Angiotensin II Differentially Regulates Interleukin-1-β-inducible NO Synthase (iNOS) and Vascular Cell Adhesion Molecule-1 (VCAM-1) Expression

ROLE OF p38 MAPK

Received for publication, December 24, 2003, and in revised form, February 17, 2004
Published, JBC Papers in Press, March 3, 2004, DOI 10.1074/jbc.M314172200

Angiotensin II is implicated in pathophysiological processes associated with vascular injury and repair, which include regulating the expression of numerous NF-κB-dependent genes. The present study examined the effect of angiotensin II on interleukin-1β-induced NF-κB activation and the subsequent expression of inducible NO synthase (iNOS) and vascular cell adhesion molecule-1 (VCAM-1) in cultured rat vascular smooth muscle cells. Neither NF-κB activation nor iNOS or VCAM-1 expression was induced in cells treated with angiotensin II alone. However, when added together with interleukin-1β, angiotensin II, through activation of the AT₁ receptor, inhibited iNOS expression and enhanced VCAM-1 expression induced by the cytokine. The inhibitory effect of angiotensin II on iNOS expression was associated with a down-regulation of the sustained activation of extracellular signal-regulated kinase (ERK) and NF-κB by interleukin-1β, whereas the effect on VCAM-1 was independent of ERK activation. The effect of angiotensin II on iNOS was abolished by inhibition of p38 mitogen-activated protein kinase (MAPK) with SB203580, but not by inhibition of PI3 kinase with wortmannin or stress-activated protein kinase/c-Jun NH₂-terminal kinase (JNK) with JNK inhibitor II. Thus, angiotensin II, by a mechanism that requires the participation of p38 MAPK, differentially regulates the expression of NF-κB-dependent genes in response to interleukin-1β stimulation by controlling the duration of activation of ERK and NF-κB.

Angiotensin II (Ang II) is an important vasoactive peptide that physiologically regulates vascular tone and maintains normal vessel structure and function. However, increased levels of Ang II have been implicated in pathophysiological processes that include atherosclerosis, cardiac hypertrophy, nephropathy, vascular injury, and remodeling (1, 2). Angiotensin-converting enzyme inhibitors and Ang II type-1 receptor (AT₁) antagonists alleviate atherosclerotic lesions in patients (3–7) and experimental animal models (8) and also prevent neointimal proliferation after balloon injury in rats (9). It is possible that Ang II may influence the response of vascular cells to inflammatory agents, including cytokines such as interleukin-1β (IL-1β). IL-1β is an important cytokine known to activate nuclear factor (NF)-κB and to induce the expression of numerous NF-κB-dependent genes, including those encoding inducible NO synthase (iNOS), cyclooxygenase-2 (COX-2), and vascular cell adhesion molecule-1 (VCAM-1) (10–12). These inflammation-related effects have pivotal roles in various models of vascular injury and repair. Although reports in the literature are conflicting, Ang II has been suggested to regulate the expression of NF-κB-dependent genes, including up-regulation of the expression of VCAM-1, intracellular adhesion molecule-1, and monocyte chemoattractant protein-1 (6, 13, 14), and down-regulation of iNOS (15–18). However, the signaling mechanism(s) by which Ang II affects NF-κB activation and how Ang II differentially regulates the expression of different NF-κB-dependent genes remains largely unknown.

In previous studies, we examined the involvement of the extracellular signal-regulated kinases (ERK) in regulating the IL-1β signaling pathway in rat vascular smooth muscle cells (VSMCs) (12, 19, 20). In those studies we described an early but transient increase in NF-κB activation in response to IL-1β, followed by a more persistent activation that was sustained for up to 24 h. We showed that inhibition of ERK effectively attenuated the more persistent activation of NF-κB, but had no effect on the early activation. The different duration of NF-κB activation was associated with changes in the selective induction of NF-κB-dependent genes, with VCAM-1 expression being regulated solely by the early activation of NF-κB, whereas both iNOS and COX-2 expression were dependent on persistent activation (12). In a separate study, we showed that platelet-derived growth factor (PDGF) and epidermal growth factor each enhanced the IL-1β -induced iNOS gene expression in VSMCs and showed that this effect was by means of an ERK-dependent mechanism responsible for the persistent activation of NF-κB (20). These results suggested that vasoactive agents that regulate ERK activity might modulate the duration of NF-κB activation and the expression of selective NF-κB-dependent genes induced by IL-1β.
ship between IL-1β and Ang II with regard to ERK activation, temporal control of NF-κB, and the expression of iNOS and VCAM-1, two genes representative of those that respond to the persistent or transient activation of NF-κB, respectively. The study provides strong evidence that IL-1β-induced expression of VCAM-1 and iNOS is differentially regulated by Ang II through ERK-dependent temporal control of NF-κB activation. In contrast, p38 MAPK plays an important role in mediating the effect of Ang II. This novel mechanism reveals a potentially important integration of the signaling by vasoactive agents and IL-1β that may be critical in regulating inflammatory responses.

**EXPERIMENTAL PROCEDURES**

**Materials**—Dulbecco’s modified Eagle’s medium/Ham’s F12 medium (DMEM/F12) and fetal calf serum were purchased from Invitrogen. Recombinant human IL-1β (specific activity: 1.9 x 10^5 units/mg) was kindly provided by Dr. Aurigemma, National Cancer Institute. PDGF (BB isoform) was obtained from Upstate Biotechnology; Ang II was from Sigma; SB203580, JNK inhibitor II, and wortmannin were from Calbiochem; and antibodies against iNOS and COX-2 were obtained from Transduction Laboratories. Antibodies against phospho-p44/42 MAPK (Thr183/Tyr185), p42/44 MAPK, phospho-p38 MAPK (Thr180/Tyr182), p38 MAPK, phospho-SAPK/JNK (Thr183/Tyr185), SAPK/JNK, MEK-1/2, IκBα, and phospho-NF-κB p65 (Ser536) were from Cell Signaling. Antibody against VCAM-1 was obtained from Santa Cruz Biotechnology; NF-κB consensus oligonucleotide was from Promega; and [γ-32P]ATP was obtained from PerkinElmer Life Sciences. All other materials used were commercial products of the highest grade available.

**Cell Culture**—Rat VSMCs were isolated from the thoracic aorta and cultured as described previously (21). Cells were used between passages 5 and 9. When confluent, the cells were washed with serum-free medium and then maintained in DMEM/F12 with 0.1% fetal calf serum for 24–48 h. The medium was refreshed just before treatment. The cells were then incubated with or without additions (IL-1β, PDGF, Ang II, inhibitors, or vehicle) for designated times as indicated under “Results.”

**Adenoviral Constructs and Infection**—Adenovirus expressing an IκBα mutant (S2A/S36A) (Adv-IκBαM) and adenovirus expressing a dominant-negative MEK-1 (S218A/S222A) (Adv-MEK1dn) were generated as described previously (12). When confluence was reached, the cells were washed once with serum-free medium and then cultured for 24 h in DMEM/F12 with 0.1% fetal calf serum either with or without adenovirus. The medium was refreshed and the cells were further incubated with or without additions as indicated in the text.

**Western Blot Analysis and EMSA**—Whole-cell lysates were prepared and Western blot analysis was performed as described previously (22). Protein content of the cell lysates was determined with BCA protein assay reagent (Pierce), with bovine serum albumin used as a standard. The images were obtained and analyzed by using a Model GS-700 imaging densitometer (BioRad). Nuclear extracts were prepared and DNA-binding activity was assessed by EMSA using an NF-κB consensus oligonucleotide as described previously (22).

**Reverse Transcription (RT)-PCR**—Total RNA was extracted from cells by using TRIzol reagent as described previously (22). The first-strand cDNA was synthesized from 1 μg of total RNA using oligo (dT)20-M4 adapter primers and avian myeloblastosis virus reverse transcriptase (Takara Shuzo Co.). Synthetic gene-specific primer sets used in PCR were: (i) iNOS forward 20-mer, 5'-GGTAGAACTTCCAGACCAAGCAATC-3', and reverse 20-mer, 5'-TGCGGAGGTCAGTAT-3', which amplified a 430-bp sequence between +2510 and +2950 of rat iNOS cDNA; (ii) VCAM-1 forward 21-mer, 5'-ACATACTCCCCCAAGATAAGC-3', and reverse 21-mer, 5'-GCTCATCTCACAACACCCGAG-3', which amplified a 497-bp sequence between +1644 and +2141 of rat VCAM-1 cDNA; and (iii) GAPDH forward 20-mer, 5'-GCCATACCATCCCTTTGAC-3', and reverse 20-mer, 5'-GGATCTTCTCTCACACCTT-3', which amplified a 702-bp sequence between +881 and +951 of rat GAPDH cDNA. PCR was performed using the following schedule: denaturation, annealing, and extension at 94, 57, and 72 °C for 40 s, 30 s, and 1 min, respectively, for 26 cycles. PCR products were electrophoresed on 1.2% agarose gels containing ethidium bromide and visualized by UV-induced fluorescence.

**Immunofluorescent Staining**—VSMCs were cultured on four-well Lab-Tek II chamber slides (Nalge Nunc Intl) under the same conditions described above. After treatment, the cells were washed with cold PBS, fixed for 8 min in methanol at −20 °C, and air dried at room temperature.

**Fig. 1.** Ang II inhibits iNOS but enhances VCAM-1 expression induced by IL-1β. A–C, VSMCs were treated for 24 h with or without IL-1β (5 ng/ml) in the absence or presence of either Ang II (A, 100 nM; C, 0.1–100 nM) or PDGF (B, 10 ng/ml). Whole-cell lysates (20 μg protein/ lane) were used for Western blot analysis of iNOS and VCAM-1. Bar graphs in A–C represent densitometric analyses of the immunoblots, with the arbitrary units from IL-1β-treated cells as 1-fold. Data shown are mean ± S.D. (A and B, n = 3; C, n = 2). Statistical analysis was performed by Student’s t test. C, *p < 0.05 versus cells treated with IL-1β alone. D, VSMCs were treated for 16 h with or without IL-1β (3 ng/ml) in the absence or presence of Ang II (100 nM). Total RNA was extracted and used for RT-PCR to determine mRNA levels of iNOS, VCAM-1, and GAPDH. The results shown in D are representative of two separate experiments.

**RESULTS**

**Ang II Inhibits IL-1β-induced iNOS but Not VCAM-1 Expression**—Treatment of VSMCs with IL-1β for 24 h induced the expression of both iNOS and VCAM-1 (Fig. 1A). In contrast, 100 nM Ang II, when added alone, did not induce iNOS, nor did it affect the basal level of VCAM-1 expression compared with untreated cells. However, Ang II added together with IL-1β (500 ng/ml) induced iNOS expression, but was without effect on IL-1β-induced iNOS expression, but was without effect on VCAM-1 expression (Fig. 1B). The ability of 24-h treatment with Ang II to modulate IL-1β-induced gene expression was dose-dependent, with the most marked effects observed between 10–100 nM (Fig. 1C). As shown in Fig. 1D, Ang II reduced IL-1β-induced iNOS mRNA levels, but had little effect on IL-1β-enhanced VCAM-1 mRNA.
Ang II Inhibits Prolonged but Not Transient NF-κB Activation Induced by IL-1β—To examine whether the differential regulation of iNOS and VCAM-1 expression by Ang II could be due to an effect on NF-κB activation, VSMCs cultured in chamber slides were treated with IL-1β for 1 h or 16 h in the absence or in the presence of Ang II, and the translocation of NF-κB was determined by immunofluorescent staining of the NF-κB p65 subunit. Fluorescence microscopy shows clearly the nuclear localization of p65 at both 1 and 16 h after IL-1β treatment, reflecting the early and persistent activation of NF-κB, respectively (Fig. 2A). When added with IL-1β, Ang II markedly attenuated the nuclear localization shown at 16 h, but had no effect on response at the earlier 1 h time point, indicating that Ang II selectively reduced the prolonged activation of NF-κB induced by IL-1β. The addition of Ang II alone did not cause nuclear translocation of p65 at either 1 or 16 h.

Because other studies have indicated that Ang II could directly activate NF-κB in cultured cells (23–25), we further tested this possibility using our experimental conditions. As shown by EMSA (Fig. 2B), in contrast to the clear activation of NF-κB by either IL-1β or tumor necrosis factor-α, Ang II added alone had no effect on NF-κB activation at either early (1 h) or late (16 h) time periods over a broad concentration range (1 and 100 nM).

An additional criterion for the activation of NF-κB is the phosphorylation of NF-κB p65 on Ser536 (26), an event measurable by Western blot analysis. The data in Fig. 2C show that Ang II attenuated the IL-1β-induced phosphorylation of NF-κB p65 (Ser536) at 24 h in a dose-dependent manner. Using a broad range of concentrations and time points, Ang II added alone had no effect on p65 phosphorylation (data not shown). Thus, the data in Fig. 2 establish clearly that Ang II attenuates the prolonged activation of NF-κB initiated by IL-1β, yet under our experimental conditions, has no effect on NF-κB activation when added without the cytokine.

Ang II Reduces Prolonged ERK Activation Induced by IL-1β—Previously we have shown that prolonged activation of ERK is required for IL-1β to induce persistent activation of NF-κB and subsequent iNOS expression (12, 19, 20). Therefore, we examined whether the IL-1β-induced prolonged activation of ERK was influenced by Ang II. As shown in Fig. 3, A and B, the prolonged phosphorylation of ERK (24 h after IL-1β addition) was suppressed by the addition of Ang II (100 nM), whereas the addition of PDGF (10 ng/ml) clearly enhanced ERK phosphorylation. The effective concentrations for Ang II to suppress ERK phosphorylation were 10 and 100 nM (Fig. 3C), which is consistent with the results observed for Ang II to inhibit IL-1β-induced p65 phosphorylation and iNOS induction. Fig. 3D summarizes the inhibitory effect of Ang II on the phosphorylation of both ERK1 and ERK2 induced by IL-1β. Although Ang II down-regulated the IL-1β-induced prolonged phosphorylation of ERK observed at the 24 h time-point (see Fig. 3, A–C), the phosphorylation levels of ERK at earlier time-points were ob-
Fig. 4. AT1 receptors mediate the effect of Ang II on persistent ERK activation and on the ERK-dependent expression of iNOS and COX-2 induced by IL-1β. A, VSMCs were uninfected or infected for 24 h with AdvMEK1dn (lanes 3 and 4, 0.5 or 1.0 × 10^10 viral particles/ml, respectively), AdvIxBaM (lanes 5 and 6, 1.3 or 2.6 × 10^10 viral particles/ml, respectively), or control virus, AdvLacZ (lane 7, 1.0 × 10^10 viral particles/ml) and then treated for 24 h with IL-1β alone (lanes 2–7, 3 ng/ml) or IL-1β plus Ang II (lane 8, 100 nM). B, VSMCs were ununtreated or treated with IL-1β (3 ng/ml) and Ang II (100 nM), or both for 24 h. Losartan (AT1 antagonist, 10 μM) or PD123319 (AT2 antagonist, 10 μM) was added 1 h before IL-1β and Ang II. Whole-cell lysates were used for Western blot analysis. The overexpression of MEK1dn and I-xBaM was confirmed by immunoblotting with antibodies against MEK-1/2 and I-xBa, respectively (shown in A; small arrows indicate the mutants). Results shown are representative of two separate experiments each.

Fig. 5. Ang II activates multiple signaling pathways in cultured rat VSMCs. VSMCs were uninfected or treated with 100 nM of Ang II (A), 3 ng/ml of IL-1β or both (B) for the indicated times. Whole-cell lysates (20 μg of proteins/lane) were used for Western blot analysis of various protein kinases, as indicated. Results shown are representative of two separate experiments each, p-ERK, phosphorylated ERK; p-p38 MAPK, phosphorylated p38 MAPK; p-p54/p46 JNK, phosphorylated p54/p46 SAPK/JNK; and p-Akt, phosphorylated Akt.

An important finding in this study is that Ang II differentially influenced the expression of iNOS and VCAM-1 in rat VSMCs that occurred in response to IL-1β. Ang II inhibited IL-1β-induced iNOS (and also COX-2) expression by down-regulating the prolonged activation of ERK and NF-κB. The
Ang II down-regulated the sustained activation of ERK by acute and transient manner. However, at later time points, Ang II itself potently activated ERK in rat VSMCs in an
means of sustained activation of ERK and NF-κB (20). It is known that Ang II acts through AT1 receptors to evoke multiple intracellular signals such as calcium mobilization, activation of protein kinase C, PI3 kinase/Akt, and MAPKs, including ERK, JNK and p38 MAPK (1). Interestingly, the p38 MAPK inhibitor SB203580 abolished the inhibitory effect of Ang II on IL-1β-induced sustained activation of ERK and NF-κB, and restored the expression of iNOS, suggesting an important role of p38 MAPK in the mechanism by which Ang II regulates ERK and thereby, NF-κB.

Although Ang II modulated the prolonged NF-κB activation in response to IL-1β, Ang II added alone did not activate NF-κB in rat VSMCs under our culture conditions. This result was documented in cells treated solely with Ang II by the absence of (i) NF-κB DNA-binding activity as determined by EMSA, (ii) NF-κB p65 nuclear localization measured by immunofluorescent staining, and (iii) inducible p65 phosphorylation using Western blot analysis. This inability of rat VSMCs to respond to Ang II by activation of NF-κB was a consistent observation made in several separate preparations of our cells. In contrast to our findings, several groups have published data showing that Ang II activated NF-κB directly in human and rat VSMCs (23–25). The reason for this discrepancy is unclear, but one notable difference between our results and the other reports is that there was no detectable NF-κB DNA-binding activity in untreated VSMCs used in the present study. It is possible that Ang II may enhance NF-κB activation in cells that have a detectable basal level of activation caused by an unknown factor(s). Another possibility is that, in the human cell studies, the required growth factors for their culture might affect the response to Ang II. In addition, several cancer cell lines have been observed to be capable of constitutively expressing IL-1 (27–29), which may account for a basal and persistent activation of NF-κB in these cell lines. However, there is no evidence currently available indicating that VSMCs in culture could be transformed to a phenotype that constitutively expresses cytokines such as IL-1. We do find that Ang II does indeed enhance the expression of some NF-κB-dependent genes induced by IL-1β, as shown for VCAM-1.

Among the multiple signaling pathways activated by Ang II, p38 MAPK seemed to be a major component mediating the inhibitory effect of Ang II on IL-1β induction of iNOS. Inhibition of p38 MAPK by SB203580 restored not only iNOS expression but also the persistent activation of ERK and NF-κB. IL-1β alone only slightly activated p38 MAPK in VSMCs, consistent with our previous report, in which we also have shown that SB203580 did not influence IL-1β-induced nitrite accumu-
I-1/β activation of p38 MAPK in a serum-dependent manner by an unknown mechanism and that the activation of p38 MAPK prevents iNOS induction by I-1/β (30). Such p38 MAPK-mediated inhibition of iNOS expression seems cell-type-dependent, because it has been reported, in contrast, that in murine astrocytes and macrophages, bovine cartilage-derived chondrocytes, and rat pancreatic islets, p38 MAPK activation is required for iNOS induction (31–34). It has been reported recently that the different isoforms (α, β, γ, and δ) of p38 MAPK play different roles in iNOS induction (35), which may suggest the involvement of the different p38 MAPK isoforms in the different cell types studied. The data in the present study suggest the possibility that one of the mechanisms for p38 MAPK inhibiting iNOS induction may relate to the down-regulation of prolonged activation of ERK, leading to the reduction of persistent activation of NF-κB.

In contrast to iNOS, VCAM-1 expression induced by I-1/β was ERK-independent and did not require the persistent activation of NF-κB, a distinction we have reported recently (12), and which is further emphasized by the findings in the present study. Although Ang II down-regulated the I-1/β-induced prolonged activation of ERK and NF-κB, Ang II actually enhanced the IL-1/β-induced VCAM-1 expression. By contrast, PDGF enhanced the IL-1/β-induced prolonged activation of ERK and NF-κB, but it did not influence IL-1/β-induced VCAM-1 expression. These findings are consistent with the earlier and transient activation of NF-κB being required for VCAM-1 expression in rat VSMCs in response to cytokine stimulation.

Thus, based on the present study and previous findings (12, 19, 20), we suggest the hypothesis summarized schematically in Fig. 8. Upon IL-1/β stimulation, an early but transient activation of NF-κB is mediated through a mechanism requiring I-κBα degradation that is not affected by the ERK pathway. In contrast, prolonged or persistent activation of NF-κB by IL-1/β is initiated at least partially, by an I-κBα degradation-mediated process that is attenuated when the ERK pathway is inhibited. In both cases, the activation of I-κBα kinases is a prerequisite. Growth factors such as PDGF may not trigger I-κBα kinase activation, but may enhance IL-1/β-induced persistent activation of NF-κB by enhancing prolonged ERK phosphorylation. When the IL-1/β-induced ERK cascade is attenuated, either pharmacologically or by endogenous substances such as Ang II, expression of the genes requiring persistent NF-κB activation may be prevented, as in the case of iNOS and COX-2, whereas expression of the genes dependent solely upon the transient NF-κB activation may not be negatively affected, as in the case of VCAM-1. Although other signaling pathways and nuclear factors may also be involved, such ERK-dependent and ERK-independent regulation of NF-κB activation apparently has a pivotal role in controlling the pattern of cytokine-inducible gene expression and may represent a novel mechanism by which growth factors, Ang II, and other vasoactive agents regulate cytokine effects and influence inflammatory processes.
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J. Biol. Chem. 2004, 279:20363-20368. doi: 10.1074/jbc.M314172200 originally published online March 3, 2004

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