Angiotensin II Activates Nuclear Transcription Factor \( \kappa B \) Through AT\(_1\) and AT\(_2\) in Vascular Smooth Muscle Cells

Marta Ruiz-Ortega, Oscar Lorenzo, Mónica Rupérez, Sven König, Burghardt Wittig, Jesús Egido

Abstract—Nuclear factor-\( \kappa B \) (NF-\( \kappa B \)) regulates many genes involved in vascular physiopathology. We have previously observed in vivo NF-\( \kappa B \) activation in injured vessels that diminished by angiotensin-converting enzyme inhibition. In the present work, we investigated the effect of angiotensin II (Ang II) on NF-\( \kappa B \) activity in rat vascular smooth muscle cells, evaluating the molecular mechanisms and the specific receptor subtype involved. Ang II increased NF-\( \kappa B \) DNA binding (5-fold, \( 10^{-9} \) mol/L at 1 hour; electrophoretic mobility shift assay), nuclear translocation of p50/p65 subunits, and cytosolic inhibitor \( \kappa B \)α (1kBα) degradation. Ang II elicited NF-\( \kappa B \)--mediated transcription (transfection of a reporter gene) and expression of NF-\( \kappa B \)--related genes (monocyte chemoattractant protein-1 and angiotensinogen). AT\(_1\) (DUP753) and AT\(_2\) (PD123319 and CGP42112) receptor antagonists inhibited Ang II–induced NF-\( \kappa B \) DNA binding in a dose-dependent manner (\( \approx 85\% \) for each one; \( 10^{-5} \) mol/L at 1 hour). The AT\(_2\) agonist \( \alpha \)-aminophenylalanine\(^6\)--Ang II augmented NF-\( \kappa B \) binding (4.6-fold, \( 10^{-9} \) mol/L at 1 hour), p65 nuclear levels, and transcription of an NF-\( \kappa B \) reporter gene. AT\(_1\), antagonist markedly inhibited NF-\( \kappa B \)--mediated transcription and gene expression. Some differences between AT\(_1\)/AT\(_2\) intracellular signals were found. Antioxidants and ceramide inhibitors, but not protein kinase C inhibitors, diminished NF-\( \kappa B \) activation elicited by both Ang II and the AT\(_2\) agonist, while tyrosine kinase inhibitors only decreased Ang II–induced NF-\( \kappa B \) activity. Our results demonstrate that Ang II activates NF-\( \kappa B \) via AT\(_1\) and AT\(_2\), although NF-\( \kappa B \)--mediated transcription occurred mainly through AT\(_1\). Both receptors share some signaling pathways (oxygen radicals and ceramide); however, tyrosine kinases only participate in AT\(_1\)/NF-\( \kappa B \) responses. These data provide novel insights into Ang II actions, suggesting a potential implication of the AT\(_2\) in the pathobiology of vascular cells.

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Key Words: angiotensin II ■ nuclear factor-\( \kappa B \) ■ receptors ■ vascular smooth muscle cell

Angiotensin II (Ang II) is the main effector peptide of the renin-angiotensin system that plays an important role in several cardiovascular diseases associated with vascular smooth muscle cell (VSMC) growth and inflammation, including hypertension, atherosclerosis, restenosis after balloon injury, and myocardial infarction.\(^1\) Ang II exerts its biological effects through the stimulation of specific receptors located on the cell surface. The use of selective antagonists has revealed the heterogeneity of Ang II receptors (AT-Rs),\(^2\) and different cDNAs corresponding to each receptor have been isolated.\(^2,3\) AT\(_1\) mediates many important cardiovascular responses, such as vasoconstriction, vascular and cardiac remodeling, and cell survival/death\(^1-3\) and evokes several intracellular signals such as calcium mobilization and activation of protein kinases, including protein kinase C (PKC) and mitogen-activated protein (MAP) cascade.\(^4\) AT\(_2\) is involved in some Ang II actions, including apoptosis and inflammatory cell recruitment, and elicits different second messengers, such as MAP phosphatase activation and kinase inhibition.\(^3,5\)

Recent studies have shown that Ang II activates some nuclear transcription factors. In VSMCs, Ang II activates the signal transducer and activator transcription factor (STAT) and activator protein-1 (AP-1) through AT\(_1\).\(^6,7\) We have recently demonstrated that Ang II activates nuclear factor-\( \kappa B \) (NF-\( \kappa B \)) in VSMCs and mesangial cells.\(^8,9\) However, AT-R subtype and molecular mechanisms of this process have not been elucidated. NF-\( \kappa B \) could play an important role in cardiovascular pathophysiology through the regulation of several genes, including cytokines, adhesion proteins, NO synthase, and angiotensinogen, as well as other products involved in atherosclerosis, inflammation, proliferation, and immune response.\(^10,11\) Elevated tissular NF-\( \kappa B \) activity has been described in an experimental model of atherosclerosis, correlated with increased macrophage infiltration and monocyte chemoattractant protein-1 (MCP-1) expression, which...
Ang II Activates NF-κB via AT<sub>1</sub> and AT<sub>2</sub>

Results

Ang II Activates NF-κB Through AT<sub>1</sub> and AT<sub>2</sub> in Cultured Rat VSMCs

Ang II increased NF-κB DNA binding activity at as early as 30 minutes, peaked at 1 hour, and declined by 2 hours. The maximal response was observed with 10<sup>-9</sup> mol/L Ang II (5-fold over control, n = 10, P < 0.05), with an intensity similar to that of 100 U/mL tumor necrosis factor-α (TNF-α) (Figure 1A). VSMCs were preincubated with specific AT-R antagonists, DUP753 for AT<sub>1</sub> and PD123319 for AT<sub>2</sub>. Both antagonists partially blocked the Ang II–induced NF-κB DNA binding activity at all time points. The maximal inhibitory effect was found after 30 minutes (Figure 1B). The inhibitory effect of each antagonist was dose dependent, being maximal with 10<sup>-3</sup> mol/L (88% and 84% inhibition versus Ang II alone, for DUP753 and PD123319, respectively, after 1 hour; n = 6, P < 0.05) (Figure 1C). When both antagonists were added simultaneously, a marked inhibition of the Ang II effect was observed (96% inhibition, 10<sup>-5</sup> mol/L, n = 3). Neither DUP753 nor PD123319 alone significantly affected NF-κB activation in unstimulated cells (0.94- and 1-fold over control, respectively; n = 6, P = NS) (Figure 1C). In VSMCs, CGP42112 has been used to demonstrate AT<sub>2</sub> binding sites. CGP42112 alone activated NF-κB, maximal at 10<sup>-5</sup> mol/L (4.5-fold over control; n = 4, P < 0.05) showing an agonist effect, as previously reported. CGP42112 blocked Ang II action (10<sup>-5</sup> mol/L, 82% inhibition versus Ang II alone, at 1 hour; n = 4, P < 0.05). To further demonstrate that the receptor subtype was associated with NF-κB activation, we used an AT<sub>2</sub> agonist, p-aminophenyllalanine–Ang II (pNH<sub>2</sub> FAII). Treatment for 1 hour with pNH<sub>2</sub> FAII increased NF-κB DNA binding activity in a dose-dependent manner (Figure 1D), with a maximal response at 10<sup>-7</sup> mol/L (4.6-fold, n = 4, P < 0.05). The specificity was demonstrated by the fact that only PD123319 blocked the pNH<sub>2</sub> FAII-induced NF-κB activation (Figure 1D). On the whole, these results suggest that Ang II–induced NF-κB DNA binding activity was mediated by both AT<sub>1</sub> and AT<sub>2</sub>.

We have also observed that, in our experimental conditions, VSMCs express AT<sub>1</sub> at gene and protein levels (see online Materials and Methods, available at http://www.circresaha.org). These data are in agreement with previous studies showing detectable AT<sub>2</sub> binding sites in cultured VSMCs.14

Ang II Translocates p50/p65 NF-κB Complexes Into the Nuclei and Degrades Cytosolic Inhibitor kB (IκB)

We have studied the composition of NF-κB complexes induced by Ang II in rat VSMCs (see online Materials and Methods, available at http://www.circresaha.org). By super-shift assays, we have observed that the NF-κB complex activated is a p50/p65 heterodimer. After 1 hour of Ang II stimulation, a translocation of p50 and p65 subunits from cytosol to nuclei was observed (immunofluorescence and Western blot). pNH<sub>2</sub> FAII upregulated nuclear p50 and p65 levels, with a maximal response at 10<sup>-9</sup> mol/L and with an intensity and kinetics similar to those of Ang II. These data suggest that AT<sub>2</sub> is involved in the transcriptional regulation of NF-κB–controlled genes. NF-κB activation involves dissociation of IκB by phosphorylation and subsequent degradation. On Ang II stimulation, cytosolic IκBα was rapidly degraded, whereas IκBβ remained unchanged. This effect was closely correlated with the time course of Ang II on NF-κB activation and with the translocation of p50/p65 to the nuclei. After 2 hours, Ang II treatment increased cytosolic IκBα levels, probably because of new protein synthesis.

Materials and Methods

Thoracic aortic rat VSMCs were serum starved for 48 hours and used for experiments. AT<sub>1</sub> and angiotensinogen mRNA expression were analyzed by reverse transcriptase–polymerase chain reaction (RT-PCR), and MCP-1 expression was analyzed by Northern blot.

Western Blot and Immunohistochemistry for AT-R

Total proteins were resolved in 12% SDS-PAGE gels, transferred, blocked, and incubated with specific AT<sub>1</sub> and AT<sub>2</sub> antibodies for 18 hours at 4°C. Detection was performed with peroxidase-conjugated secondary antibody and developed using an enhanced chemiluminescence kit (Amersham). For immunoperoxidase staining, cells were fixed in methanol/acetone at –20°C and incubated with primary antibodies and then with peroxidase-conjugated secondary antibody.

NF-κB DNA Binding Activity

Nuclear and cytosolic extracts were prepared by homogenization and centrifugation. NF-κB activity was determined in nuclear extracts by binding with labeled NF-κB consensus and analyzed by electrophoretic mobility shift assay (EMSA). To quantify nuclear p50 and p65 levels and cytosolic IκBα and IκBβ, Western blot analyses were done. For immunofluorescence staining, cells were fixed in paraformaldehyde for 10 minutes on ice followed by 0.1% Triton X-100 for 1 minute and then incubated with antibodies against p50/p65 subunits and with FITC-labeled IgG as secondary antibody.

Transient Transfections and Luciferase Assay

Double transient transfections of growth-arrested VSMCs with NF-κB/luc and thymidine kinase (TK)–Renilla were performed by particle-mediated gene transfer, with the Biolistic PDS-1000/He System (Bio-Rad Laboratories) and gold microcarriers coated with DNA, into quiescent VSMCs. After transfection, cells were serum starved for 24 hours before stimulation. Lysates were assayed for luciferase and Renilla activities (Promega).

Statistical Analysis

Results are expressed as n-fold increase over control in densitometric arbitrary units and as mean ± SEM of the experiments performed. Significance was established using the GraphPAD Instat program with the Student t test; differences were considered significant if the P value was < 0.05.

An expanded Materials and Methods section is available online at http://www.circresaha.org.
When cells were pretreated with either AT₁ or AT₂ antagonists, an inhibition of Ang II–induced IκBα degradation was observed (Figure 2), suggesting that both receptors participate in this process.

**Molecular Mechanisms of Ang II–Induced NF-κB Activation**

We next investigated which intracellular signaling responses elicited by Ang II could be involved in NF-κB activation in VSMCs, trying to elucidate differences between AT₁ and AT₂. For this reason, we have used different inhibitors (see online Materials and Methods, available at http://www.cirresaha.org). PKC inhibitors did not modify the NF-κB activation induced by Ang II or the AT₂ agonist (Figure 3A), suggesting that PKC is not involved in this process. Phosphotyrosine kinase (PTK) inhibitors caused a marked reduction in Ang II–induced NF-κB DNA binding activity (genistein, 95% inhibition, at 10⁻⁶ mol/L; n=4; P<0.05).

**Figure 1.** A, Effect of Ang II on NF-κB activity in VSMCs. VSMCs were incubated with Ang II (10⁻⁹ mol/L) for 30, 60, and 120 minutes. In parallel experiments, cells were treated for 60 minutes with Ang II (10⁻⁷ and 10⁻⁹ mol/L) and TNF-α (100 U/mL). Competition assays with a 100-fold excess of unlabeled or mutant NF-κB and unrelated (AP-1) oligonucleotides show specific NF-κB complexes. Ang II activates NF-κB through AT₁ and AT₂. B, Time course. Cells were preincubated for 30 minutes with the AT₁ DUP753 (10⁻⁶ mol/L) and the AT₂ PD123319 (10⁻⁵ mol/L) antagonists and then stimulated with 10⁻⁹ mol/L Ang II for 30 and 60 minutes. C, Dose response. Cells were pretreated for 30 minutes with DUP753 and PD123319 alone (10⁻⁵ to 10⁻⁷ mol/L) or in combination (10⁻⁵ mol/L), and then stimulated with 10⁻⁹ mol/L Ang II for 60 minutes. Both antagonists at 10⁻⁵ mol/L had no effect on NF-κB activity in control cells. D, AT₂ agonist activates NF-κB. VSMCs were incubated with pNH₂FAII (10⁻⁷ to 10⁻¹¹ mol/L) for 60 minutes. At some points, cells were pretreated with DUP753 or PD123319 (10⁻⁵ mol/L). Upper panels show representative autoradiograms from 7 to 10 different EMSA experiments with similar results. Positions of specific NF-κB complexes and free oligonucleotide are indicated (arrows). Lower panels show values of mean±SEM obtained by densitometric analysis. *P<0.05 vs control; †P<0.05 vs Ang II; ‡P<0.05 vs pNH₂FAII.

**Figure 2.** Role of AT-R antagonists on IκBα. Cells were preincubated for 30 minutes with DUP753 (10⁻⁶ mol/L) and PD123319 (10⁻⁵ mol/L) and then stimulated with 10⁻⁹ mol/L Ang II for 60 minutes. Upper panels, Representative experiment of 4 with comparable results; lower panel, mean±SEM. *P<0.05 vs control.

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In contrast, they had no effect on the AT<sub>2</sub> agonist (Figure 3B), suggesting that activation of PTK could be involved in Ang II–induced NF-κB response via AT<sub>1</sub>. NF-κB activation is also mediated by active oxygen radicals. Structurally diverse antioxidants markedly diminished the NF-κB activation elicited by Ang II and the AT<sub>2</sub> agonist pNH<sub>2</sub>FAII (Figure 3C). Another possible signaling pathway of NF-κB activation could involve ceramide production. The inhibitor of ceramide synthase fumonisin B<sub>1</sub> inhibited the NF-κB activation induced by Ang II and pNH<sub>2</sub>FAII (Figure 3D), suggesting that ceramide could be a mediator of Ang II/AT<sub>2</sub>–induced NF-κB activation.

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**Figure 4.** Transient transfection of the NF-κB promoter in quiescent VSMCs. Quiescent cells were treated with 10<sup>-7</sup> mol/L Ang II, PMA, or pNH<sub>2</sub>FAII for 24 hours. Cells were pretreated for 30 minutes with DUP753 and PD123319 alone (10<sup>-7</sup> mol/L) or in combination and then stimulated with 10<sup>-7</sup> mol/L Ang II for 24 hours. Data are expressed as n-fold increase vs control, corrected by the Renilla values. Data are mean±SEM of 4 experiments performed in triplicate. *P<0.05 vs control; †P<0.05 vs Ang II.

**Effect of Ang II on NF-κB–Mediated Gene Transcription**

To investigate the effects of the Ang II receptor antagonists on NF-κB–mediated gene expression, two different strategies were followed; these were transient transfection with a reporter plasmid containing NF-κB promoter binding sites and gene expression analysis of NF-κB–related genes.

Growth-arrested VSMCs were cotransfected with NF-κB/ luc and TK-Renilla (internal control) by Biolistic gene transfer. Then, cells were serum starved for 24 hours before stimulation with 10<sup>-7</sup> mol/L Ang II or phorbol 12-myristate 13-acetate (PMA) for an additional 24 hours, and luciferase activity was measured. Ang II activated the expression of the reported NF-κB/luc plasmid (6-fold versus control; n=5, P<0.05), with a similar response to PMA. No increase was seen with the control plasmid (not shown). pNH<sub>2</sub>FAII also increased luciferase activity (3.7-fold, P<0.05; Figure 4).

When cells were pretreated with either AT<sub>1</sub> or AT<sub>2</sub> antagonists, a partial decrease of Ang II–induced NF-κB–mediated transcription was observed that was completely blocked when both antagonists were added together (Figure 4, 95% inhibition versus Ang II alone; n=5, P<0.05). Interestingly, the inhibitory effect of DUP753 was higher than that of PD123319, suggesting that although Ang II increases NF-κB–mediated transcription through AT<sub>1</sub> or AT<sub>2</sub>, the AT<sub>1</sub>/NF-κB pathway seems to be more active.

We further evaluated the role of AT-R in some NF-κB–controlled genes. Ang II stimulation increased MCP-1 mRNA...
These data suggest that MCP-1 and angiotensinogen gene expression (Figure 5), whereas PD123319 only pro-
duced a slight decrease. pNH²FAII also increased MCP-1 and diminished after 24 hours, as previously shown.⁸,⁹

Ang II acts through two specific receptor subtypes, AT₁ and AT₂. AT₁ predominates in vascular tissues and is responsible for most of the physiopathological actions of Ang II.²,³ AT₂ is mainly present in fetal tissues and is reexpressed in patho-
logical conditions, such as myocardial infarction and cardiac hypertrophy,³ as well as in cultured VSMCs,¹⁴ as shown in this article. Pharmacological and molecular tools are contrib-
ting to the elucidation of the AT₁/AT₂ functions. Specific receptor antagonists have demonstrated that AT₁ is involved in cell proliferation, production of cytokines, and matrix proteins,²,³,¹⁸ and in the pathogenesis of Ang II–induced hypertension and cardiac hypertrophy.¹⁹,²⁰ AT₂ participates in cell growth inhibition, neointimal formation after vascular injury, trophic effects of VSMCs, and blood pressure control.³ Some Ang II responses could be mediated by both receptors, including NO release, collagen synthesis and α2-adrenoreceptor activity.²¹–²³ In VSMCs we have demonstrated that both AT₁ and AT₂ activate nuclear NF-κB DNA binding activity that is functional in its ability to transactivate κB-containing promoters. The AT₁ antagonist markedly inhibited NF-κB–mediated gene transcription. Ang II via AT₁ increases interleukin (IL)–6 and angiotensinogen mRNA through an NF-κB–mediated transcriptional mechanism.¹¹,¹⁸ In the same way, Ang II–induced MCP-1 mRNA was diminished by AT₁ antagonist and by different NF-κB inhibitors. However, AP-1 and NF-κB cooperate in the MCP-1/ IL-1β response,²⁴ showing a potential role for other transcription factors. Ang II–induced hypertension in rats is characterized by marked monocyte infiltration and vascular cell adhesion molecule and MCP-1 expression in the aor-
ta.²⁵,²⁶ ACE inhibitors reduce the presence of monocyte/mac-
rophages in the vessel wall of hypertensive rats.²⁷ In an experimental model of atherosclerosis, ACE inhibition dimin-
ished NF-κB activity and chemokine expression in the lesion.⁸ All of these data suggest that Ang II could contribute to inflammatory events in atherosclerosis and hypertension through vascular inflammatory genes, by the AT₁/NF-κB pathway. In contrast, we have observed that an AT₂ agonist increased NF-κB DNA binding and mediated gene transcription. AT₂ upregulates the chemokine RANTES and renal inflammatory cell recruitment,⁵ showing a possible gene target for the AT₂/NF-κB pathway. These data suggest that Ang II, acting mainly via AT₁, and in particular conditions through AT₂, could regulate several NF-κB–related genes involved in the pathogenesis of cardiovascular diseases.

NF-κB has been implicated in the transcription of genes mediating cell growth control, but its role in growth regulation remains to be established. The development of athero-
sclerotic fibrous plaques is due to activation of VSMCs, which proliferate and increase matrix deposition. Activation of NF-κB has been observed in human atherosclerotic lesions and in cultured VSMCs in a proliferative state.⁶,¹² Apoptosis of VSMCs and macrophages has been found in atheroscle-
rotic lesions.²⁸ In most cell types, NF-κB mediates cell survival signals, protecting cells from apoptosis, but under certain conditions it may also induce apoptosis.²⁹ In vitro experiments suggest that Ang II may cause growth via AT₁ and apoptosis via AT₂. In vivo, stimulation of AT₁ or AT₂ causes apoptosis in the media of blood vessels.³⁰ Among the intracellular mechanisms elicited by AT₂, ceramide produc-

**Discussion**

Ang II acts through two specific receptor subtypes, AT₁ and AT₂. AT₁ predominates in vascular tissues and is responsible

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**Figure 5.** A, Analysis of MCP-1 mRNA expression in VSMCs. Quiescent cells were treated with 10⁻⁷ mol/L Ang II for 6 hours. TNF-α (100 U/mL) was used as positive control. Cells were pre-
treated for 30 minutes with DUP753 and PD123319 and then stimulated with 10⁻⁻⁷ mol/L Ang II for an additional 6 hours. Left panels, Effect of NF-κB inhibitor (MG132; 10⁻⁵ mol/L) on MCP-1 mRNA expression induced by Ang II and AT₂ agonist, MCP-1 (upper panels) and GAPDH (middle) mRNA expression was determined by Northern blot. Shown is a representative autoradiogram from 5 different experiments with identical results. Lower panels, Mean±SEM obtained by densitometric analysis. *P<0.05 vs control values. B, Ang II increased angiotensinogen gene expression via AT₁. Quiescent VSMCs constitutively express angiotensinogen (Ao) mRNA as shown by RT-PCR. Cells were stimulated for 6 hours with 10⁻⁻⁷ mol/L Ang II. Shown is a representative RT-PCR of 2 performed; GAPDH was used as internal control.

levels at as early as 3 hours, being maximal at 6 hours (10⁻⁻⁷ mol/L; 5-fold versus control, n=5, P<0.05, Northern blot) and diminished after 24 hours, as previously shown.⁸,⁹ DUF753 significantly diminished Ang II–induced MCP-1 gene expression (Figure 5), whereas PD123319 only pro-
duced a slight decrease. pNH²FAII also increased MCP-1 mRNA but to a lesser extent than Ang II (Figure 5). In addition, inhibitors of NF-κB activation, MG132 (Figure 5B), glitoxin, and pyrrolidine dithiocarbamate (not shown) di-
minated MCP-1 mRNA induction caused by Ang II. By RT-PCR, Ang II upregulated angiotensinogen mRNA at 6 hours, which was only diminished by DUP753 (Figure 5B). These data suggest that MCP-1 and angiotensinogen gene expression were mainly mediated by AT₁.
A similar behavior was observed with TNF-α, which increases ceramide production; activates NF-κB; and, depending on cell culture conditions, causes cell proliferation or apoptosis. Although many studies have been done, future work is necessary to completely understand the in vivo relation between NF-κB, cell growth/apoptosis, and the role of AT-R in these processes.

AT₁ and AT₂ are coupled to G proteins and belong to the 7-transmembrane-domain receptor family. 2 Although the intracellular signalings elicited after activation of the AT₁ and AT₂ are different, our data suggest that both receptors share a common molecular pathway: the activation of NF-κB. Many agents activate NF-κB, but the mechanisms are not well understood. 10 Recently, 2 different pathways leading to NF-κB activation have been suggested, because sanguinarine blocked NF-κB activation caused by okadaic acid, PMA, TNF-α, and IL-1β, but not by ceramides and H₂O₂. 32 Reactive oxygen metabolites (reactive oxygen species; ROS) serve as common intracellular agents for NF-κB by a wide range of stimuli. We have demonstrated that a variety of structurally diverse antioxidants blocked the activation of NF-κB elicited by Ang II and AT₂ agonist, suggesting that ROS act as activators of IκBα degradation and subsequent liberation of active NF-κB. Many studies have shown that ROS act as mediators of proinflammatory cytokines, such as IL-1β and TNF-α, providing a point for “cross talk” between Ang- and cytokine-activated second messenger pathways, and supporting the emerging idea of Ang II as a true cytokine. Ang II-mediated gene transcription occurred mainly through AT₁, because the AT₁ antagonist markedly inhibited NF-κB-mediated gene transcription and Ang II–induced overexpression of related genes, such as angiotensinogen, chemokines (MCP-1), cytokines, 22 and adhesion molecules, 26 involved in the development of atherosclerosis. Finally, our data also showed a novel action of AT₂, the activation of NF-κB, which suggests a potential involvement in the pathogenesis of cardiovascular diseases.

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Methods

Materials

All culture reagents were purchased from Gibco BRL (Paisley, Scotland, UK). Dup753 was provided by MSD (Spain). NF-κB consensus oligonucleotide 5'-AGTTGAGGGACTTTCCAGGC-3' and mutant NF-κB 5'-AGTTGAGGCTCCTTCCAGGC-3' were from Promega Corp. (Madison, WI). The antibodies against NF-κB proteins, p65, c-Rel and IkB/MAD-3 and AT-R, were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA) and p50 from Chemikon (Temecula, CA). Secondary HRPO-conjugated and FITC-labeled antibodies were from The Binding Site Inc. (Birmingham, UK). α-[32P]dCTP (3000 Ci/mmol) and γ-[32P]CTP (3000 Ci/mmol) were from Amersham (Buckinghamshire, UK). All other chemicals were from Sigma Chemicals (St Louis, MO). None of the compounds was cytotoxic for VSMCs at the concentrations used, as determined by trypan blue staining (not shown).

Cell culture

Rat thoracic aortic VSMCs were isolated from male Wistar rats, as described6. Fat, connective and adventitial tissue were removed by blunt dissection from the thoracic aorta. Vessels were opened longitudinally and incubated 30 min with 4 mg/ml collagenase (type II, 290 U/mg). Cells were counted and seeded at a concentration of 10⁴ cells/cm² in DMEM (Roswell Park Memorial Institute, Buffalo, NY) with 100 U/mL penicillin, 100 μg/mL streptomycin and 2 mmol/L glutamine. Cells were harvested for passaging at 2-weeks intervals and used between the 2nd and 7th passage. Cells were characterized by phase contrast microscopy and immunofluorescence staining. VSMCs were positive for smooth muscle α-actin and negative for von Willebrand factor (excluding endothelial cells).

RNA extraction, Northern blot and PCR

Total RNA from 48h serum deprived VSMCs was extracted by the Chomczynski and Sacchi method11. The quality of RNA was determined by electrophoresis in a 1% agarose-formaldehyde gel and ethidium bromide staining.

AT₂-R and angiotensinogen mRNA expression was analyzed by RT-PCR, as previously published32-35. The primers used were: AT₂-R (sense: 5'-CTGACCCCTGAACATGTGTC-3', antisense 5'-GGTGTCATTCTCTAAGAG-3'), angiotensinogen (sense: 5'-TTCAAGGCGAAGACCTCCC-3', antisense: 5'-CCGGCAGGAGTGCAGT-3') and G3PDH (sense: 5'-AATGCATCCTGACCACCAA-3', antisense: 5'-GTAGCCATTCCATTGTCATA-3'), that yielded products of 710bp, 308 bp and 515 bp, and PCR (1 min at
58°C/63°C/54°C, 1 min at 68°C and 1 min at 94°C; 35 cycles) respectively. Control experiments were done with RNA samples, but without AMV reverse transcriptase. The DNA products were analyzed on 1.5% agarose gel and ethidium bromide staining.

MCP-1 mRNA was studied by Northern blot analysis as previously described. Blots were prehybridized for 4 h at 42°C in hybridization solution (50% formamide, 1% SDS, 5x SSC, 1x Denhardt’s solution, 0.25 mg/ml denatured salmon sperm DNA and 50 mM sodium phosphate buffer pH 6.5), and hybridization was carried out at 42°C overnight with 20% dextran sulfate and α-[32P]-denatured probe. The filters were washed using a 2x SSC, 0.1% SDS, at room temperature for 30 min and then twice with 0.2x SSC, 0.1% SDS, at 55°C for 15 min. Results were expressed as arbitrary densitometric units relative to G3PDH.

Determination of NF-κB DNA binding activity

Nuclear extracts were obtained and NF-κB activity was determined by binding assay of nuclear proteins to labeled oligoconsensus and electrophoretic mobility shift assay (EMSA). Nuclear and cytoplasmic extracts were prepared as previously described. After the incubation period, cells were trypsinized and resuspended in buffer A (10 mmol/L HEPES pH 7.8, 15 mmol/L KCl, 2 mmol/L MgCl₂, 0.1 mmol/L EDTA, 1 mmol/L DTT, 1 mmol/L PMSF) and homogenized. Nuclei and cytosolic fraction were separated by centrifugation at 1000xg for 10 min, the nuclei were washed twice in buffer A and resuspended in the same buffer, with a final concentration of 0.39 mol/L KCl. Nuclei were extracted for 1 h at 4°C and centrifuged at 100,000xg for 30 min. Supernatant was dialyzed in buffer C (50 mmol/L HEPES pH 7.8, 50 mmol/L KCl, 10% glycerol, 1 mmol/L PMSF, 0.1 mmol/L EDTA and 1 mmol/L DTT) and then cleared by centrifugation and stored at -80°C. Protein concentration was determined by the BCA method. NF-κB consensus oligonucleotide was [32P] end-labeled with [γ-32P]ATP and T4 polynucleotide kinase (Promega). Nuclear extracts (5-10 μg) were equilibrated for 10 min in a binding buffer [4% glycerol, 1 mmol/L MgCl₂, 0.5 mmol/L EDTA, 0.5 mmol/L DTT, 50 mmol/L NaCl, 10 mmol/L Tris-Cl, pH 7.5, and 50 μg/mL of poly(dl-dC)] (Pharmacia LKB, Uppsala, Sweden), then the labeled probe (0.35 pmols) was added and incubated for 20 min at room temperature. Negative controls without cellular extracts and competition assays with a 100-fold excess of unlabeled NF-κB, mutant NF-κB and AP-1 (unrelated) oligonucleotides were performed to establish the specificity of the reaction. HeLa cell nuclear extracts were used as a positive control of the technique (not shown). When competition assays were done, the unlabeled probe was added to this buffer 10 min prior to the addition of the labeled probe. For supershift assays 1 μg of anti-p65, anti-c-rel or anti-p50 antibodies were added and incubated with nuclear extracts for 1 h prior to addition of labeled probe. The reaction was stopped by adding gel loading buffer (250 mmol/L Tris-HCl, 0.2% bromophenol blue, 0.2% xylene cyanol and 40% glycerol) and run on a non-denaturing, 4% acrylamide gel in TBE. The gel was dried and exposed to X-ray film.
Western blotting

Nuclear fractions (20 μg) were employed to quantify p50 and p65 levels. Cytosolic fractions (40 μg) were analyzed for IκBα and IκBβ. Both fractions were obtained as described above. Total proteins were used for AT-R. Proteins were resolved in 12% SDS-PAGE gels and electrophoretically transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). After blocking (buffer: 0.01 mmol/L Tris pH 7.5, 0.4 mol/L NaCl, 0.1% Tween-20, 1% BSA and 5% milk), membranes were incubated in the same buffer with the corresponding primary antibody for 18 h at 4°C. After washing, detection was made by incubation with peroxidase-conjugated antirabbit IgG and developed using an ECL chemiluminescence kit (Amersham). As control, competition studies with blocking peptides were done (not shown).

Immunofluorescence staining for p50 and p65

Cells were grown in eight well Titer-Tek slides (Costar, Cambridge, MA) and serum starved for 48h. After stimulation, cells were washed, fixed in methanol/acetone at-20°C for 20 min, and in 3% PFA, 10 min on ice, and then treated with 0.1% Triton-X100 for 1 min on ice to permeabilize nuclear membranes. To block unspecific binding, cells were incubated with 10% rabbit antiserum and 1% BSA in PBS for 1 h at 37°C. Polyclonal antibodies against p50 and p65 subunits (10 μg/ml) were used as primary antibody and a 1/200 dilution of FITC-labeled antirabbit IgG as secondary antibody. Controls were stained with non-immune serum or with the secondary antibody alone. Preparations were mounted in 70% glycerol solution and examined under a microscope. Images were photographed and printed at equivalent exposures.

Immunoperoxidase staining for AT-R was done: Quiescent VSMC, grown in eight-well, were fixed in methanol/acetone at-20°C, incubated with primary antibodies (AT1 and AT2) and then with peroxidase-conjugated secondary antibody. Controls were stained with non-immune serum or with the secondary antibody alone.

Transient transfections and luciferase assay

To evaluate whether AngII could increase the transcriptional activity of genes under the control of the NF-κB promoter we employed a luciferase reporter plasmid (NF-κB/luc) that contains five copies of the recognition site for NF-κB sequence. Double transient transfections of growth-arrested VSMC with NF-κB/luc and internal control TK-renilla (Stratagene) were performed by ex vivo via particle mediated (biolistic) gene transfer, with the Biolistic PDS-1000/He System (BIO-RAD Laboratories, CA, USA). The helium pressure (2200 p.s.i.) and vacuum circuits in the system accelerate 0.8-1.6 um sized AU-165 gold microcarriers (ABC R, Karlsruhe, Germany) coated with DNA (1mg Gold coated with 1 μg of NF-KB DNA
and 1 μg of renilla DNA) into quiescent VSMC. After transfection, cells were serum-starved for 24h before stimulation. Lysates were assayed for luciferase and renilla activities (Promega).

**Results**

We determined whether VSMC in our experimental conditions present AT$_2$ receptor at gene and protein levels. VSMC were serum-deprived for 48h, and then treated with 10%FCS or insulin/transferrin/selenite (ITS) for additional 24h. Then, RNA was extracted and AT$_2$ receptor gene expression was analyzed by RT-PCR. In unstimulated cells and in response to ITS, a band corresponding to AT$_2$ receptor mRNA expression was observed, while in VSMC in proliferative state (10%FCS), no detectable levels of AT$_2$ receptor gene expression were noted (Figure 1A). In growth-arrested VSMC, we evaluated the protein levels of AT$_2$ receptors by Western blot using a specific anti-AT$_2$ receptor antibody (Figure 1B). These cells presented a band of 42 KDa corresponding to the predicted size, as previously described. These results were confirmed by immunohistochemistry (Figure 1C) and data are in agreement with previous studies showing detectable AT$_2$ binding sites in cultured VSMC. These cells also possess AT$_1$ receptors (figure 1 B and 1C) as previously demonstrated. AngII translocates p50/p65 NF-κB complexes into the nuclei and degrades cytosolic (expanded text).

The diversity of dimeric complexes formed by Rel factors requires definition of NF-κB system in each specific cell type. To determine the composition of NF-κB complex induced by AngII in rat VSMC, we performed supershift assays, immunofluorescence analysis and Western blot experiments employing specific antibodies against the NF-κB protein subunits.

For supershift assays, nuclear extracts from VSMC treated with 10$^5$ mol/L AngII for 60 min were used. Binding assay was done by preincubation with 1 μg of the antibodies against NF-κB subunits p50, p65 and c-Rel, and protein complexes were resolved by electrophoresis. Antibodies to p50 and p65 shifted the band to a higher molecular weight. However, no effect was seen with the anti c-Rel antibody (Figure 2A). These data suggest that p50 and p65, but not c-Rel, are present in the active NF-κB complex in rat VSCMs and are in agreement with that in human VSMC.

By immunofluorescence, we determined the intracellular localization of p50 and p65 subunits. In control cells a diffuse cytoplasmic fluorescence was seen with both antibodies (Figure 2B). After treatment with 10$^9$ mol/L AngII for 1h, the immunohistochemical-staining pattern for both subunits changed from a diffuse cytoplasmic to a prominent nuclear pattern (Figure 2B). Positive (100 U/mL TNF-α) and negative (absence of primary antibody) controls were also done (not shown).

By Western blot, we quantified p50 and p65 proteins in nuclear extracts. Unstimulated VSMC constitutively expressed p50 in nuclear proteins and increased in AngII-treated cells (3-fold, 10$^9$ mol/L after 60
min, n=3, p<0.05) (Figure 2C). The p65 subunit is responsible for the transcriptional activity of NF-κB. p65 was nearly undetectable in nuclear extracts of unstimulated cells. Stimulation with AngII (10^{-9} \text{ mol/L}) for 1h resulted in nuclear expression of p65 (9-fold, n=3, p<0.05) (Figure 2C). All these data suggest that NF-κB complex activated by AngII consists of a heterodimer of 50- and 65-kDa subunits. To further confirm the role of the AT2 receptor, we evaluated the effect of the AT2 agonist pNH2FAII on p50/p65 nuclear levels. Treatment of quiescent VSMC with pNH2FAII (10^{-7} to 10^{-11} \text{ mol/L}) for 60 min upregulated nuclear p50 and p65, with a maximal response at 10^{-9} \text{ mol/L} and with an intensity similar to AngII (2- and 5-fold, respectively, n=3, p<0.05) (Figure 2C). These data suggest that the AT2 receptor is involved in the upregulation of transcriptional activity of some genes controlled by NF-κB.

Activation of NF-κB involves degradation of IκBα and/or IκBβ.65 We evaluated whether AngII affected cytosolic IκBα and IκBβ levels. VSMC were stimulated with 10^{-9} \text{ mol/L} AngII for 30, 60 and 120 min, and cytosolic extracts were immunoblotted for IκB proteins. In control cells, IκBα was found as a protein of around 39 KDa. After 1h of AngII stimulation this band disappeared, suggesting a degradation of IκBα (Figure 3). This effect was closely correlated with the time course of AngII on NF-κB activation, and with the translocation of p50 and p65 subunits to the nuclei. Upon AngII stimulation, cytosolic IκBα was rapidly degraded, while IκBβ remained unchanged. These biological differences were also seen with various inducers of NF-κB. Thus, IL-1 and LPS caused a rapid IκBβ degradation, but neither TNFα nor phorbol ester modified cytosolic IκBβ levels.66 Conversely, rapid IκBα degradation was induced by all four stimuli.66 We observed restored cytosolic IκBα levels after 2h (Figure 3). Since IκB gene is also controlled by NF-κB,66 these data suggest that transactivation by NF-κB increases IκBα synthesis, which can then restore the inactive state of the NF-κB complex.

**Molecular mechanisms of AngII-induced NF-κB activation (expanded text)**

We next investigated which intracellular signaling responses elicited by AngII could be involved in NF-κB activation in VSMC, trying to elucidate differences between AT1-R and AT2-R. VSMC were preincubated for 1h with several inhibitors (see table 1) and then stimulated for 60 min with 10^{-9} \text{ mol/L} AngII, or with the AT2 agonist pNH2FAII.

AngII, through AT1 receptors, activates several kinases, including protein kinase c (PKC) and phosphotyrosine kinases (PTK).22 None of the PKC inhibitors (staurosporine, H7 and BIP) modify the NF-κB activation induced by AngII or the AT2 agonist (figure 5A, printed manuscript), suggesting that PKC is not involved in this process. In contrast, these inhibitors abolished PMA-induced NF-κB activation (figure 5A, printed manuscript). Some difference between AT1/AT2 receptors were observed with PTK inhibitors. Genistein caused a marked reduction in NF-κB activity in response to AngII (95% inhibition vs AngII alone, at 10^{-6} \text{ mol/L} n=4, p<0.05) (Figure 5B, printed manuscript). Similar results were observed with 10^{-6} \text{ mol/L} erhbstatin and
herbimycin A (not shown). In contrast, PTK inhibitors had no effect on the AT$_2$ agonist induced NF-$\kappa$B DNA binding activity (figure 5B, printed manuscript). AT$_2$-R is linked to activation of protein-phosphotyrosine phosphatase-2A (PP2A), we also tried to study the role of PP2A, but treatment of VSMC with the phosphatase inhibitor okadaic acid activates NF-$\kappa$B (data not shown), as occurs in other cell types.

NF-$\kappa$B activation is also mediated by active oxygen radicals. Pretreatment of VSMC with antioxidants markedly diminished the NF-$\kappa$B activation elicited by AngII and the AT$_2$ agonist pNH$_2$FAll (Figure 5C, printed manuscript). VSMC were also preincubated with several NF-$\kappa$B inhibitors. Pyrrolidine dithiocarbamate abolished the NF-$\kappa$B activation elicited by AngII and the AT$_2$ agonist pNH$_2$FAll (Figure 4). The proteasome inhibitor of IκB degradation, MG132, markedly diminished NF-$\kappa$B DNA binding of both compounds (Figure 4). Similar results were also observed with the immunosuppressor gliotoxin (10$^{-5}$ mol/L) (not shown). When VSMC were exposed for 1h to fumonisin B$_1$ an inhibition in the NF-$\kappa$B activation induced by AngII and pNH$_2$FAll (Figure 5E printed manuscript) was observed, suggesting that ceramide could be a mediator of AngII/AT$_2$ induced NF-$\kappa$B activation.
<table>
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Figure 1. AT₂ receptor in VSMC. (A) Analysis of gene expression by RT-PCR. Quiescent cells (after 48h serum-starvation), and cells treated with insulin/transferrin/selenite (ITS) expressed AT₂ receptor mRNA. By contrast, this gene was not observed in VSMC in proliferative state (10%FCS). Figure shows a representative experiment of RT-PCR of 4 made. (B) Determination of AT₁ and AT₂ receptors by Western blot. Total proteins from quiescent VSMC were analyzed by electrophoresis and immunoblotting with a specific AT₂ antibody, showing a band of 42kDa corresponding to the predicted size. After washing, the membranes were also blotted with an anti-AT₁ antibody detecting a band of around 50kDa. (C) Localization of Ang receptors. Quiescent VSMC express both receptors as shown by immunohistochemistry with specific antibodies. As negative controls, samples were incubated in the absence of primary antibody. Magnification 100x.

Figure 2. Characterization of NF-κB complexes induced by AngII in rat VSMC. (A) The nuclear extracts of AngII-treated VSMC were preincubated with antibodies against the NF-κB subunits p50, p65 and c-Rel. Supershifted bands are observed with anti-p50 and anti-p65 antibodies (marked by arrows, longer exposition shown in the left part). The position of the NF-κB complexes and free-oligonucleotide is also indicated. Figure shows a representative EMSA of five experiments. (B) Localization of NF-κB subunits. In control cells a diffuse cytoplasmic immunostaining was seen with anti-p50 and anti-p65 antibodies. When cells were treated for 1h with 10⁻⁹ mol/L AngII an intense nuclear fluorescence was observed with both antibodies, showing a translocation of the p50 and p65 subunits to the nuclei. (C) Nuclear levels of p50 and p65. VSMC were incubated with AngII (10⁻⁷ to 10⁻⁹ mol/L) and pNH₂FAII (10⁻⁹ mol/L) for 60min. Nuclear extracts were isolated and analyzed by Western blot. In the upper part figure shows a representative experiment of 3 with comparable results, and in the lower values of means ± SEM.*p<0.05 vs control. White and black bars represent p50 and p65, respectively.

Figure 3. Effect of AngII on cytosolic IκB levels in VSMC. Cells were treated with 10⁻⁹ mol/L AngII for increasing periods of time. A rapid degradation of I-κBα (apparent molecular mass of 39 KD) followed by resynthesis was seen with AngII, while I-κBβ was not modified. Cytosolic extracts were electrophoresed under reducing, denaturing conditions, stained with affinity-purified anti-IκBα or anti-IκBβ antibodies and visualized by enhanced chemiluminescence. Ponceau S stained show equal loading and transfer of samples. Results are from one of three comparable series of experiments.

Figure 4. Molecular mechanisms of AngII-induced NF-κB activation. VSMC were preincubated for 1h with the NF-κB inhibitors: PDTC (10⁻⁴ mol/L) and MG132 (10⁻⁵ mol/L) and then stimulated with 10⁻⁹ mol/L AngII and/or pNH₂FAII for 60 min. The figure shows a representative EMSA of three made. Values of means ± SEM
References (supplementary). See also printed version.


FIGURE 1
FIGURE 2
FIGURE 3
FIGURE 4