The proinflammatory actions of Angiotensin II are dependent on p65 phosphorylation by the IκB kinase complex

The vasoactive hormone angiotensin II (Ang II) probably triggers inflammatory cardiovascular diseases by activating transcription factors such as NF-κB. We describe here a novel mode of NF-κB activation in cultured vascular smooth muscle cells exposed to Ang II. Ang II treatment resulted in an increase in the phosphorytansferase activity of the IKK complex, which was mediated through the AT1 receptor subtype. The typical phosphorylation and proteasome-dependent degradation of the NF-κB inhibitor IκBα were not observed. Rather, Ang II treatment of vascular smooth muscle cells led to the phosphorylation of p65 on serine 536, a signal detected in both the cytoplasm and the nuclear compartments. The use of pharmacological inhibitors that inhibit the activation of MEK by Ang II revealed that phosphorylation of p65 on serine 536 did not require the MEK-ERK-RSK signaling pathway. On the other hand, specifically targeting the IKKβ subunit of the IKK complex by overexpression of a dominant negative version of IκKB (IKKβ K44A) or silencing RNA technology demonstrated that the IκKB subunit of the IKK complex was responsible for the detected phosphoryserine 536 signal in Ang II-treated cells. Characterization of the signaling pathway leading to activation of the IKK complex by Ang II revealed that neither epithelial growth factor receptor transactivation nor the phosphatidylinositol 3-kinase-AKT signaling cascade were involved. Collectively, our data demonstrate that the proinflammatory activity of Ang II is independent of the classical pathway leading to IκBα phosphorylation and degradation but clearly depends on the recruitment of an IKK complex signaling cascade leading to phosphorylation of p65 on serine 536.

Hypertension is a classical risk factor for the development of atherosclerosis (for review, see Ref. 1). Epidemiological studies showed that among hypertensive patients, those who have an activated renin-angiotensin-aldosterone system have a higher incidence of myocardial infarction than other forms of hypertension (2–6). The octapeptide angiotensin II (Ang II)6 is the multifunctional effector of the renin-angiotensin system. It binds to two distinct receptor subtypes, designated AT1 and AT2 (7). Most of the known effects of Ang II are relayed through binding of the AT1 subtype, and studies in models of vascular injury have demonstrated that blockade of the renin-angiotensin system or gene disruption of the AT1 receptor can prevent the development of atherosclerosis (8–12). In addition to its vasoconstrictive role, Ang II may also act locally as a growth factor. Indeed, it is a hypertrophic factor for vascular smooth muscle cells (VSMC) and cardiac myocytes and has mitogenic effects for different cell types, such as cardiac fibroblasts (for review, see Refs. 13–15). Interestingly, other studies have demonstrated that Ang II has proinflammatory actions by promoting an increase in the expression level of vascular cell adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1) and several chemokines such as monocyte chemoattractant protein (MCP)-1, regulated upon activation, normal T-cell expressed and secreted (RANTES), interleukin (IL)-8, IL-6, and interferon-γ-inducible protein 10 (for reviews, see Refs. 16 and 17). However, the molecular understanding of the proinflammatory actions of Ang II is still unclear.

Activation of the Ang II AT1 receptor (AT1R) in VSMC is functionally linked to the recruitment and activation of cytosolic kinases such as the classical Ras/mitogen-activated protein kinase kinase-1 (MEK1)/extracellular signal-regulated kinase (ERK)/ribosomal S6 kinase (RSK), protein kinases C, calmodulin (CAM), killing, and Janus kinases. These kinases can in turn lead to the activation of transcription factors involved in gene regulation and the growth promoting effect of Ang II (15, 18). The transcription factor nuclear factor κB (NF-κB) is a key regulator of inflammation, immune response, and cellular survival (19) and the proinflammatory actions of Ang II have been attributed to its activation (16, 20–26).

The NF-κB family is represented by five members: p50, p65(ReA), c-Rel, p52, and RelB. In resting cells, they exist as homo- or heterodimers that are sequestered in the cytoplasm in an inactive form through their association with one of several inhibitory molecules, namely IκBα, -β, -ε, p105, and p100. These inhibitors mask the nuclear localization sequence in the Rel homology domain of NF-κB, thereby preventing it from accumulating in the nucleus (for recent reviews, see Refs. 27 and 28). IκBα is the most characterized member in this family. It is composed of three domains: an N-terminal signal responsive

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6 The abbreviations used are: Ang II, angiotensin II; IKK, IκB kinase; RANTES, regulated upon activation, normal T-cell expressed and secreted; EGFR, epidermal growth factor receptor; VSMC, vascular smooth muscle cells; TNF-α, tumor necrosis factor-α; EGF, epithelial growth factor, IL-6, interleukin-6; siRNA, silencing RNA; AT1R, Ang II AT1 receptor; MEK, mitogen-activated protein kinase kinase/extracellular signal-regulated kinase; ERK, extracellular signal-regulated kinase; RSK, ribosomal S6 kinase; VCAM-1, vascular cell adhesion molecule 1; ICAM-1, intercellular adhesion molecule 1; MCP, monocyte chemoattractant protein; CAM, calmodulin; PI, phosphatidylinositol; GST, glutathione S-transferase.
domain, a central ankyrin repeat domain that interacts with NF-κB, and a C-terminal PEST domain that is responsible for the basal turnover of the protein (29). In the classical pathway, the first phase of NF-κB activation mainly consists of the regulated degradation of IκBα and is triggered by prototypical activators such as tumor necrosis factor (TNF)-α, lipopolysaccharide, IL-1β, and phorbol 12-myristate 13-acetate. These stimuli induce the phosphorylation of IκBα at Ser32 and Ser36 in the N-terminal signal responsive domain by the canonical IκB kinase (IKK) complex, which is composed of two catalytic subunits called IKKα and IKKβ and one regulatory subunit IKKγ. Phosphorylated IκBα is subsequently polyubiquitinated and targeted to the 26 S proteasome complex, resulting in the release and nuclear accumulation of NF-κB, which can now stimulate target gene transcription. Studies report that Ang II can stimulate degradation of IκBα, but the decrease in its expression level is very small suggesting the existence of other pathways to regulate NF-κB activity (24, 26).

Notably, several lines of evidence suggest the existence of a second phase of NF-κB activation. Part of this second phase involves phosphorylation of the p65 subunit, which plays a key role in determining both the strength and duration of the NF-κB-mediated transcriptional response (for recent reviews, see Refs. 30 and 31). The sites of phosphorylation reported to date are Ser527 and Ser531 in the Rel homology domain and three phosphoacceptor sites located in the transactivation domain, Ser569, Ser529, and Ser536. Several candidate kinases that phosphorylate each serine residue have been identified, such as protein kinase A and mitogen- and stress-activated protein kinase-1 for Ser276 (32, 33), protein kinase Cδ for Ser311, glycogen synthase kinase-3β for Ser569, casein kinase II (CKII) for Ser529 (34), and IKKα/β as well as RSK for Ser536 (35–37). Importantly, phosphorylation at Ser536 was recently shown to reduce the ability of p65 to bind IκBα (36) and to allow the recruitment of TAFI131, a component of the basal transcriptional machinery (37). Phosphorylation at Ser536 is also responsible for recruitment of coactivators such as p300 (38) and also triggers its rapid turnover in the nucleus (39). All these evidences support the importance of p65 phosphorylation at Ser536 in the function of NF-κB. Notably, a recent study proposed that Ang II induces NF-κB through a RSK-dependent pathway leading to Ser536 phosphorylation of p65 (40). In light of these interesting findings, we have set up experiments to identify more precisely the molecular mechanism by which Ang II induces proinflammatory action in VSMC and now describe a series of studies dissecting the novel mechanism by which Ang II activates NF-κB. We show that following binding to the AT1R, Ang II leads to an increase in the phosphotransferase activity of the IKK complex, which leads to phosphorylation of p65 on Ser536. Because IκBα is not degraded in response to Ang II, we propose a model where it is the phosphorylation of p65 on Ser536 that is likely to be involved in the proinflammatory actions of Ang II in VSMC.

**EXPERIMENTAL PROCEDURES**

Reagent, Antibodies, and Plasmids—Ang II was purchased from Hubakel Scientific (St. Laurent, Quebec, Canada). EGF and TNF-α were from BioSource (Camarillo, CA). The tyrosin kinase AG1478, a selective EGFR kinase inhibitor, the PI 3-kinase inhibitor LY294002, the MEK1/2 inhibitors U0126 and PD98059 were all from Biomol (Plymouth Meeting, PA). G418 was from Invitrogen. Commercial antibodies were from the following suppliers: anti-IKKγ (SC-8330), anti-IκBα (SC-371), anti-p65 (SC-372), and anti-IκKα (SC-7218) were from Santa Cruz Biotechnology (Santa Cruz, CA); anti-β-actin clone AC-74 (A5316) was from Sigma; anti-IKKβ antibody, phospho-IκBα (Ser53) antibody (number 9241), phospho-AKT (Ser473) antibody (number 9271), phospho-ERK1/2 (Thr202–Tyr204) antibody (number 9101), phospho-p65 (Ser536) antibody (number 3031), and phospho-RSK (Ser380) antibody (number 9341) were from Cell Signaling Technology (Beverly, MA); anti-phosphotyrosine, clone 4G10, and anti-Lamin A/C were from Upstate Cell Signaling Solution (Lake Placid, NY). An antibody that recognizes both p105 and p50 was a generous gift of Dr. John Hiscott (McGill University, Montreal, Quebec). Glutathione S-transferase (GST)-IκBα (1–54) has been described (41). GST-p65 (354–551) was produced by subcloning PCR-amplified fragments in pGEX-KG. The resulting construct were transformed in *Escherichia coli* and following isopropyl β-D-thiogalac- topyranoside induction (1 mM for 3 h at 37°C), purified over a glutathione-agarose column (Amersham Biosciences). The expression plasmids pTrack-Flag-IKKβ (K44A) and pCDNA3.1-HA-AT1R were provided by Drs. John Hiscott and Stephane Laporte (McGill University), respectively.

**Cell Types and Transfection**—Rat VSMC and VSMC overexpressing a dominant-negative version of EGFR (HERCD533) (42) were obtained from Dr. Sylvain Meloche (University of Montreal, Montreal, Quebec) and Darren Richard (Laval University, Quebec, Quebec) and grown in low glucose Dulbecco’s modified Eagle’s medium supplemented with 10% calf serum, 2 mM-glutamine, and antibiotics (50 units/ml penicillin and 50 μg/ml streptomycin). Cultures were maintained at 37°C in a humidified atmosphere of 95% air, 5% CO₂. All experiments were conducted on cells at passage levels 9–14. Quiescent VSMC were obtained by incubation of 95% confluent cell cultures in serum-free Dulbecco’s modified Eagle’s medium–Ham’s F-12 (1:1) supplemented with 15 mM Hepes (pH 7.4), 0.1% bovine serum albumin, and 5 μg/ml transferrin for 48 h. For experiments with pharmacological inhibitors, the cells were treated with vehicle alone or with the indicated concentrations of inhibitors for 30 min before addition of Ang II. 293T cells were purchased from American Type Culture Collection (ATCC), cultured in minimal essential medium containing 10% fetal bovine serum, and transfected using the calcium phosphate coprecipitation method. All cells tested negative for mycoplasma contamination.

**Small Interfering RNA (siRNA)**—Two 21-nucleotides siRNA duplexes with 2-nucleotides (2-deoxy)-thymidine 3’ overhangs were obtained from Dharmacon Research (Lafayette, CO) and directed against rat IKKβ: IKKβ number 1 (nucleotide 1460 to 1478 (AAACCGCATCCAGATTGA)) and IKKβ number 2 (nucleotide 2028 to 2046 (GAATGTTCTCGACTTATG))). A siCONTROL non-targeting silencing RNA (siRNA) (number D-001210-01-20) was as described previously (41). For transfection, cells were trypsized and seeded into six-well plates (8.0 × 10⁴ cells/well) without antibiotics. After 24 h, cells were transfected with siRNA using Lipofectamine2000 (Invitrogen) according to the manufacturer’s specifications. siRNA were used at a concentration of 120 nM in transfections. Cells were harvested for analysis 72 h after transfection.

**Retrovirus Construction, Transduction, and Generation of Rat VSMC Overexpressing IκBα2NΔ4**—The pMSCVneo IκBα2NΔ4 retrovirus was a kind gift of Dr. John Hiscott (McGill University) and has been described (43). The Phoenix Amphototropic packaging cell line was transiently transfected with plasmids pMSCVneo and pMSCVneo IκBα2NΔ4. At 48 h post-transfection, retrovirus-containing medium was harvested and used to infect rat VSMC. VSMC were infected twice at 24-h intervals in the presence of 10 μg/ml Polybrene (Sigma); selection with 800 μg/ml G418 (Invitrogen) was started 5 days later. After 14 days, a population of resistant VSMC was selected. For selection of clones from both rat VSMC (Neo) and rat VSMC (IκBα2NΔ4), cloning rings were used, and expression of the transgene was verified by immuno blot analysis.
Reverse Transcriptase-PCR Analysis—Total RNA was isolated using TRIzol (Invitrogen). DNase-treated (Ambion, Austin, TX) cellular RNA (1 μg) was reverse transcribed with avian myeloblastosis virus reverse transcriptase (Sigma) in a 20-μl reaction volume according to the manufacturer’s instructions. PCRs were subsequently performed per the manufacturer’s specifications with the following primers: rat IL-6 forward and reverse, 5’-TGGTGTGGACAGCCACTGC-3’ and 5’-TTTCAGAGATGATTGGATGGTC-3’, respectively; rat MCP-1 forward and reverse, 5’-CAGGTCTCTGTACGCTTCT-3’ and 5’-AGTATTCATGGAGGGAATAG-3’, respectively, and rat RPL32 forward and reverse, 5’-GTGAAGCCCAAGATCGTC-3’ and 5’-GAACAACACACACAC-3’, respectively. PCR products were electrophoresed on 1.2% agarose gels containing ethidium bromide and visualized using a gel documentation device (Typhoon scanner 9410).

Immunoblot Analysis—After the different treatments, cells were washed twice with ice-cold phosphate-buffered saline, and whole cell extracts were prepared using Triton X-100 lysis buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 50 mM sodium fluoride, 5 mM EDTA, 40 mM β-glycerophosphate, 1 mM sodium orthovanadate, 10−8 M phenylmethylsulfonyl fluoride, 10−6 M leupeptin, 10−7 M pepstatin A, 1% Triton X-100) for 30 min at 4 °C. Lysates were clarified by centrifugation at 13,000 × g for 10 min and equal amounts of lysate proteins (30–75 μg) were subjected to electrophoresis on 7.5, 10, or 12% acrylamide gels. Proteins were electrophoretically transferred to Hybond-C nitrocellulose membranes (Amersham Biosciences) in 25 mM Tris, 192 mM glycine, and fixed for 10 min in methanol/acetic acid/glycerol (40:7:3). Immunoblot analysis of IκBα and β-actin were accomplished as described previously (41). Phospho-IκBα (Ser32), phospho-AKT (Ser473), phospho-ERK1/2 (Thr202/Tyr204), phospho-p65 (Ser536), and phospho-RSK (Ser380) were used following the manufacturer’s instructions. Both anti-p65 and anti-IKKβ were used at 1 μg/ml. Rabbit anti-p105/p50 and monoclonal antibody 4G10 were used at 1:1000 and 1:5000, respectively.

In Vitro Kinase Assays—The phosphotransferase activity of the IKK complex was measured as described previously (41). Briefly, 250 to 400 μg of whole cell extracts were incubated for 4 h at 4 °C with specific antibody to IKKγ (anti-IKKγ (SC-8330)) preabsorbed to protein A-Sepharose beads. The immune complexes were washed three times with lysis buffer and once with kinase buffer (20 mM Hapes, pH 7.4, 20 mM MgCl2, and 2 mM dithiothreitol). IKK complex activities were assayed by resuspending the beads in 40 μl of kinase buffer containing 1 μg of GST substrates (GST-IκBα or GST-p65) as indicated, 20 μM ATP, and 20 μCi of [γ-32P]ATP. The reactions were incubated at 30 °C for 30 min and stopped by the addition of 5 × Laemmlir’s sample buffer. The samples were analyzed by SDS-gel electrophoresis. Following Coomassie staining, the gels were dried and exposed to a Gel Documentation device (Typhoon scanner 9410, Amersham Biosciences) for imaging and quantification. In some experiments, the upper part of the gel was electrophoretically transferred to Hybond-C nitrocellulose membranes and immunoprecipitated IKK complex was revealed by immunoblotting.

Phosphor 32 Labeling and Immunoprecipitation—Quiescent VSMC in 100-mm Petri dishes were metabolically labeled for 4 h at 37 °C in bicarbonate and phosphate-free Hapes-buffered Dulbecco’s modified Eagle’s medium containing 0.75 mCi/ml [32P]phosphoric acid. The cells were then stimulated by the addition of 100 nM Ang II or 20 ng/ml TNF-α to the medium for the indicated periods of time. Following a quick wash with ice-cold phosphate-buffered saline, whole cell extracts were prepared as described above and precleared for 1 h with 10 μl of normal rabbit serum and incubated for 4 h at 4 °C with anti-p65 antibody preabsorbed to protein A-Sepharose beads. Immune complexes were washed six times with lysis buffer. Protein complexes were eluted by heating at 95 °C for 5 min in denaturing sample buffer and analyzed by SDS-gel electrophoresis on 10% acrylamide gels. The proteins were then electrophoretically transferred to polyvinylidene difluoride membranes (Millipore) in 25 mM Tris, 192 mM glycine, and 20% methanol, and exposed to a gel documentation device (Typhoon scanner 9410, Amersham Biosciences) for imaging and quantification. The membranes were used in immunoblotting experiments using anti-p65 antibody to verify the immunoprecipitated p65.
IKK-dependent Phosphorylation of p65 by Ang II

**Bioisynthetic Labeling Experiments**—To examine the turnover of IkBa protein, quiescent VSMC in 100-mm Petri dishes were pulse-labeled for 1 h with 166 μCi/ml of [35S]methionine and [35S]cysteine and then chased for the indicated times in serum-free medium containing excess methionine and cysteine and either Ang II or TNF-α. The cells were then washed twice with ice-cold phosphate-buffered saline and lysed in Triton X-100 lysis buffer. Lysates (500 μg of protein) were precleared for 1 h with 5 μl of normal rabbit serum and the resulting supernatants were incubated with protein A-Sepharose beads preabsorbed with 2 μg of anti-IkBα for 4 h at 4 °C. Immune complexes were washed five times with Triton X-100 lysis buffer. Proteins were eluted by heating at 95 °C for 5 min in denaturing sample buffer and analyzed by SDS-gel electrophoresis on 12% acrylamide gels. The IkBa protein was detected by fluorography and visualized using a gel documentation device (Typhoon scanner 9410, Amersham Biosciences).

**Electromobility Shift Assays**—Preparation of cytosolic and nuclear extracts as well as electromobility shift assays was accomplished as described previously (44).

**RESULTS**

**NF-κB-dependent Chemokine Gene Induction by Ang II in VSMC**—Because controversy exists in the literature as to know whether Ang II has the ability to activate NF-κB transcription factor (45, 46), we first verified the state of activation of known NF-κB-regulated chemokines, namely IL-6 and MCP-1, in VSMC stably overexpressing the super-repressor version of IkBa, IkBa2NΔ4. This mutant molecule has been shown to abrogate totally NF-κB induction by different stimuli. It involves mutation of the IkB serine phosphorylation sites to alanine (S32A/S36A) and removal of the C-terminal PEST domain to generate a form of IkB (IkBa2NΔ4). The basal turnover of this mutated IkB is diminished but most importantly, IkBa2NΔ4 is no longer responsive to inducer-mediated phosphorylation and degradation, and thus acts as a transdominant repressor of the NF-κB pathway (by sequestering NF-κB subunits in the cytoplasm) (43). Following selection of the stable transfectant, clones were isolated and analyzed for transgene expression in Western blot analysis (see Fig. 1A). Note that when the level of IkBa2NΔ4 is high, the expression of endogenous IkBa is diminished due to the fact that the IkBa gene is itself regulated by the NF-κB transcription factor (Fig. 1A, compare clones IkBa2NΔ4 number 1 with 4). This illustrates that overexpression of IkBa2NΔ4 titrates out any free NF-κB subunits thus allowing reduction in basal NF-κB transcription of target genes such as IkBa. Following Ang II stimulation, induction of both MCP-1 and IL-6 transcripts was observed in the two clones of rat VSMC expressing only the neomycin cassette (clones neo 3 and 6). However, the presence of a high level of IkBa2NΔ4 in clone 4 strongly diminished but most importantly, IkBa2NΔ4 abrogated the induction of these transcripts by Ang II (Fig. 1B). The clone expressing a moderate level of IkBa2NΔ4 (clone 1) had an intermediate effect on the induction of IL-6 and MCP-1. Together, these data clearly demonstrate a role of NF-κB in Ang II-induced proinflammatory genes in VSMC.

**IKK Complex Activation by Ang II in VSMC**—Given the essential role of the IKK in the activation of NF-κB, we next verified the phosphoryltransferase activity of this complex when VSMC were exposed to Ang II. Following immunoprecipitation of the scaffolding protein of the IKK complex, IKKγ, we observed that Ang II treatment of VSMC resulted in a time-dependent (Fig. 2A) and dose-dependent (Fig. 2B) stimulation of the IKK complex as observed by an increase in phosphoryltransferase activity toward the *in vitro* substrate GST-IκBα. The maximal increase in the phosphoryltransferase activity was observed at 1 μM Ang II and sustained for at least 60 min starting at 1 min post-stimulation (Fig. 2, A and B). Importantly, the observed IKK complex stimulation was completely abrogated when cells were preincubated with the AT1 antagonist Irbezartan, demonstrating that activation of the IKK complex by Ang II is AT1-dependent (Fig. 2B). The use of the recombinant protein GST-IκBα2N, where Ser32 and Ser36 were mutated to alanine, showed no phosphorylation under our conditions thus confirming the specificity of the immunoprecipitated IKK complex in VSMC (Fig. 2C).

**Nonclassical Activation of NF-κB by Ang II in VSMC**—Through phosphorylation-dependent degradation of IkB proteins by the proteasome, activation of the IKK complex is a rate-limiting step in the activation of the NF-κB transcription factor (27, 47). Notably, several groups have published data showing that Ang II induces weak or strong degradation of IkB proteins in VSMC (22, 24). However, another recent study reported no degradation of the IkBa/β NF-κB inhibitors in the same model exposed to Ang II (40). Given that we observed a significant increase in the phosphoryltransferase activity of the IKK complex in VSMC stimulated with Ang II (Fig. 2), we wanted to revisit the possibility that the expression level of IkB proteins might be reduced following Ang II treatment. Western blot analysis clearly demonstrated no degradation of the two NF-κB inhibitors, IkBα and IkBβ, nor phosphorylation of IkBα on Ser32 when cells were exposed to Ang II (see Fig. 3A, *panels a–c*) as opposed to TNF-α-treated cells where a specific IkBα phosphoserine 32 signal was observed after 5 min of stimulation (Fig. 3A, *panel a, lanes 11 and 12*) followed by a net decrease of the IkBα isofrom after 10 min (Fig. 3A, *panel a, lane 12*). To confirm this observation, we next determined the rate of IkBα turnover by pulse-chase experiments on VSMC treated with Ang II and TNF-α. When com-
pared with untreated cells, stimulation of cells with Ang II did not affect the stability of IκBα, as opposed to stimulation with TNF-α where 90% of NF-κB inhibitor was degraded after 15 min of treatment (Fig. 3B).

Albeit the lack of IκBα degradation by Ang II, we observed a significant DNA binding activity of p50/p65 heterodimers in VSMC (see supplemental data Fig. 1). Ang II has pleiotropic actions at multiple points in the signaling cascades activated by Ang II and leading to p65 phosphorylation in VSMC without affecting the specific phosphoserine 536 signal (Fig. 4A). Using a phosphospecific antibody, we next addressed if p65 phosphorylation was significantly increased its phosphorylation level (Fig. 4B, lane 1) and exposure to Ang II or TNF-α significantly increased its phosphorylation level (Fig. 4A). Using a phosphospecific antibody, we next addressed if p65 phosphorylation was occurring on an IKK phosphorylation site, namely Ser536. The use of pharmacological inhibitors that target specifically MEK 1/2, namely PD98059 and U0126, completely abrogated Ang II-induced RSK1 activation in VSMC without affecting the specific phosphoserine 536 signal (Fig. 5A). Inhibition of the MEK-ERK-RSK signaling cascade for up to
Ang II-induced Ser^536 phosphorylation of p65 is dependent of IKK. A, pharmacological inhibition of MEK blocks RSK activation by Ang II but not phosphorylation of p65 on Ser^536. Quiescent VSMC were pretreated for 30 min with 30 μM PD98059, 10 μM U0126, or 0.01% Me_2SO (DMSO), and then stimulated with 100 nM Ang II for 10 min. Whole cell extracts were prepared and subjected to immunoblot analysis using anti-phospho-p65 (Ser^536) antibody (upper panel), anti-p65 antibody (middle panel), and anti-phospho-RSK (Ser^380) antibody (lower panel). Membranes were stripped between each blotting experiment. B, sustained inhibition of the MEK-ERK-RSK signaling cascade does not inhibit phosphorylation of p65 on Ser^536 by Ang II. Quiescent VSMC were pretreated for 30 min with 10 μM U0126 or 0.01% Me_2SO and then stimulated with 100 nM Ang II for the indicated times. Whole cell extracts were prepared and subjected to immunoblot analysis using anti-phospho-p65 (Ser^536) (panel a), anti-p65 (panel b), anti-phospho-ERK1/2 (Thr202-Tyr204) (panel c), or anti-phospho-RSK (panel d) antibodies. C, the kinetic of Ser^536 phosphorylation of p65 paralleled the activation of the IKK complex. Quiescent VSMC were stimulated for the indicated times with Ang II. Whole cell extracts were prepared and subjected to an IKK complex kinase assay (a) as described in the legend to Fig. 2. For immunoblotting (lower panel), the upper part of the gel was probed with an anti-IKK antibody to confirm equal amounts of the immunoprecipitated kinase complex (b). WCE were also analyzed by immunoblotting using anti-phospho-p65 (Ser^536) antibody (c) and anti-p65 antibody (d). Membranes were stripped between each blotting experiment. D, Ang II treatment leads to phosphorylation of p65 on Ser^536 in the cytoplasm. Quiescent VSMC were stimulated for the indicated periods of time with Ang II. Cytoplasmic and nuclear extracts were prepared and analyzed by immunoblotting using anti-phospho-p65 (Ser^536) antibody and anti-p65. The purity of the nuclear fractions was demonstrated by the virtual exclusion of p105. Using a higher sensitivity setting on the documentation device allowed us to better appreciate the presence of p65 phosphorylated on Ser^536 in the nucleus (lower right panel). E, overexpression of a dominant negative version of IKKβ in 293T abrogates the phosphorylation of p65 on Ser^536 by Ang II. 293T cells were cotransfected with AT1R in the presence of either pFlag-IKKβ (K44A) or the vector alone. 30 h post-transfection, cells were incubated in serum-free medium for the next 18 h before stimulation with Ang II (100 nM) for the indicated periods of time. Whole cell extracts were prepared and subjected to immunoblot analysis using anti-phospho-p65 (Ser^536) antibody, anti-FLAG antibody, and anti-p65 antibody as indicated. F, reducing the expression level of IKKβ in VSMC transfected with a siCONTROL non-targeting silencing RNA (control) or two different RNA duplexes designed to specifically target IKKβ as indicated, 24 h post-transfection, cells were incubated in serum-free medium for 48 h and then stimulated with Ang II for the indicated times. Whole cell extracts were prepared and analyzed by immunoblotting using the indicated antibodies. Membranes were stripped between each blotting experiment.

FIGURE 5. Ang II-induced Ser^536 phosphorylation of p65 is dependent of IKKβ.
1 h did not prevent the rapid induction nor the sustained phosphorylation of p65 on Ser536 by Ang II (Fig. 5B). To verify the possible role of the IKK complex in Ang II-induced p65 phosphorylation on Ser536, we next verified if the activation of the IKK complex followed a kinetic similar to the phosphoserine 536 signal. A detailed kinetics in VSMC revealed that the activation of the IKK complex by Ang II was rapid and sustained for at least 24 h (Fig. 5C, panel a). Interestingly, the activation of the IKK complex followed the same kinetic of phosphorylation of p65 on Ser536 (Fig. 5C, panel c). As predicted, stimulation of the IKK complex by Ang II also resulted in an increase in phosphotransferase activity toward the in vitro substrate GST-p65-(354–551) (see supplemental data Fig. 3). Because IKK-induced p65 phosphorylation was previously shown to occur in the cytoplasm (51, 52), we next addressed in which cellular compartments phosphorylation of p65 occurred in Ang II-treated cells. Fig. 5D shows the presence of a strong inducible phosphoserine 536 signal that was predominantly detected in the cytoplasmic compartment. Interestingly, a significant amount was also observed in the nucleus. To further substantiate the hypothesis of a possible involvement of the IKK complex in Ang II-mediated p65 phosphorylation, we took advantage of a point mutant version of IKKβ (IKKβ K44A), which lacks the ATP binding site and therefore acts in a dominant negative fashion. When co-expressed with the AT1R in 293T cells, it totally prevented the phosphorylation of p65 by Ang II (Fig. 5E). To directly ask whether the IKKβ subunit of the IKK complex was responsible for the detected p65 phosphoserine 536 signal in Ang II-treated cells, we next used RNA silencing technology. Upon transfection of the indicated siRNA duplexes, the expression levels of IKKβ were down-regulated by 90% and correlated with a net decrease of the phosphoserine 536 signal in Ang II-treated cells (Fig. 5F). Silencing IKKβ in VSMC did not, however, affect the phosphorylation of the ERK isoforms by Ang II. Altogether, these data demonstrate that the IKKβ subunit of the IKK complex plays an essential role in Ser536 phosphorylation of p65 by Ang II.

IKKβ is a cytoplasmic kinase and the above results suggest that it is the cytoplasmic phosphorylation of p65 that triggers its nuclear accumulation, possibly through a process involving loss of affinity for IκBα without the requirement for its degradation (36). Consequently, to further substantiate the hypothesis of a dispensable role of the IκB inhibitors in Ang II-induced nuclear accumulation of phosphorylated p65, VSMC were pretreated with the proteasome inhibitor MG-132 and again a significant amount of phosphorylated p65 was found in the nucleus of Ang II-treated cells (see supplemental data Fig. 4).

Molecular Characterization of AT1 Receptor Signaling to the IKK Complex—Because we observed an important role of the IKK complex in the proinflammatory actions of Ang II, we next wanted to verify what might be the signaling pathways coupling the AT1R to the IKK complex. Using pharmacological as well as molecular approaches, we address the roles of the EGFR receptor and the PI 3-kinase-AKT pathway in Ang II-mediated IKK complex activation in VSMC. Fig. 6A shows that pre-treatment with the tyroptostatin AG1478, a selective EGFR kinase inhibitor that efficiently blocks the activation of ERK1 by Ang II (Ref. 41 and data not shown), did not antagonize the activation of the IKK complex. This observation is in agreement with activation of the phosphotransferase activity of the IKK complex in rat VSMC overexpressing a dominant-negative version of EGFR (HERCD533) (42) (Fig. 6B). Inhibition of the PI 3-kinase pathway by use of the specific inhibitor LY294002 inhibited activation of AKT by Ang II (data not shown), but was also without any consequence on Ang II-induced activation of the IKK complex in VSMC (Fig. 6C). Together, these data show that AT1R-mediated transactivation of the EGFR and the PI 3-kinase-AKT pathway are not involved in the induction of the IKK complex by Ang II in VSMC.

**DISCUSSION**

After primary injury of the vasculature, the inflammation response that follows is characterized by three key events: 1) increment of vascular permeability; 2) infiltration of inflammatory cells; and 3) tissue repair and remodeling (for review, see Ref. 16). Through the induction of a repertoire of NF-κB-regulated genes such as IL-6, MCP-1, IL-8, RANTES, VCAM-1, and ICAM-1, it is now well appreciated that Ang II participates in these key events of the inflammatory response leading to the development of cardiovascular diseases like atherosclerosis (16). Whereas the pathways leading to NF-κB activation following treatment with prototypical activators, such as TNF-α, lipopolysaccharide, or IL-1β, is well characterized, the molecular understanding of the signaling pathways that are involved in the coupling of G protein-coupled receptors, such as AT1R or AT2R to NF-κB, is still unclear. The results presented in this report show that Ang II activates NF-κB in cultured VSMC, and independently of IκBα phosphorylation and IκBα/β degradation (see Fig. 3). These observations strongly suggest that the classical pathway of NF-κB activation is not triggered by Ang II in VSMC. Moreover, other mechanisms described as being responsible for Ang II-induced NF-κB activation, namely p105 processing and tyrosine phosphorylation of IκBα (24, 48), are not observed in Ang II-treated VSMC. Instead, we now propose a new model where Ang II treatment leads to an AT1R-dependent activation of the IKK complex. The activated IKK complex then phosphorylates Ser536 of p65 (see Fig. 7 for the detailed mechanism). The extent to which the present mechanism can account for the known proinflammatory actions of Ang II in the cardiovascular system is unknown at the moment as other transcription factors are also involved in the proinflammatory actions of the peptide, such as CREB and AP-1 (ATF-2/c-Jun) (53, 54). However, p65 phosphorylation on Ser536 has also been observed in Ang II-treated cultured adipocytes as well as in pressure-dependent activation of NF-κB in arterial segments (55, 56). Thus, a possible molecular link may exist between two known risk factors for the development of atherosclerosis, namely obesity and hypertension, for the activation of NF-κB through p65 phosphorylation.
No Degradation of IκBα in Ang II-treated VSMC—Many studies have looked at IκB degradation when VSMC are exposed to Ang II. Intriguingly, no studies have addressed if the canonical IKK complex was activated by Ang II. Using in vitro kinase assays, our report clearly demonstrates a rapid and sustained phosphotransferase activity of the IKK complex, which was mediated by the AT1R and dependent on the concentration of Ang II used (see Figs. 2 and 5C). In response to prototypical activators of NF-κB, activation of the IKK complex normally leads to phosphorylation and degradation of IκBα. Why then does Ang II not induce the degradation of IκBα? One possible scenario that might account for this observation is the recent observation demonstrating recruitment of β-arrestin proteins to IκBα when cells are exposed to β2-adrenergic receptors (57, 58). Interestingly, this association between β-arrestin proteins and IκBα was shown to abrogate TNF-α-induced IκBα phosphorylation and degradation (57). Thus, experiments are underway to try to verify if the stimulation of VSMC with Ang II promotes the recruitment of β-arrestin proteins to IκBα, a process that could explain the incapacity of Ang II to induce the degradation of IκBα.

Phosphorylation of p65 by IKKβ—A recent report by Zhang and colleagues (40) demonstrated phosphorylation of p65 on Ser536 in VSMC exposed to Ang II. Using the MEK 1/2 inhibitor U0126 as well as silencing RNA technology, they suggested that this was essentially occurring in a RSK-dependent manner. However, several observations from our study suggest a more important contribution of the IKK complex in this process. First, our study clearly shows that the two MEK 1/2 inhibitors, PD98059 and U0126, significantly abrogated the activation of RSK by Ang II without affecting the phosphosereserine 536 signal (Fig. 5D). Second, the kinetic of the IKK complex activation follows the same kinetic as p65 Ser536 phosphorylation (Fig. 5C). Third, it was shown that IKKβ-dependent phosphorylation of Ser536 of p65 occurs in the cytoplasm (51, 52), similar to what we observed in Ang II-treated cells (Fig. 5D), whereas RSK-dependent phosphorylation of p65 happens exclusively in the nuclear compartment (36). Fourth, overexpression of a dominant negative version of IKKβ completely abrogated Ang II-induced phosphorylation of p65 on Ser536 in 293T cells expressing the AT1R (Fig. 5E). Fifth, down-regulation of IKKβ expression by siRNA technology has allowed us to conclude that activation of the IKK complex is responsible for the detected phosphosereserine 536 signal observed in Ang II-treated VSMC (Fig. 5F). We do not have an explanation for the difference between the report by Zhang and colleagues (40) and this study. Notably, the two MEK 1/2 inhibitors U0126 and PD98059 had no effect on the phosphorylation of p65 on Ser536 by Ang II in VSMC derived from Sprague-Dawley or Wistar-Kyoto rats (data not shown).

Pleiotropic Roles for the Phosphorylation of p65 on Ser536—Although the exact roles of this covalent modification of p65 are not precisely characterized, it seems to affect several aspects of NF-κB signaling. Thus, Ser536 phosphorylation of p65 has been implicated in many nuclear effects cumulating to activation of NF-κB target genes (37, 38). Through an increased association of a phosphomimetic form of p65 with TAFII31 (37), and the role of this phosphoacceptor site in recruiting RSK (38), it has become clear that phosphorylation of p65 may play a major role in NF-κB-mediated transcripational response. Ser536 phosphorylation of p65 could also affect its ability to associate with the IκBα inhibitor. Notably, p53 was recently reported to induce NF-κB activity without IκBα degradation (36) and a model has been proposed to explain the enhanced nuclear localization and increased DNA binding of NF-κB. In this model, p65 phosphorylated at Ser536 loses its affinity for IκBα and alters the basal nucleocytoplasmic shuttling properties of the NF-κB/IκBα complex favoring nuclear retention. Based on this model, our data suggest that a subpopulation of p65 phosphorylated on

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**FIGURE 7. Proposed model for the activation of NF-κB by Ang II in VSMC.** Following binding to AT1R, Ang II induces the transactivation of the EGFR that then transmits downstream signals to two different kinase modules: the Raf/MEK/ERK and the PI 3-kinase/PDK/AKT modules (42, 59, 60). These signaling cascades are not involved in the activation of the canonical IKK complex by Ang II. Activated IKK complex is likely not able to engage IκBα because of the possible recruitment of β-arrestin to the latter (57, 58). Instead, the IKK complex induces the phosphorylation of p65 at Ser536 in a complex formed of IκBα/p50/p65 in the cytoplasm (52). This complex shuttles in and out from the nucleus. A subpopulation of p65 phosphorylated at Ser536 loses its affinity for IκBα (36) thereby allowing a significant nuclear accumulation of p65 and engagement of the latter with coactivators (CBP/p300) (38) to the β-response element found on NF-κB-regulated genes. Phosphorylation of p65 on Ser536 could also be involved in the increase turnover of the protein (39) thus regulating the overall NF-κB response.
Ser536 loose their affinity for IkBα and accumulate into the nuclear compartment (see Fig. 5D and supplemental data Fig. 4). Finally, phosphorylation of p65 on Ser536 also triggers its rapid turnover in the nucleus (39). Interestingly, we also noticed a prolonged nuclear signal of p65 phosphorylated on Ser536 in the presence of MG132 (compare Figs. 5D and supplemental data Fig. 4). Thus, it is likely that the induction of phosphorylation of p65 by Ang II regulates the overall NF-κB response through both positive inputs (increase in nuclear accumulation and p65 transcriptional activity) as well as negative inputs (increase in p65 turnover).

**ATIR Coupling to the IKK Complex**—The mechanisms by which G protein-coupled receptor are able to transduce a signal from the cellular environment to the cytoplasm have been studied for decades. Albeit the essential roles of heterotrimeric G proteins in this process, it has now become well appreciated that G protein-coupled receptors use tyrosine kinase receptors to activate multiple signaling pathways, most of which influence cell growth and survival. Thus, for ATIR, it has been clearly established that transactivation of the EGFR is essential for activation of the Raf/MEK/ERK as well as PI 3-kinase/PDK/AKT modules (42, 59, 60). In addition to ERKs and AKT, activation of NF-κB is also linked to cellular growth and survival, and with the observations that both PDK1 and AKT can act as IKK-activating kinases (61, 62), it was therefore important to address if EGFR transactivation and the PI 3-kinase/PDK/AKT module were involved in the activation of the IKK complex by Ang II. A pharmacological approach using EGFR kinase and PI 3-kinase inhibitors and a molecular approach using VSMC overexpressing a dominant negative version of EGFR have allowed us to conclude that neither EGFR transactivation nor the PI 3-kinase pathway were involved in ATIR-mediated activation of the IKK complex.

In conclusion, our data suggest that ATIR is coupled to a nonclassical mode of activation of NF-κB in VSMC involving an IKK complex/p65 Ser536 signaling cascade. Understanding the role of inflammation in atherosclerosis might pave the way for the development of new therapeutics to treat this cardiovascular disease such as IKKβ inhibitors (28). While our paper was submitted, a related paper was published (63) indicating a role of IKKβ in p65 phosphorylation by Ang II.

**Acknowledgments**—We thank Drs. Sylvain Meloche, John Hiscott, and Darren Richard for reagents used in this study and Dr. Guy Servant for helpful discussions.

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