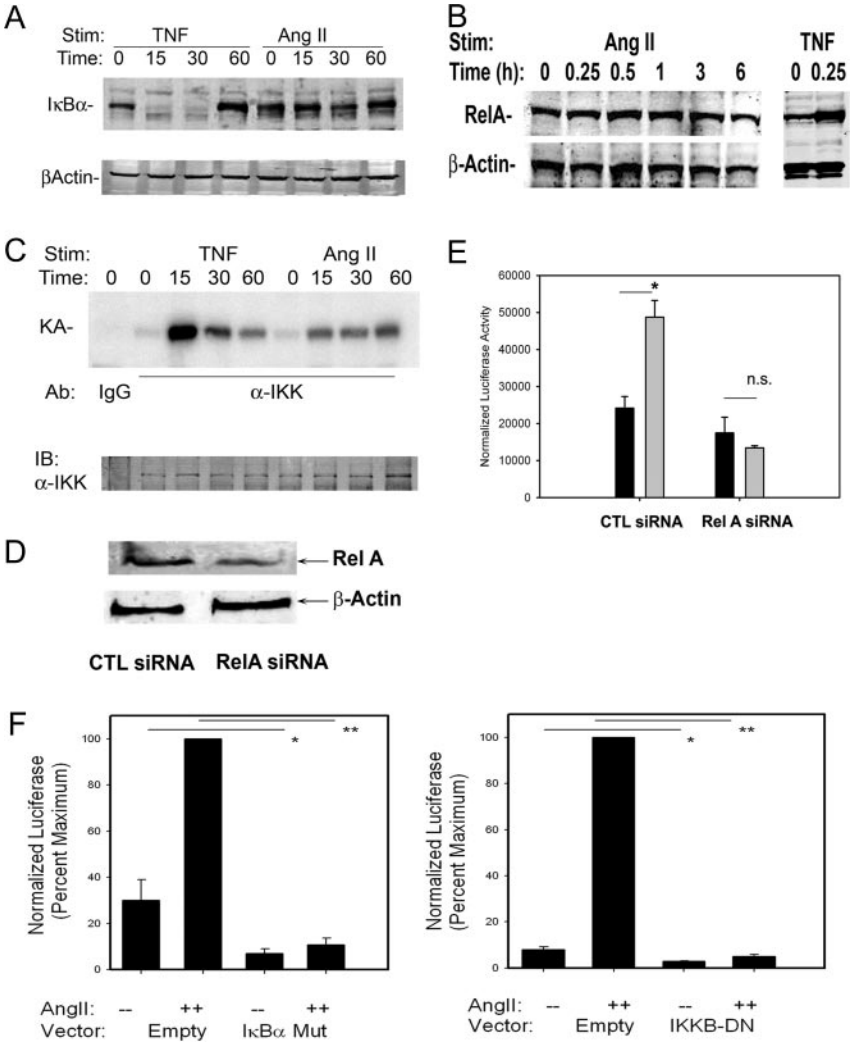

Fig. 1. Requirement of RelA for Ang II-Inducible Transcription

A, Assay for canonical NF-κB pathway activation. HepG2 cells were treated with TNF (20 ng/ml) or Ang II (1 μM) for indicated times (in minutes) before fractionation into cytoplasmic extract (CE). *Top panel*, Western immunoblot of CE using anti-IκBα Ab. *Bottom panel*, Membrane was reprobed with anti-β-Actin Ab as internal control. In contrast to the nearly complete IκBα proteolysis induced by TNF, Ang II had a minor effect. A dose-response curve for IκBα is shown in supplemental Fig. 1. B, Effect of Ang II on RelA translocation. Nuclear extract (NE) using an established sucrose cushion gradient fractionation (15, 60). *Top panel*, NE was assayed for changes in RelA abundanced by Western immunoblot. *Bottom panel*, Anti-β-Actin Ab as internal control. A dose-response curve for RelA is shown in supplemental Fig. 2. In contrast to a strong induction of RelA translocation induced by TNF as assayed by Western blot in these cells (27, 33, 60, 62), Ang II produces less than a 1.5-fold change in nuclear RelA abundance. C, Immunoprecipitation-kinase assay. AT_1-complemented HepG2 cells were stimulated with nothing, TNF (20 ng/ml), or Ang II (1 μM) for 30 min and cell lysates were prepared. IKKβ was immunoprecipitated and KA was measured by incorporation of $\gamma[^{32}P]$ATP into its substrate IκBα (1–51). *Top panel* is autoradiogram of kinase assay. TNF induces a 21-fold induction of KA after 15 min of stimulation, which rapidly decays. By contrast, Ang II induces a weaker, delayed IKK activity, peaking at 6.6-fold after 1 h of stimulation. Negative control lane using nonimmune IgG as the immunoprecipitating Ab shows no KA. *Bottom panel*, Membrane was probed with anti-IKKβ Ab as a recovery control. D, siRNA mediated RelA knockdown. Western immunoblot of HepG2 cell extracts transfected with control (CTL) or RelA (p65)-specific siRNA (50 nM, 48 h). *Top panel*, Blot is probed with anti-RelA Ab; *bottom panel*, blot is probed with anti-β Actin Ab as loading control. Relative to β actin, a 70% reduction in RelA expression is observed. E, Transient transfection assay. HepG2 cells transfected with control (CTL) or RelA (p65)-specific siRNA were retransfected with NF-κB-dependent APRE-LUC reporter vector (15, 30). Cells were stimulated 48 h later with Ang II for 6 h. Shown is normalized luciferase reporter activity. Note that Ang II-inducible APRE-LUC reporter activity is reduced in cells depleted of RelA. F, Requirement of nuclear RelA. *Left*, HepG2 cells were transfected with APRE-LUC in the absence or presence of pcDNA3-IκBα ($Ser^{32}Ala/Ser^{36}Ala$) expression vector. Cells were either unstimulated or stimulated with Ang II as indicated. To compare basal activities, shown is percent luciferase reporter activity relative to the maximal Ang II stimulated value for each experiment. *Right*, HepG2 cells were transfected with APRE-LUC in the absence or presence of pEF-IKKβ ($Lys^{44}Met$). Percent luciferase is shown. *, $P < 0.05$; **, $P < 0.01$ (two-tailed *t* test). Stim, Stimulation; IB, immunoblot; Mut, mutant.

lysates were prepared. IKKβ kinase activity (KA) was then measured after immunoprecipitation (IP) by incorporation of γ[^{32}P]ATP into its substrate IκBα(1–51). In the absence of anti-IKKα/β antibody (Ab), no phosphorylation product was produced (Fig. 1C). As expected, TNF strongly induced IKKβ activity by 21-fold 15 min after stimulation, which rapidly returned to near basal activity 60 min later (27, 33). By contrast, Ang II only weakly activated IKKβ KA, with an activation profile distinct from that produced by TNF, gradually increasing to 6-fold induction after 60 min of stimulation. Together these data were surprising because although Ang II potently induces NF-κB transcription in HepG2 cells, it does not strongly activate the canonical NF-κB pathway.

In our earlier studies using a specific microaffinity DNA-binding capture assay in which the binding of individual NF-κB isoforms could be specifically detected and quantified, we found that Ang II induced NF-κB1 isoform binding to the NF-κB binding site but produced less than 2-fold increase in RelA binding (15). Because NF-κB1 lacks a COOH-terminal transactivation domain, this finding made it difficult to understand how gene expression could be activated. We therefore sought to test whether constitutive nuclear RelA mediated the Ang II-induced transcription. For this purpose, HepG2 cells were transfected with either RelA-specific short-interfering RNA (siRNA) or nonspecific control siRNA. Whole-cell extracts were prepared 48 h and 72 h post transfection and knockdown was determined by Western immunoblot. We consistently produced an approximately 70% decrease in steady-state RelA abundance 48 h after transfecting 50 nM RelA-specific siRNA compared with that of control siRNA (Fig. 1D). Importantly, RelA was selectively down-regulated as indicated by internal control staining with β-actin (Fig. 1D). Experiments using higher siRNA concentrations or longer times did not appreciably increase the knockdown effect further (data not shown). We therefore selected 50 nM concentrations of siRNA and 48-h exposure for subsequent experiments.

To determine the role of RelA in Ang II-inducible transcription, cells transfected with RelA siRNA were subsequently retransfected with NF-κB-dependent reporter (APRE WT-LUC; Refs. 15, 30). As seen in Fig. 1E, Ang II treatment increased NF-κB-dependent APRE luciferase reporter activity about 2-fold in control siRNA-transfected cells. In the RelA siRNA-transfected cells, basal APRE WT-LUC was decreased slightly, but this reduction was not statistically significant compared with control siRNA-treated cells. However, Ang II-inducible APRE-LUC reporter activity was completely abolished (Fig. 1E). This experiment demonstrates that NF-κB activation in Ang II treatment requires constitutive nuclear RelA.

RelA is a dynamic protein that undergoes constitutive nucleocytoplasmic shuttling (35), a phenomenon regulated by the availability of IκBα (36) and basal IKK activity (37). To further demonstrate the requirement of nuclear RelA for the Ang II effect, we perturbed its constitutive nucleocytoplasmic shuttling of RelA using two independent methods. First, ectopic expression IκBα is known to produce cytoplasmic RelA sequestration because the constitutive nuclear export signal of RelA makes it available for IκBα association, where it is trapped in a nondegradable cytoplasmic complex (38). Indeed we found that expression of the nondegradable pcDNA-IκBα (Ser^{32}Ala/Ser^{36}Ala) potently inhibited basal APRE-LUC activity to levels less than 30% of that seen by cotransfection of empty pcDNA vector, indicating that IκBα (Ser^{32}Ala/Ser^{36}Ala) was sequestering RelA from the nucleus (Fig. 1F, *left*). In this situation, Ang II-inducible transcription was abolished (Fig. 1F). Similarly, expression of a dominant negative IKKβ (Lys^{44}Met) also potently inhibits its basal and Ang II-inducible gene expression (Fig. 1F, *right*). Together, these data indicate that although Ang II does not produce detectable changes in nuclear RelA abundance, its presence in the nucleus is required for its transcriptional effect.

Ang-II Induced Phospho Ser^{536} RelA Formation and Complex Formation with NIK

To further understand the mechanism for Ang II-induced NF-κB activation despite no consistently detectable change in RelA nuclear translocation, we examined its effect on RelA phosphorylation. We were intrigued by recent findings that transcriptional activation of RelA was inducibly regulated by phosphorylation at Ser residue 536 in its COOH-terminal TAD in a manner independent of the canonical pathway (39). To determine whether RelA was phosphorylated on Ser^{536}, and whether Ang II affected this, a time course of Ang II-stimulated HepG2 cells was subjected to Western immunoblot with anti-phospho-Ser^{536} RelA Ab. Here, we observed a consistent approximately 3-fold increase in RelA Ser^{536} phosphorylation within 15 min, which persisted for up to 1 h after Ang II stimulation (Fig. 2A).

To date, two major kinases have been shown to phosphorylate RelA at Ser^{536} including the IKKβ kinase (40) and NIK (41). Because Ang II did not strongly activate IκBα proteolysis and RelA translocation (Fig. 1), we focused on evaluating the role of NIK in Ang II-induced RelA Ser^{536} phosphorylation. For this purpose, we used nondenaturing coimmunoprecipitation (Co-IP) to investigate whether endogenous NIK and RelA interacted, or whether this interaction was Ang II inducible. Extracts from Ang II-stimulated HepG2 cells were immunoprecipitated by anti-RelA Ab, and the presence of NIK in the immune complex was determined by Western immunoblot (Fig. 2B). Here we found that Ang II stimulation induces NIK-RelA complex formation, peaking 30 min after Ang II stimulation. These data suggested the existence of an Ang II-inducible RelA·NIK complex and suggested a potential role for NIK in Ang II-induced NF-κB activation.

Involvement of NIK in Ang II-Induced NF-κB Activation

To analyze the functional involvement of NIK in Ang II-induced promoter activity, HepG2 cells were co-

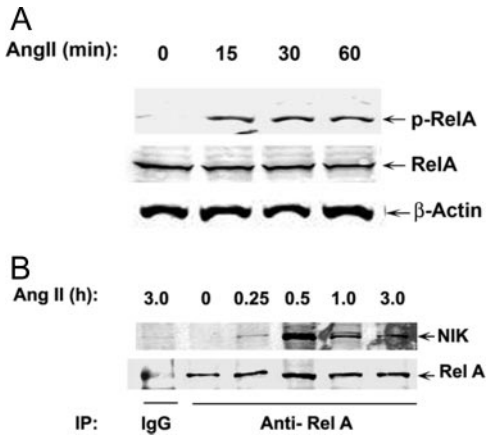

Fig. 2. Ang II Induces RelA Phosphorylation at Ser 536

A, Western blot of phospho-RelA formation. HepG2 cells were treated with Ang II for indicated times (in minutes) before denaturing lysis. Fractions were assayed for formation of phospho-Ser^{536} RelA by Western immunoblot using phospho-Ser^{536}-specific Ab (Cell Signaling). Staining with anti-RelA Ab (*middle*) or anti-β Actin Ab (*bottom*) were used as loading controls. B, Effect of Ang II on NIK-RelA complex. Co-IP experiment of HepG2 cells stimulated with Ang II for indicated times (in hours). Lysates were immunoprecipitated (IP) with either IgG or anti-RelA Ab and washed, and Western blot assays were performed on the immune complexes. *Top*, Western immunoblot of immune complexes using anti-NIK Ab. *Bottom*, Western using anti-RelA Ab as a recovery control. RelA is complexed with NIK in the absence of stimulation; this fraction begins to increase after 30 min and strongly complexes after 1 h, decreasing thereafter.

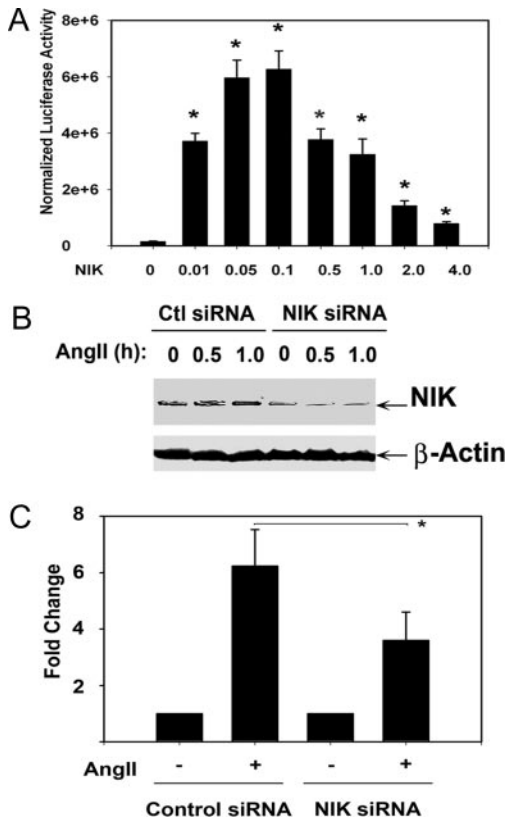

Fig. 3. NIK Requirement for Ang II-Induced Transcriptional Activity

A, Effect of NIK DN on Ang II-induced transcription. HepG2 cells were transfected with APRE-LUC reporter and increasing concentrations of expression vector encoding NIK DN. Empty plasmid was used to keep amounts of DNA equivalent for each transfection condition. NIK DN inhibits Ang II-induced transcription by 50% at 1 μg. B, siRNA-mediated NIK knockdown. HepG2 cells were transfected with 50 nM control or NIK siRNA and harvested 72 h afterward. *Top*, Western blot using anti-NIK Ab. *Bottom panel*, Membrane was reprobed with β-Actin as internal control. A 50% reduction in the steady-state concentration of NIK was produced. C, Effect of NIK knockdown on Ang II-induced transcription. HepG2 cells transfected with siRNA as in Fig. 3B were retransfected with the APRE-LUC reporter. Cells were stimulated 72 h later, with Ang II for 6 h. Shown is normalized luciferase activity of a representative experiment. Each point was performed in triplicate; the experiment was repeated twice with similar results. *, $P < 0.01$. Ctl, Control.

transfected with APRE-LUC reporter and increasing amounts of dominant negative (DN) NIK expression constructs. Empty vector was used to ensure similar amounts of DNA were transfected for each point. Cells were stimulated 36 h later in the presence or absence of Ang II and reporter activity was measured (Fig. 3A). Here, Ang II induced APRE-LUC approximately 7-fold 6 h after stimulation (note this induction is greater than the result shown in Fig. 1E because, in this latter experiment, cells were sequentially transfected with siRNA and plasmid, a manipulation that reduces the Ang II effect). The cotransfection of NIK-DN significantly reduced APRE-LUC reporter activity in a dose-dependent manner, producing more than 50% inhibition at 1 μg of NIK-DN expression vector. This finding suggests that Ang II-induced NF-κB-dependent promoter activity is partly dependent on NIK activity.

Because overexpression of DN kinases can have nonspecific effects, we sought to independently confirm the observation that NIK was required for Ang II-induced NF-κB transcription. For this purpose, siRNA-mediated knockdown was performed. HepG2 cells transfected with NIK siRNA had reductions of 50% relative to control transfectants (Fig. 3B). To determine its effect on Ang II-induced transcription, control and NIK siRNA transfected cells were subsequently retransfected with APRE WT-LUC and stimulated with Ang II. The partial NIK knockdown consistently and significantly reduced Ang II-induced NF-κB transcription (Fig. 3C), indicating that NIK mediated, at least in part, RelA-dependent transcription.

To determine whether constitutively active (CA) NIK was sufficient for NF-κB-dependent activation, NIK-CA was cotransfected with APRE-LUC and reporter activity was determined. NIK-CA was a potent transactivator of APRE-LUC, inducing luciferase reporter activity by more than 20-fold compared with empty vector control over concentrations of 10–500 ng (Fig. 4A). At higher concentrations, reporter activity fell, due to NIK-induced apo-

ptosis (42). Together these data indicated that exogenous NIK was sufficient to activate APRE reporter activity.

RelA Activation by NIK Is Mediated by Its COOH-Terminal TADs

To further establish that RelA was the target of NIK-induced transcription, and map the protein domains involved, yeast one-hybrid assays were performed. In this experiment, a luciferase reporter gene driven by the GAL4 response element termed the upstream activating sequence (UAS-LUC) was cotransfected with expression vectors encoding fusion proteins of the GAL4 DNA-binding domain with various NH_2-terminal deletions of RelA in the absence or presence of NIK-CA. As shown in Fig. 4B, GAL4-RelA fusions have detectable transcriptional activity consistent with their constitutive nuclear translocation driven by the GAL4 nuclear localization sequences (30, 43). Cotransfection of NIK-CA strongly increases transcriptional activity of full-length RelA-GAL-4 fusion, GAL-4-RelA(1–551). However, maximum induction was observed with two NH_2-terminal deletions of RelA that were unable to interact with IκBα (amino acids 255–51 and 313 −551) that still contained both TADs-1 and -2. In fact, the NIK-dependent transactivation of GAL4-RelA(313–551) is dose dependent and can be seen with as little as 10 ng of NIK-CA (Fig. 4C). Together these experiments indicate that NIK activity can increase the transactivation potential of RelA TAD in a manner independent of IκBα interaction or induction of the canonical pathway.

To further map the RelA TAD mediating NIK interaction, GAL4-RelA(313–551) and its N- and C-terminal deletion mutants were cotransfected with UAS-LUC in the absence or presence of NIK-CA. Luciferase activity obtained was normalized to the transcriptional activity of GAL4-RelA(313–551) as 100%. As shown in Fig. 4D, which tabulates the relative NIK-induced transcriptional activity of these deletion mutants, the presence of both RelA TADs is sufficient for NIK-induced transcriptional activity, where GAL4-RelA(428–551) is transactivated at similar levels as GAL4-RelA(313–551). Deletion of TAD-1 (GAL4-RelA 313–485) reduces NIK-inducible transcription to 47% of parent construct, whereas deletion of TAD-1 and -2 reduces the activity to 20%. Similar qualitative results are found with the RelA NH_2-terminal deletions. Further, a single Ser-to-Ala point mutation at residue 536 ($S^{536}A$) significantly abrogated the transcriptional activity to 37% that of the parent construct. Thus, these experiments implied that the functional RelA-NIK interaction is complex, with intact TAD-1, and -2 and Ser^{536} being essential for NIK-regulated GAL4-RelA activity.

These data suggested that NIK interacts with TAD-2 domain, with the requirement for the TAD-1 being mediated through phosphorylation. To determine the RelA domains involved in NIK complex formation, cells were transfected with expression vectors encoding Myc-NIK and FLAG-tagged RelA(313–551), RelA(428–551), RelA(313–485), and RelA(486–551). In whole-cell lysates of the transfectants, all proteins were expressed with some truncation fragments being seen in expression vectors containing the TAD-1 domain (Fig. 4E). After nondenaturing Co-IP with anti-MYC and Western immunoblot with anti-FLAG, we could specifically identify RelA(313–551), RelA(428–551), and RelA(313–485) association with NIK, whereas RelA(486–551) did not bind. These data indicated that RelA TAD-2 was required for stable protein interaction with NIK.

To further explore the nature of the NIK-RelA interaction, we tested whether phosphorylation of Ser^{536} in TAD-1 was required for stable interaction. For this purpose, HepG2 cells were transfected with expression plasmids encoding FLAG epitope-tagged enhanced cyan fluorescent protein (ECFP)-RelA(WT) or the RelA(Ser^{536}Ala) site mutation. The ability of these proteins to complex with endogenous NIK was then determined by nondenaturing Co-IP. After primary IP using anti-NIK Ab, the immune complexes were assayed for the presence of ECFP-RelA (Fig. 4F). Although the ECFP-RelA was detectable in the IgG control, a significant enrichment was seen in the IPs using anti-NIK Ab. Moreover, both ECFP-RelA WT and RelA(Ser^{536}Ala) associated with NIK, leading us to conclude that phospho-Ser^{536} is not required for NIK interaction with the RelA TADs.

Ang II-Induced NIK Activity Is Required for IKKα and RelA Phosphorylation

Our findings suggested that NIK mediates Ang II-inducible transcription and forms an inducible complex with the RelA TADs. To determine whether Ang II induced NIK activity, we developed a specific NIK IP-kinase assay. Whole-cell lysates were prepared from Ang II-stimulated HepG2 cells and IPed using anti-NIK Ab. The immune complexes were then incubated with γ[^{32}P]ATP in the presence of recombinant glutathione-*S*-transferase (GST)-IKKα T (activation) loop (44). Ang II induced a 4-fold induction of NIK activity within 15 min of stimulation that persisted for 60 min (Fig. 5A). Importantly, the Ang II-mediated induction of NIK KA was coincident with the induction of IKK KA (compare with Fig. 1C).

To confirm that the induction of NIK activity is associated with changes in phosphorylation of its endogenous target, we examined the effect of Ang II on phosphorylation of the major NIK cytoplasmic target, IKKα (44). Cell extracts from a time course of Ang II-stimulated HepG2 cells were assayed for activation loop phosphorylation at Ser^{176}. Consistently, we observed Ang II induced a 2.5-fold transient increase in phospho-Ser^{176} IKKα formation after Ang II treatment (Fig. 5B). To further confirm the function of activated NIK and phospho-IKKα, we examined the production of nuclear p52 in Ang II-stimulated HepG2 cells. We found that Ang II induced 2-fold nuclear accumulation of p52 over the same time course as the induction of KA and phospho-IKKα formation (Fig. 5C).

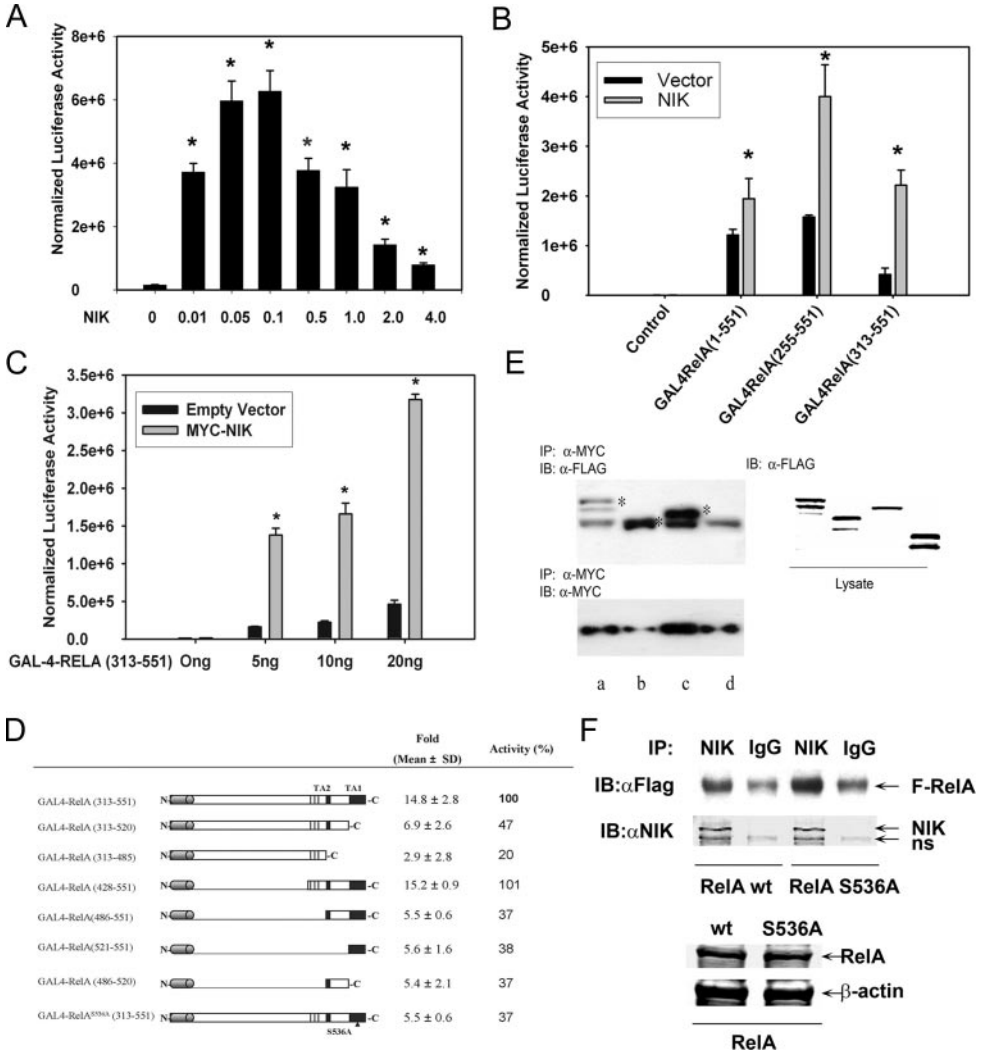

Fig. 4. NIK Transactivates RelA

A, Effect of NIK constitutively active (CA) expression on APRE LUC reporter activity. HepG2 cells transfected with APRE-LUC in the presence of increasing amounts of NIK-CA expression vector. Empty plasmid was used to keep amounts of DNA equivalent for each transfection condition. Shown is normalized luciferase reporter activity 48 h after transfection. NIK-CA produces potent APRE-LUC transactivation detectable with as little as 10 ng of expression vector. B, NIK-CA enhances activity of the RelA transactivation domain. HepG2 cells were cotransfected with UAS-LUC reporter, indicated GAL4-RelA fusion vectors [control GAL4 DNA binding domain, full-length RelA amino acids (aa) 1–551, or RelA COOH-terminal deletions from aa 255–551 or aa 313–551], in the absence or presence of NIK-CA expression vector. Shown is normalized luciferase reporter activity. NIK enhances RelA transactivation activity. C, Dose dependence of RelA TAD activation on NIK. Cells were cotransfected with UAS-LUC reporter, GAL4-RelA(313–551), and increasing amounts of NIK-CA expression vector. Empty plasmid was used to maintain identical DNA concentrations. As little as 5 ng of NIK-CA vector potently transactivates GAL4-RelA(313–551). *, $P < 0.01$. D, Functional domains of RelA in NIK-inducible transcription. Various GAL4-RelA fusions were tested for NIK-inducible transcription by cotransfection of NIK-CA and UAS-LUC. Shown are schematic diagram of the RelA transcription factor with approximate locations of the transactivation domains (TA) indicated. *Middle*, Fold change in UAS-LUC reporter activity (mean ± SD) induced by NIK relative to empty vector. At *right*, activity of various deletions were expressed as percentage of wild-type GAL4-RelA(313–551). E, RelA-interacting domains in NIK association. Selected FLAG-RelA fusions were cotransfected with full-length Myc epitope-tagged NIK expression vector. Plasmids used were: RelA(313–551), a; RelA(428–551), b; RelA(313–485), c; RelA(486–551), d. *Left panel*, Nondenaturing IP performed with anti-Myc Ab. Immunoprecipitates were fractionated and analyzed for RelA association by Western immunoblot (IB) using anti-FLAG Ab (*top*), and anti-Myc (*bottom*) as a recovery control. Specific FLAG-RelA interacting proteins are identified by *. *Right panel*, Cells were lysed and Western immunoblot (IB) was performed with anti-FLAG Ab on whole-cell extracts (WCE). Note that although Myc-NIK is recovered in panel D, RelA(486–551) is not. F, NIK-RelA complex formation is independent of RelA phosphorylation at Ser^{536}. Expression vectors encoding FLAG epitope-tagged ECFP-RelA WT and RelA Ser536Ala were transfected into HepG2 cells. WCE were prepared 48 h later, and IPed with IgG or anti-NIK as indicated. *Top*, The washed immune complexes were then assayed for the presence of FLAG-ECFP-RelA using anti-FLAG Ab by Western. *Middle*, The membrane was reprobed with anti-NIK to ensure recovery. *Bottom panel*, FLAG-ECFP-RelA expression was measured in transfected lysates by anti-FLAG Western blot. β-Actin was measured as a loading control. Both proteins are expressed at similar levels. ns, Nonspecific band; wt, wild type.

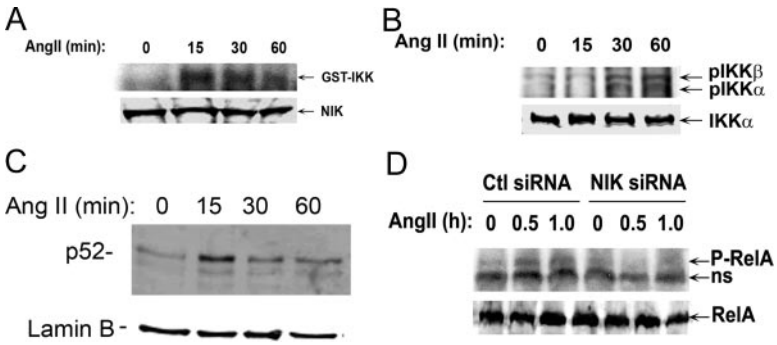

Fig. 5. Ang II Induces NIK Activity

A, Ang II induces NIK activity. AT_1-complemented HepG2 cells were stimulated with nothing or Ang II (1 μM) for indicated times (at *top*) and cell lysates were prepared. NIK was immunoprecipitated and KA was measured by incorporation of γ[^{32}P]ATP into its substrate, the IKK activation loop. *Top panel*, Autoradiogram of kinase assay. *Bottom panel*, Membrane was probed with anti-NIK Ab as a recovery control. Ang II induces NIK KA 4-fold within 15 min of stimulation. B, Effect of Ang II on phospho-IKKα. HepG2 cells were Ang II stimulated for indicated times and denatured in RIPA buffer. *Top panel*, Western immunoblot for phospho-Ser^{171} IKKα. *Bottom*, Total IKKα was measured as loading control. Fold change is normalized and phospho-Ser^{171} IKKα/total IKKα is expressed as fold change relative to unstimulated control. C, Ang II induces nuclear p52 formation. Nuclear extracts from Ang II-stimulated HepG2 cells were assayed for changes in p52 (*top panel*). Lamin B staining was used as protein recovery marker (*bottom panel*). D, Ang II-induced RelA phosphorylation requires NIK. HepG2 cells were transfected with control or NIK siRNA and Ang II stimulated for indicated times. *Top panel*, Western immunoblot for phospho-Ser^{536} RelA. *Bottom panel*, Total RelA staining was used as a loading control. Ns, Nonspecific band. See Fig. 3B for the effect of NIK siRNA on steady-state levels of NIK. Ctl, Control.

Together these data indicate Ang II induces NIK activity and activation of downstream IKKα-dependent processing activity.

We next sought to determine the requirement of NIK in RelA Ser^{536} phosphorylation. HepG2 cells were down-regulated for NIK expression using siRNA-mediated transfection and stimulated with Ang II. The time-dependent formation of phospho-Ser^{536} RelA in response to Ang II stimulation was assayed by Western blot (Fig. 5D). In the NIK down-regulated cells, a significant inhibition of phosphor-Ser^{536} RelA was observed. These data indicated that NIK is required for Ang II-induced phosphorylation of the IKKα activation loop and the RelA TAD-1 domain.

Involvement of Rho Family of GTPases in Ang II-Induced NF-κB Activation

Ang II activates the Rho family of small GTPases including RhoA, Rac, and cdc42 in vascular smooth muscle cells and myocardiocytes (45, 46). In addition, others have found that Rac-induced NF-κB activation is NIK dependent in colonic cancer cells (47). We therefore were interested in determining whether Rho GTPases mediated Ang II-induced NF-κB promoter activity.

To establish the role of RhoA in NF-κB activation, APRE-LUC-transfected HepG2 cells were cotransfected with increasing concentrations of a eukaryotic expression vector encoding *Clostridium botulinum* C3 transferase. C3 transferase catalyzes the ADP ribosylation of RhoA, thereby inhibiting its ability to exchange GTP (48). Transfectants were then stimulated with either TNF or Ang II and reporter activity was measured. Although C3 transferase expression did not have a significant effect on TNF-induced APRE-LUC activity (Fig. 6A, *top*), C3 significantly inhibited Ang II-induced APRE luciferase activity at all concentrations used in the experiment (0.1–1.0 μg), thereby demonstrating the selective involvement of RhoA in the Ang II signaling pathway.

We next evaluated whether RhoA was required for phospho-Ser^{536} RelA formation. HepG2 cells were electroporated in the absence or presence of C3 and stimulated with Ang II for 0–60 min before denaturing cell lysis. The relative changes in phospho-Ser^{536} RelA formation were then determined using Western blot. Under these conditions, we found that the rapid approximately 3-fold induction of phospho-Ser^{536} RelA formation was significantly inhibited in the presence of C3 transferase (Fig. 6B). This finding indicated that RhoA is required for both Ang II-inducible transcription and phospho-Ser^{536} RelA formation.

The implication of the RhoA GTPase prompted us to further investigate the role of other Rho GTPases because it was earlier shown that Rac could induce NF-κB activation through NIK mediated by the Rac effector, p21-activated kinase (PAK1) (47). Therefore, the effect of Rac-CA (Rac Val^{12}) was determined on Ang II-induced transcription. We observed a dose-dependent increase in Ang II-induced reporter activity, achieving a maximum 8-fold by transfection of 0.5 μg of Rac-CA (Fig. 6C). Conversely, Ang II-induced NF-κB reporter activity was significantly decreased by Rac-DN (Rac Asn^{17}, Fig. 6D). These experiments suggest Rac was also an important mediator in Ang II-induced NF-κB activation in HepG2 cells.

Ang II Induces phospho-Ser^{536} RelA Formation and Target Gene Activation *in Vivo*

Although cultured HepG2 cells have been used extensively for investigation of acute-phase reactant syn-

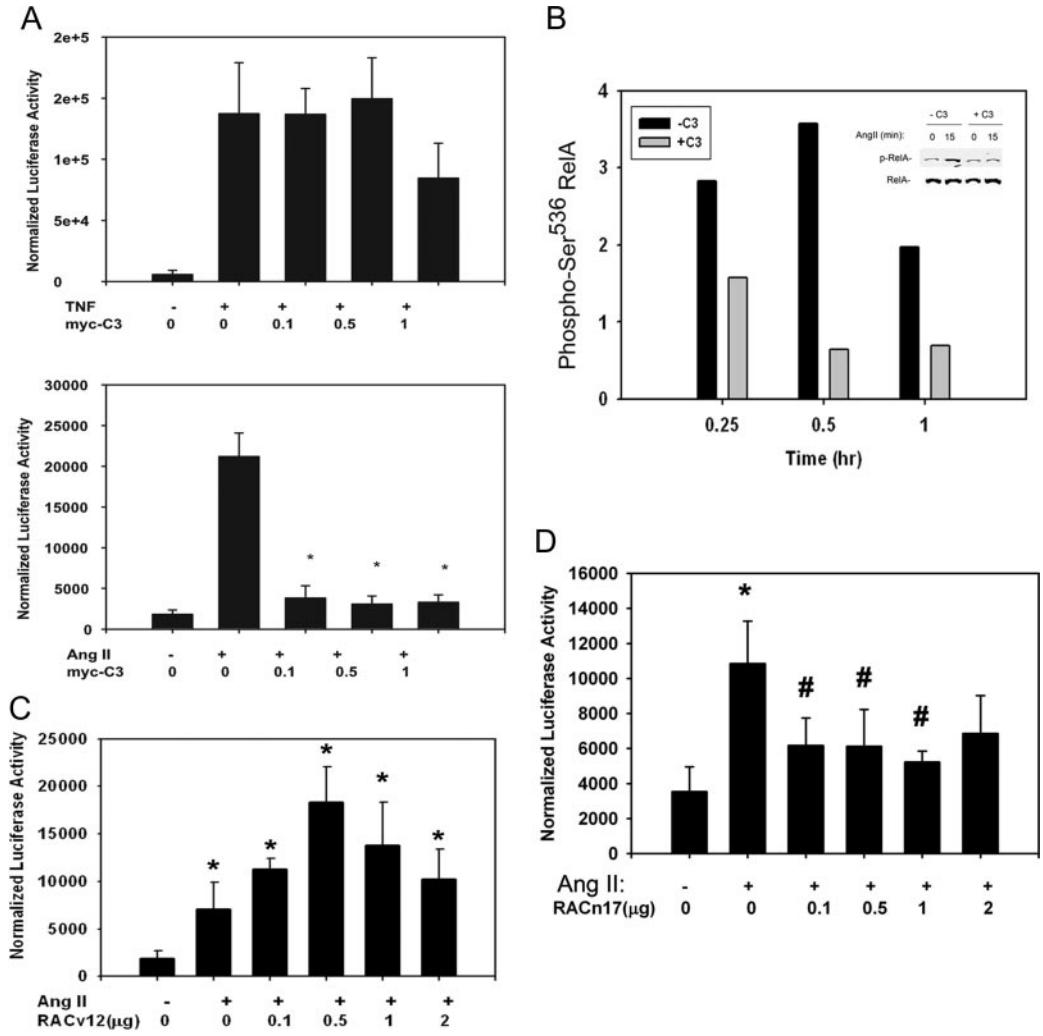

Fig. 6. Ang II-Induced Transcription Is Dependent on Rac and Rho GTPases

A, Effect of the Rho A inhibitor, C3 transferase, on NF-κB-dependent transcription. HepG2 cells cotransfected with APRE-LUC and increasing concentrations of expression vector encoding C3 transferase were stimulated with 20 ng/ml TNF (*top*), or 1 μM Ang II (*bottom*). Shown is normalized luciferase reporter activity as a mean of three independent experiments. *, $P < 0.01$ compared with stimulated values in the absence of C3. B, C3 transferase on phospho-Ser^{536} RelA formation. AdAT1a-transduced HepG2 cells were electroporated in the absence or presence of C3 transferase (*Materials and Methods*). Cells were stimulated 48 h later for indicated times in the absence or presence of Ang II (1 μM). Shown is quantitation of Western blot using near-infrared detection of phospho-Ser^{536} RelA formation normalized to β-actin as a loading control. C, Transient transfection with Rac-CA [Rac Val (12)]. HepG2 cells were cotransfected with APRE-LUC and increasing amounts of Rac-CA expression vector. Empty plasmid was used to keep amounts of DNA equivalent for each transfection condition. Rac-CA (0.5 mg) increases Ang II-induced NF-κB transcription by 2-fold. *, $P < 0.01$. D, Transfection with Rac-DN [Rac Asn (17)]. HepG2 cells were cotransfected with APRE-LUC and increasing amounts of Rac-DN expression vector. All concentrations of Rac-DN significantly inhibit Ang II-induced transcription. *, $P < 0.01$, compared with unstimulated; #, $P < 0.05$, compared with Ang II stimulated.

thesis, we sought to confirm that the Ang II-phospho-Ser^{536} RelA pathway was also active *in vivo*. For this purpose, we measured phospho-Ser^{536} RelA formation in liver isolated from Ang II-infused rats. Rats were infused with Ang II at modest pressor doses (49, 50), and abundance of phospho-Ser^{536} RelA was quantitated by sandwich ELISA. This assay has been validated to reproduce the abundance of Phospho-Ser^{536} RelA by Western blot (51). We observed an approximately 3.3-fold increase in phospho-Ser^{536} RelA formation 1 h after Ang II infusion (Fig. 7A). To further determine whether the NF-κB pathway was activated, we measured expression of endogenous IL-6, a well-established NF-κB-dependent gene downstream of Ang II signaling (14, 16). IL-6 mRNA was strongly and rapidly induced, first detectable after 15 min after Ang II infusion and peaking at 73-fold 30 min later (Fig. 7B). Together, these findings validate that Ang II induces phospho-Ser^{536} RelA formation and activation of NF-κB-dependent genes *in vivo*.

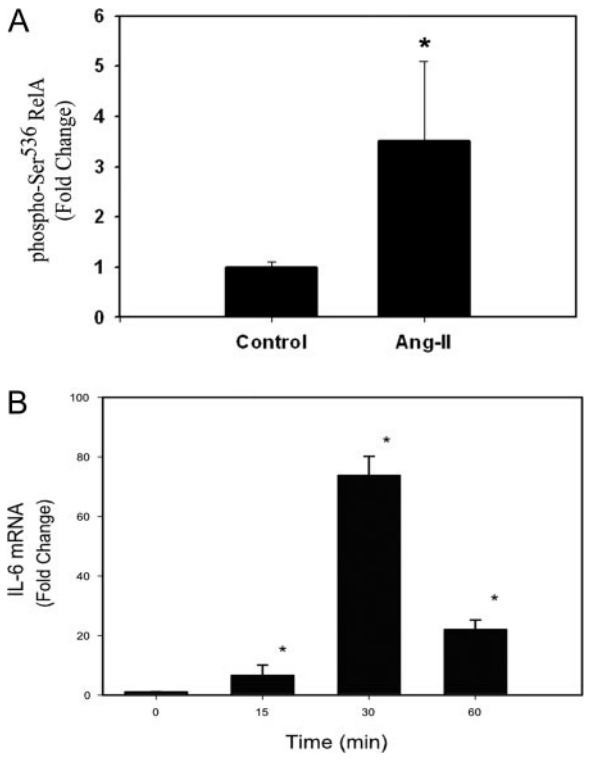

Fig. 7. Ang II Induces Hepatic phospho-Ser^{536} RelA Formation and IL-6 Expression *in Vivo*

A, Phospho-Ser^{536} RelA formation *in vivo*. Sprague Dawley rats were transiently infused with Ang II at modest pressor doses, and changes in phospho-Ser^{536} RelA formation were measured by sandwich ELISA. Shown is fold change over control values. B, IL-6 induction. Q-RT-PCR assay for IL-6 formation from the same experiment as in panel A. Shown is fold change over unstimulated values. *, $P < 0.05$.

DISCUSSION

Produced by local and circulating renin angiotensin systems, the potent vasopressor Ang II mediates inflammatory and fibrotic responses in the hepatocyte by its ability to activate the NF-κB transcription factor and control the expression of downstream gene networks (4). In this manuscript, we extend our previous findings on how Ang II signals to NF-κB in a mechanism largely independent of the canonical NF-κB activation pathway. Despite inducing no detectable changes in RelA translocation, the presence of RelA is required for mediating Ang II-induced NF-κB activation because siRNA-mediated RelA knockdown completely inhibits Ang II-induced NF-κB reporter activity, and reduction of the basal nuclear RelA by cytosolic sequestration abolishes Ang II-induced transcription. Instead, Ang II induces RelA·NIK complex formation and RelA phosphorylation at Ser^{536}. Our data indicate that NIK mediates RelA activation because NIK-DN is a potent inhibitor of Ang II-induced NF-κB activity, and NIK-CA activates the RelA TAD in the context of the GAL4 fusion proteins. Overall, we interpret our data to indicate that Ang II induces NF-κB-dependent transcription through a mechanism distinct from the canonical pathway, being independent of IκB proteolysis, but mediated by Rho/Rac-NIK-dependent Ser^{536} RelA phosphorylation and NIK complex formation.

Because of its central role in atherosclerosis, a number of studies (from our laboratory and others) have examined the effect of Ang II on NF-κB activation in vascular smooth muscle cells (VSMCs). A consistent finding is that Ang II does not strongly induce the canonical pathway at levels sufficient to detectably induce IκBα (or β) proteolysis, having been reported by at least three independent laboratories (51–53). These findings are consistent with our observations presented here (Fig. 1), and from our previous work (15) in HepG2 cells. Although we note that other laboratories have observed IκBα proteolysis in HepG2 cells (54), the reason(s) for this discrepancy is not readily apparent to us and will require further resolution. Nevertheless, constitutive nuclear RelA is found in unstimulated cells and is a consequence of its active nucleocytoplasmic shuttling. Basal RelA can be detected by Western blots (Fig. 1), and gel shift and affinity pull-down assays (15) and can be found associated with endogenous gene targets in chromatin immunoprecipitation assays (51). Our findings that expression of a nondegradable IκBα inhibits Ang II-inducible transcription, even under conditions in which no IκBα degradation can be observed (Fig. 1F), reproduces the findings of others (52). We interpret this result to mean that IκBα Mut expression inhibits basal RelA nucleocytoplasmic shuttling, thereby depleting its nuclear pool. Moreover, this apparently uninducible nuclear RelA pool is required for Ang II-inducible transcription because we find here that its selective inhibition by siRNA-mediated knockdown abrogates NF-κB-dependent transcription, establishing RelA as the NF-κB isoform that mediates the Ang II effect. Instead of canonical pathway activation, our present findings rather suggest that the Ang II effect is primarily mediated by inducing the nuclear RelA's transcriptional potential. This result is directly supported by our GAL4 experiments showing that NIK can transactivate RelA independent of its association with IκBα. Specifically, the RelA mutants (255–551 and 313–551), which are constitutively nuclear and unable to bind IκBα, can be strongly transactivated, clearly separating the contribution of the canonical pathway from the acquisition of transcriptional activation.

The NF-κB transcription factors are nuclear phosphoproteins, a modification that affects their subcellular localization, posttranslational processing, and transcriptional activity (55). RelA can be phosphorylated at multiple sites, including Ser^{276} by the catalytic subunit of protein kinase A and mitogen- and stress-activated protein kinase-1 (56, 57) Ser^{311} by protein kinase C ζ (58), at Ser^{529} by casein kinase II (59) in a stimulus- and cell type-dependent manner, leading either to an increase in RelA DNA binding and/or transcriptional activity. Of relevance here, RelA phosphorylation at Ser^{536} has been reported in response to NIK (41), IKKβ (40), and the S6 ribosomal kinase [RSK (53)]. Our data showing that Ang II induces RelA Ser^{536} phosphorylation in HepG2 cells

are consistent with the findings of our laboratory and others showing Ser^{536} phosphorylation in VSMCs (51, 61). Our data here are the first to implicate Ang II-inducible RelA Ser^{536} phosphorylation in HepG2 and demonstrate that the phenomenon occurs *in vivo* in response to Ang II. Although one study has shown that Ser^{536} phosphorylation is apparently downstream of RSK (53) in VSMCs, this finding has not been replicated in the same cells by inhibiting RSK via its upstream activating kinase, MAPK kinase (MEK)1/2 (52). Instead our siRNA knockdown experiments have indicated that NIK is required for Ang II-induced Ser^{536} phosphorylation in HepG2 cells. Our findings, of course, do not prove that NIK is the kinase that directly phosphorylates RelA; it remains possible that NIK activates IKKα or other downstream kinase that is the proximal RelA kinase. More work will be required to understand the relationship between NIK, IKKα, and RSK in response to Ang II stimulation.

NIK, also known as MEKK14, is a member of the MAP3K serine-threonine kinases that activate multiple steps in the NF-κB signaling pathway (63). In the noncanonical pathway NIK is required for phosphorylation and complex formation with IKKα to induce p100 processing into 52-kDa NF-κB2. This liberates the RelB-NF-κB2 complex to activate target genes important in lymphocyte signaling (64). The role of NF-κB2 outside of the lymphocyte has not been well defined. Although we find that NF-κB2 formation and nuclear accumulation are induced by Ang II stimulation in HepG2 cells, this isoform is unable to compensate for RelA deficiency (Fig. 1E), indicating that NF-κB2 is not a major Ang II-inducible transactivating species for this reporter gene. The role of NF-κB2 and its genetic targets in Ang II signaling will require further investigation. In a separate pathway initiated by the TNF-related peptide, lymphotoxin β, NIK is thought to mediate RelA Ser^{536} phosphorylation (41). Our data suggest that Ang II activates a pathway that results in NIK-dependent RelA Ser^{536} phosphorylation in a manner largely independent of IκBα proteolysis and RelA translocation. In further support of this newly defined pathway, recent work in phorbol ester- and ionomycin-stimulated Jurkat T lymphocytes has shown that Ser^{536} phosphorylation occurs in a RelA pool that does not bind IκBα (39). Here, the major target for Ser^{536} phosphorylation is free (non-IκBα complexed) RelA, which is undergoing nucleocytoplasmic shuttling (34).

Although NIK plays these important roles in the NF-κB pathway by serving as a scaffolding protein and functioning as a kinase, our IP-kinase assays directly demonstrate that NIK activity itself is Ang II responsive. Currently, it is known that the signaling activity of NIK is inducible by several mechanisms. In response to cytokine stimulation NIK is activated by threonine phosphorylation at Ser^{559} in its activation loop mediated by the TGFβ-activated kinase, producing subsequent oligomerization and activation of its KA (65). A separate pathway, described in response to *Helicobacter pylori* infection, Rac associates with PAK1, thereby inducing a conformational change in PAK1 allowing autophosphorylation of the autoregulatory loop of PAK1. Active PAK1, in turn, interacts with the NIK C-terminal domain, resulting in NIK activation through autophosphorylation (66). Of importance, Ang II is a potent inducer of Rac and Rho GTPases; more work will be needed to understand whether Ang II induces NIK by activation loop phosphorylation and whether PAK1 autophosphorylation plays a role.

We show here that siRNA-mediated NIK knockdown reduces RelA Ser^{536} phosphorylation and inhibits NF-κB transcriptional activity. Although RelA phosphorylation may affect its transcriptional activity via its ability to recruit coactivators, we have found also that Ang II induces NIK to complex with RelA. This raises an interesting possibility that RelA may recruit NIK to target genes, where it may play other roles in transcriptional activation, such as coactivator or histone phosphorylation. Although others have observed formation of a NIK-RelA complex and suggested that it largely depends on the RelA TAD-1 domain (41), our data instead indicate that complex formation is dependent on the TAD-2 domain. At present, we do not know the explanation for this discrepancy, other than this complex may contain cell type-specific proteins that may influence protein conformation or contacts required for protein-protein interaction. Comparing the kinetics of RelA Ser^{536} phosphorylation with its NIK complex formation (Fig. 2, A and B), suggests there are slight differences with maximum RelA phosphorylation preceding maximum NIK complex formation by approximately 15 min. Although one interpretation is that Ser^{536} phosphorylation may be required, in some way, to induce conformational changes or alter its subnuclear distribution in a way that is required for NIK complex formation, our data that a nonphosphorylated RelA (Ser^{536}Ala) mutant still strongly binds to NIK (Fig. 4F) excludes these possibilities. The processes responsible for RelA-NIK complex formation are presently unknown and will require further investigation. Also in this experiment, a slightly greater amount of RelA (Ser^{536} Ala) is found associating with NIK than the RelA wild type. This finding may be explained by the finding that RelA phosphorylation at Ser^{536} marks the protein for stimulus-induced proteasomal degradation (67, 68). It will be of interest to determine whether the phospho-Ser^{536} RelA bound with NIK is an intermediate precursor for proteolysis.

The Rho GTP-binding proteins, including RhoA, Rac, and cdc42, are well established mediators of G protein-coupled receptor signaling pathways, including those activated by Ang II (69). Activated G protein coupled receptors stimulate inactive Rho GTPase association with guanine nucleotide exchange factors to exchange GDP for GTP. Activated (GTP-bound) RhoA mediates signals that induce cytoskeletal reorganization, actin stress fiber formation, and focal adhesion organization (69, 70). In vascular smooth muscle cells, Ang II signaling rapidly activates Rho GTPases, inducing reactive oxygen species stress (71). However, their role in mediating NF-κB signaling has not been fully explored. Recently, RhoA has been found to mediate

inducible gene expression, where it is involved in Ang II-induced up-regulation of atrial natriuretic factor in cardiomyocytes; however, the mechanism for this effect was not elucidated (46). Our data here implicate RhoA signaling in mediating Ang II-induced NIK activation and RelA Ser^{536} phosphorylation. Our present data are the first to demonstrate that inhibition of RhoA by the C3 exotoxin inhibits RelA phosphorylation.

These findings are consistent with a third pathway (alternative canonical pathway) for NF-κB activation in Ang II-stimulated hepatocytes (Fig. 8.). Supported by the data of other studies (15, 51, 52), we find that Ang II does not appreciably induce IκBα proteolysis or RelA translocation. Our study further indicates this alternative canonical pathway requires involvement of Rho GTPases and induction of NIK activity. NIK controls two separate events, one involving phosphorylation and complex formation with Rel A TAD-2, and the second, initiating p52 processing, resulting in p52-RelB translocation. Based on our siRNA knockdown experiments, the RelA pathway represents the primary transcriptional activation complex in HepG2 cells.

In summary, Ang II is a potent inducer of the RelA transcriptional activation by Rho GTPases, NIK activation, and phosphorylation-induced RelA transcriptional activity. Further definition of the molecular targets will yield new ways to counteract the profibrotic and proinflammatory actions of Ang II in human disease.

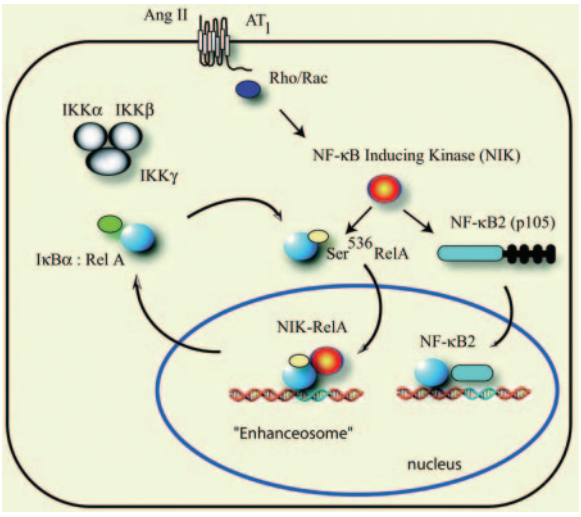

Fig. 8. Alternative Canonical NF-κB Signaling Pathway Induced by Ang II

Schematic view of the Rho/Rac-NIK-phospho-Ser^{536} RelA pathway. Ang II-induced NIK activity is mediated through the Rac/RhoA family of GTPases. An important regulatory step involves a complex formed with NIK and RelA, resulting in phosphorylation at Ser^{536} and activation of the transcriptional function of RelA. In parallel, NF-κB2 p100 processing is initiated, resulting in nuclear accumulation of NF-κB2-RelB complexes.

MATERIALS AND METHODS

Cell Culture and Treatment

The human hepatoblastoma cell-line HepG2 was obtained from American Type Culture Collection (ATCC) (Manassas, VA) and grown in DMEM (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, and antibiotics (penicillin/streptomycin/fungizone) in a humidified atmosphere of 5% CO_2. Recombinant human TNFα (30 ng/ml; Calbiochem, San Diego, CA), and Ang II (100 nM, Sigma, St. Louis, MO) were added in culture medium, and cells were incubated for the indicated time periods at 37 C. For biochemical experiments, a CsCl-purified adenovirus-expressing AT_1 receptor was used to transduce HepG2 cells (51). Cells were infected at an MOI of 5–10 in culture medium for 48 h before Ang II stimulation. Cells were serum starved in DMEM-0.1% BSA overnight before Ang II stimulation.

Plasmids and Transient HepG2 Transfections

APRE-LUC consists of the trimerized rat RelA·NF-κB1 binding sites from the rat angiotensinogen gene cloned upstream of a minimal promoter driving the expression of the firefly luciferase reporter gene (30). The eukaryotic expression vectors pRK-MycNIK and pRK-MycNIK DN, encoding NIK Thr^{559}Phe site mutant (72), the AT_1 receptor expression plasmid pEF-Bos (15), the pcDNA3-IκBα (Ser^{32}Ala/Ser^{36}Ala), and the pEF-IKKβ (Lys^{44}Met) (73) expression plasmids were previously described. Coding sequences for the FLAG epitope-tagged wild-type and Ser^{536}Ala mutation of human RelA were generated by rolling circle PCR. The coding sequences were then cloned downstream of ECFP in the pECFP-C1 plasmid (CLONTECH Laboratories, Inc., Palo Alto, CA), producing pECFP-FLAG-RelA WT, and pECFP-FLAG-RelA (Ser^{536}Ala). Plasmids were purified by ion exchange (QIAGEN, Chatsworth, CA) and sequenced to verify authenticity.

For APRE reporter assays, HepG2 cells were transfected using lipofectamine (Invitrogen, Carlsbad, CA) into triplicate 60-mm plates with a mixture of 20 μg APRE-LUC and 7.5 μg pEF-Bos into three plates, representing 6.7 μg APRE-LUC reporter, and 2.5 μg AT1 expression vector per 60-mm plate. Cells were cultured an additional 28 h, serum starved overnight, and stimulated for the indicated times (0–6 h) before harvest for independent assay of luciferase. The timing of administration of hormone was such that all plates were harvested simultaneously. Normalized luciferase was determined for each individual plate in the triplicate before calculation of the mean value for each treatment condition. Fold activation was determined by dividing the normalized luciferase activity in each treatment by the normalized value in untreated cells. For GAL4 experiments, HepG2 cells were transfected in triplicate 60-mm plates with a mixture of 6 μg UAS-LUC, 20 ng pEF-Bos, 10 ng GAL4-RelA, and 20 ng pRK NIK expression vector, representing 2 μg UAS-LUC, 6.7 ng pEF-Bos, 3.3 ng GAL4-RelA, and 6.7 ng pRK NIK per 60-mm plate. In conditions not receiving NIK, empty pRK plasmid was substituted for pRK-NIK to keep concentrations of DNA comparable.

Sucrose Density-Purified Nuclear Extracts

For nuclear purification, HepG2 cells were resuspended in buffer A [50 mM HEPES (pH 7.4), 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol (DTT), 0.1 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin A, 1 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor, 10 μg/ml aprotinin, and 0.5% Nonidet P-40]. After 10 min on ice, the lysates were centrifuged at 4000 x *g* for 4 min at 4 C. After discarding the

supernatant, the nuclear pellet is resuspended in buffer B (buffer A with 1.7 M sucrose), and centrifuged at 15,000 x *g* for 30 min at 4 C (15). The purified nuclear pellet was then incubated in buffer C [10% glycerol, 50 mM HEPES (pH 7.4), 400 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.1 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin A, 1 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor, and 10 μg/ml aprotinin] with frequent vortexing for 30 min at 4 C. After centrifugation at 15,000 x *g* for 5 min at 4 C, the supernatant is saved for nuclear extract. Both cytoplasmic and nuclear extracts were normalized for protein amounts determined by Coomassie G-250 staining (Bio-Rad Laboratories, Inc., Hercules, CA). Sucrose cushion separation results in structurally intact nuclei devoid of cytoplasmic contamination as measured by the absence of cytoplasmic markers, such as tubulin (32).

Immunoprecipitation and Western Immunoblots

Nuclear fractions corresponding to 1 mg of protein were incubated with 4 μg of indicated IgG Ab overnight at 4 C. The immunoprecipitates were captured on protein A sepharose beads, and washed four times with RIPA buffer [150 mM NaCl, 1% IGEPAL CA-630, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 50 mM Tris HCl (pH 7.5)], subjected to 10% SDS-PAGE, and transferred to Immobilon-P transfer membrane. The membrane was blotted with indicated Ab, including RelA (Santa Cruz), NIK (Sigma Aldrich). Infrared dye (IRD) conjugated donkey antirabbit IgG antibody was used for secondary detection. Western blots were imaged and quantified using Odyssey Infrared Imaging system (LICOR Biosciences, Lincoln, NE).

Immunoprecipitation-Kinase Assays

HepG2 cells transduced with Ad-AT_1 receptor were starved overnight and stimulated with TNF or Ang II for indicated times. The IKK IP-kinase assays were performed as described (74). Briefly, IKKβ immunoprecipitates were washed with PBS followed by a final wash in Kinase buffer (20 mM HEPES, pH 7.5; 10 mM $MgCl_2$; 50 mM NaCl; 20 mM β-glycerophosphate; 100 μM Na_3VO_4; 20 μM ATP; 10 μg/ml aprotinin; 2 mM DTT). The immunoprecipitates were then incubated for 30 min at 30 C with 1 μCi of [γ-^{32}P]ATP and 2 μg of recombinant purified GST-IκBα(1–51) substrate in 1× Kinase buffer. For the NIK IP-kinase assays, NIK was immunoprecipitated using anti-NIK Ab and incubated under the same conditions with 2 μg of recombinant purified GST-IKK substrate in 1× Kinase buffer. Reactions were stopped by adding 4× SDS-PAGE sample buffer and boiling for 5 min. Products were separated by 10% SDS-PAGE, electrophoretically transferred to Immobilon-P transfer membrane (Millipore Corp., Bedford, MA), and exposed to BioMax film (Eastman Kodak, Rochester, NY).

siRNA Transfection

RelA, NIK, and control siRNA (Dharmacon Smart Pools, Lafayette, CO) were transfected into HepG2 cells by TransIT-siQUEST Transfection Reagent (Mirus Corp., Madison, WI) at 50 nmol/liter final concentration. Cells were stimulated 72 h later.

Quantitative RT-PCR (Q-RT-PCR)

Q-RT-PCR assays were developed using TaqMan technology on an Applied Biosystems 7000 sequence detection system (Applied Biosystems, Foster City, CA). Applied Biosystems' 20× assay mix of primers and FAM dye-labeled IL-6 probe was used with predeveloped 18S rRNA (VIC-dye-labeled probe) TaqMan assay reagent as an internal control. Validation experiments were performed to test the efficiency of the target amplification and the efficiency of the reference amplification. All absolute values of the slope of log input amount *vs.* cycle threshold (CT) is less than 0.1. Separate tubes (singleplex) for one-step RT-PCR were performed with 50 ng RNA for both target genes and endogenous controls using TaqMan one-step RT-PCR master mix reagent kit (Applied Biosystems). The cycling parameters for one-step RT-PCR were: reverse transcription, 48 C for 30 min; AmpliTaq activation, 95 C for 10 min; denaturation, 95 C for 15 sec; and annealing/extension, 60 C for 1 min (repeat 40 times). Duplicate CT values were analyzed using the comparative CT(ΔΔCT) method. The amount of target (2^{-DDCT}) was obtained by normalizing to an endogenous reference (18S RNA) and relative to a calibrator (one control sample).

Electroporation of *Clostridium botulinum* Exoenzyme C3

HepG2 cells transduced with adAT1 were trypsinized and resuspended in DMEM. Approximately 2×10^6 HepG2 cells were electroporated in a total volume of 400 μl containing 5 μg of *C. botulinum* exoenzyme C3 (CalBiochem) in a 0.4-cm sterile cuvette (Bio-Rad Laboratories). Cells were electroporated with 450 V at 25 μF, yielding a typical time constant of 1 msec in a Bio-Rad Gene Pulser. After electroporation, cells were incubated on ice for 15 min in 1 ml of DMEM containing 10 μg/ml polymyxin-B (Sigma-Aldrich) allowing cell recovery. Cells were then rinsed three times with DMEM and cultured in complete DMEM with 10% fetal bovine serum for another 48 h before Ang II treatment.

ELISA Measurement of Phospho-Ser^{536} RelA Formation

Sprague Dawley rats (~250 gm) were anesthetized and perfused with either PBS or Ang II at 0.5 mg/kg/min in PBS in accordance with our IACUC-approved protocol. Livers were explanted 15 min later, frozen in liquid nitrogen, and pulverized. Proteins were extracted in 1× RIPA lysis buffer and Phospho-Ser^{536} RelA formation was measured by sandwich ELISA (Cell Signaling Technology, Beverly, MA) according to the supplier's recommendation. After washing, binding of horseradish peroxidase-linked secondary Ab was detected by 3,3′,5,5′ tetramethyl benzidine substrate, and absorbance was read at 450 nm. Shown is fold change of A_{450} relative to sham treatment.

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