Differential Activation of Mitogen-Activated Protein Kinases in Smooth Muscle Cells by Angiotensin II
Involvement of p22phox and Reactive Oxygen Species

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Abstract—The atherogenic effect of the renin-angiotensin system can be explained, in part, by the influence of its effector, angiotensin II (Ang II), on vascular smooth muscle cell (VSMC) growth. There is evidence that reactive oxygen species (ROS) play a role in the atherogenesis and activation of mitogen-activating protein (MAP) kinases, which are involved in proliferation and differentiation. The study was performed to further characterize the role of ROS in Ang II–mediated MAP kinase activation and the regulation of the transcription factor activator protein-1 (AP-1). Rat VSMCs were stimulated with Ang II. The activities of MAP kinases were assessed by Western blot analysis or by immunocomplex kinase assay. AP-1 binding was determined by using an electrophoretic mobility shift assay. Rat VSMCs were treated with Ang II–activated MAP kinases, extracellular signal–regulated kinase (ERK), c-Jun amino terminal kinase (JNK), p38 MAP kinase (p38 MAPK), and their downstream effector, AP-1. Interestingly, only the activation of ERK1/2, but not JNK or p38 MAPK, was tyrosine kinase, protein kinase C, and MEK1/2 dependent. Ang II also induced the rapid formation of ROS, which could be inhibited by a specific antibody as well as by antisense against the p22phox subunit of the NAD(P)H oxidase. JNK and p38 MAPK, but not ERK, activation was inhibited by an inhibitor of NAD(P)H oxidase. Antisense against p22phox also solely inhibited p38 MAPK but did not affect ERK. The results indicate that in VSMCs, Ang II activates MAP kinases and AP-1 through different pathways; the results further suggest that ROS, generated by p22phox, mediate Ang II–induced JNK and p38 MAPK activation, which may contribute to the pathogenesis of atherosclerosis. (Arterioscler Thromb Vasc Biol. 2000;20:940-948.)

Key Words: angiotensin II ■ atherosclerosis ■ reactive oxygen species ■ mitogen-activated protein kinase ■ activator protein-1

Angiotensin II (Ang II), the main peptide hormone of the renin-angiotensin system, plays an important role in the pathogenesis of cardiovascular diseases, including atherosclerosis, myocardial infarction, and hypertension.1 Ang II exerts hypertrophic and hyperplastic effects by activating a number of intracellular signal transduction pathways through a 7-transmembrane heterotrimeric G protein–coupled receptor called the Ang II type 1 (AT1) receptor.2

New data indicate that Ang II plays an important role in the generation of reactive oxygen species (ROS) by activation of NAD(P)H oxidase, a plasma membrane–bound protein.3,4 Two kinds of NAD(P)H oxidase systems have been proposed to exist, phagocytic and nonphagocytic. Phagocytic NAD(P)H oxidase consists of 4 subunits,5 whereas nonphagocytic NAD(P)H oxidase seems to be structurally related but not identical to phagocytic NAD(P)H oxidase. The mRNA of 1 of the subunits, p22phox, has been shown to be expressed in nonphagocytic cells, such as vascular smooth muscle cells (VSMCs),3,6 but expression of the other subunits in VSMCs remains controversial.

The generation of ROS in response to various external stimuli has been related to the activation of mitogen-activated protein (MAP) kinases6 and transcription factors, such as activator protein-1 (AP-1).7 Many of the signaling events relevant for cell proliferation and differentiation are mediated through activation of transcription factors by the MAP kinase family members, extracellular signal–regulated protein kinase (ERK) 1/2, c-Jun amino terminal kinase (JNK), and p38 MAP kinase (p38 MAPK), which have been shown to be also activated by Ang II.8–10 ERK1/2 is activated by agonists for tyrosine kinase–encoded receptors and G protein–coupled receptors that induce mitogenesis or cellular differentiation.11 ERKs mediate the effects of these agonists by phosphorylating and regulating the activity of a number of proteins and transcription factors, such as c-Fos and Elk-1.11,12 Unlike the related ERKs, JNK and p38 MAPK are only weakly activated by growth factors but are markedly activated in response to a variety of cellular stresses.13 JNK

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phosphorylates c-Jun, Elk-1, and ATF-2 at putative regulatory amino-terminal serine residues and increases their transcriptional activities, whereas p38 MAPK phosphorylates ATF-2 and C/EBP-homologous protein. JNK-activated c-Jun is one of the major components of the transcription factor AP-1, which regulates the expression of many genes involved in cellular growth, transformation, and differentiation.

To further elucidate the mechanisms of Ang II–mediated MAP kinase and AP-1 activation in VSMCs, we studied the activities of ERK, JNK, and p38 MAPK in the same cell system. Furthermore, we also tested the role of ROS in MAP kinase activation and formation of the AP-1 complex by Ang II. In addition, we set out to identify precisely the source of rapid ROS production in VSMCs mediated by Ang II.

Methods

Materials

Ang II and diphenylethionon (DPI) were obtained from Sigma Chemical Co. Losartan was a generous gift from Merck, Rahway, NJ. PD 123319 was purchased from BioTrend. GF19203X was obtained from Biomol. SB203580 and PD 98059 were purchased from Calbiochem. 2′,7′-Dichlorodihydrofluorescein diacetate (H2DCF-DA) was from Molecular Probes Europe. Anti–c-Jun and anti–c-Fos were purchased from Santa Cruz Biotechnology. Polyclonal anti-p22phox antibody was raised in chicken against amino acids 184 to 197 (QVNPIPVTDENV) of p22phox; preimmune chicken immunoglobulin served as a control (Davids). Peptides QVNPIPVTDENV and TPNIEQPVHVVD were synthesized by chicken immunoglobulin served as a control (Davids). Protein G–Sepharose 4 Fast Flow was obtained from Pharmacia Biotechnology. Anti–phospho-ERK1/2, c-Jun–glutathione-S-transferase were purchased from New England Biolabs. Anti–phospho-c-Jun (1–79) fusion protein bound to glutathione–Sepharose beads (O/N at 4°C). Beads were recovered by washing twice with buffer A (50 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% Triton) and then washed twice with a kinase buffer containing 25 mmol/L Tris-HCl, pH 7.5, 5 mmol/L β-glycerophosphate, 1 mmol/L Na3VO4, 1 μg/mL leupeptin, and 1 mmol/L phenylmethylsulfonyl fluoride, and then washed twice with a kinase buffer containing 100 μmol/L ATP (37°C for 30 minutes). The reaction was terminated by addition of 15 μL of 5× Laemmli sample buffer and boiling (at 100°C for 5 minutes). Samples were separated by SDS-PAGE and transferred to nitrocellulose membranes. To detect phosphorylated c-Jun, the membranes were incubated with polyclonal anti–phospho-c-Jun (1 hour at RT). The blots were then incubated with peroxidase-conjugated goat anti-rabbit IgG (1 hour at RT), and the proteins were detected by the ECL system.

Measurement of JNK Activity

The activities of JNK were measured by solid-phase kinase assay as described, with minor modifications. Cell lysates were incubated with GST–c-Jun (1–79) fusion protein bound to glutathione–Sepharose beads (O/N at 4°C). Beads were recovered by washing twice with buffer A (20 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% Triton, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L β-glycerophosphate, 1 mmol/L Na3VO4, 1 μg/mL leupeptin, and 1 mmol/L phenylmethylsulfonyl fluoride), and then washed twice with a kinase buffer containing 25 mmol/L Tris-HCl, pH 7.5, 5 mmol/L β-glycerophosphate, 2 mmol/L dithiothreitol, 0.1 mmol/L Na3VO4, and 10 mmol/L MgCl2. The beads were then incubated with 50 μL of a kinase buffer containing 100 μmol/L ATP (37°C for 30 minutes). The reaction was terminated by addition of 15 μL of 5× Laemmli sample buffer and boiling (at 100°C for 5 minutes). Samples were separated by SDS-PAGE and transferred to nitrocellulose membranes. To detect phosphorylated c-Jun, the membranes were incubated with polyclonal anti–phospho-c-Jun (1 hour at RT). The blots were then incubated with peroxidase-conjugated goat anti-rabbit IgG (1 hour at RT), and the proteins were detected by the ECL system.

Preparation of Cell Lysates for Western Blot and MAP Kinase Experiments

Quiescent VSMCs were isolated from the thoracic aortas of male Sprague-Dawley rats by enzymatic digestion as described by Chamey-Campbell et al. Cells were grown in DMEM supplemented with 10% heat-inactivated FCS, 1% l-glutamine, 100 μg/mL penicillin, and 100 μg/mL streptomycin. The cells were passaged 3 to 5 times at 70% to 80% confluence and were made quiescent by incubation in DMEM supplemented with 0.1% BSA for 48 hours before use. For antisense experiments, cells were incubated in serum-free medium and then transfected with 1 μmol/L oligonucleotides for 24 hours, as described by Hannken et al. The medium was subsequently changed, and transfected cells were stimulated with Ang II as indicated.

Preparation of Cell Lysates for Western Blot and MAP Kinase Experiments

Quiescent VSMCs were stimulated with Ang II in either the presence or absence of inhibitors. After stimulation, cells were harvested by aspirating the medium and washing twice with PBS (4°C). Cells were lysed by the addition of lysis buffer (20 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% Triton, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L Na3VO4, 1 μg/mL leupeptin, and 1 mmol/L phenylmethylsulfonyl fluoride at 4°C), scraped off the dish, sonicated, and centrifuged at 13 000 rpm at 4°C for 10 minutes. Supernatants were either used immediately or stored at −80°C. Protein concentrations were determined by using a bicinchoninic acid protein assay kit from Pierce, according to the manufacturer’s protocol.

Western Blot Analysis

Cell lysates were separated by SDS-PAGE and transferred to nitrocellulose (Schleicher & Schuell) membranes. The membranes were blocked (at room temperature [RT] for 1 hour in TBST containing 20 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 0.05% Tween 20, and 5% BSA), incubated with primary antibodies (1 hour at RT or overnight [O/N] at 4°C), and then incubated with the appropriate secondary peroxidase-conjugated antibodies (1 hour at RT). The proteins were detected by using an enhanced chemiluminescence detection system (ECL, Amersham).
gation, washed, and resuspended (30 mmol/L MES, pH 5.8, containing 120 mmol/L NaCl, 4 mmol/L MgCl₂, 1.2 mmol/L KH₂PO₄, 1 mmol/L Na₂HPO₄, and 10 µmol/L flavin adenine dinucleotide at 4°C). NADPH oxidase assays were started by the addition of 0.25 mmol/L NADPH and were terminated after 30 minutes at 37°C by adding HCl to yield a final concentration of 0.1 mmol/L. H₂O₂ was determined as described. Assays were routinely performed at 4 different protein concentrations ranging from 5 to 30 µg/mL.

Assay of Intracellular Redox State

Intracellular ROS production was measured in vital cells by the method of Obha et al., which is a nonporous compound that is converted into a nonfluorescent polar derivative (H₂DCF) by cellular esterases after incorporation into cells. H₂DCF is rapidly oxidized to the highly fluorescent 2',7'-dichlorofluorescein in the presence of intracellular hydrogen peroxide and peroxidases. Briefly, cells were incubated 30 minutes at 37°C with 5 µmol/L H₂DCF-DA in HBSS-HEPES. Cells were stimulated, and the fluorescence intensity over time for 5 groups of 15 to 20 cells was measured by fluorescence microscopy (Zeiss; excitation 488 nm, emission 513 nm). The fluorescence intensity was measured for each group, and the relative fluorescence intensity was taken as the average of 5 values.

Preparation of Nuclear Extracts

For the electrophoretic mobility shift assay, nuclear protein extracts were prepared according to the method of Schreiber et al., with minor modifications.

After Ang II stimulation, 2×10⁵ cells were washed twice with PBS (4°C) and scraped into 400 µL of hypotonic buffer (10 mmol/L HEPES, pH 7.9, 10 mmol/L KCl, 0.1 mmol/L EDTA, 0.1 mmol/L EGTA, and 2 mmol/L dithiothreitol) supplemented with proteinase and phosphatase inhibitors (5 µg/mL E-64, 1 mmol/L NaF, 0.2 mmol/L Na₂VO₃, and 0.5 mg/mL 4-(2-aminoethyl)-benzenesulfonyl fluoride and incubated for 15 minutes on ice; after which time, 25 µL of 10% Nonidet P-40 was added, and the tubes were vigorously vortexed for 10 seconds. The nuclei were recovered by centrifugation (14,000 rpm for 1 minute at 4°C). The nuclear pellets were resuspended in 50 µL of cold buffer C (20 mmol/L HEPES, pH 7.9, 0.4 mol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 2 mmol/L DTT supplemented with 5 µg/mL E-64, 1 mmol/L NaF, 0.2 mmol/L Na₂VO₃, and 0.5 mg/mL Pefabloc), and the tubes were rocked for 15 minutes at 4°C. After centrifugation (14,000 rpm for 5 minutes at 4°C), the supernatants containing nuclear protein were collected and stored at −80°C until use.

Gel Mobility Shift Assay

Nuclear extracts (2 µg each) were incubated with labeled oligonucleotide probes and 2 µg of poly(deoxyinosine-deoxycytidine)–poly(deoxyinosine-deoxycytidine) in 20 µL of binding buffer (60 mmol/L HEPES, pH 7.9, 50% glycerol, 20 mmol/L Tris-HCl, pH 8.0, 300 mmol/L KCl, 5 mmol/L EDTA, 100 µg/mL BSA, 2.5 mg/mL Pefabloc, 25 µg/mL E-64, 5 mmol/L NaF, 1 mmol/L Na₂VO₃, and 5 mmol/L dithiothreitol for 5 minutes at RT). The sequences of the double-stranded oligonucleotides used in the present study are as follows: consensus AP-1, 5'-CTGTTGAGTCTAACGCGCGGA-3'; consensus Oct-1, 5'-TGTCGAATGCAAATCACTAGAA-3'; consensus Oct-3, 5'-TGTCGATACAAATCTAGAA-3'. The oligonucleotides were labeled with [γ-³²P]ATP by using T4 polynucleotide kinase. Binding reactions were resolved on a 4% native polyacrylamide gel containing 1× TAE buffer (25 mmol/L Tris, 25 mmol/L boric acid, and 0.5 mmol/L EDTA). Gels were run at 150 V in a cold room (4°C) for 2 to 3 hours, dried, and exposed to x-ray film for 12 to 24 hours. In addition, a supershift assay for AP-1 was carried out by using rabbit polyclonal antibodies against c-Jun and c-Fos. The specific antibodies were incubated with samples after the initial binding reaction between nuclear protein extracts and [³²P]-labeled consensus oligonucleotide (1 hour at RT).

Statistics

Data are presented as mean±SD; for statistical analysis, ANOVA was used.

Results

Activation of ERK1/2, JNK, and p38 MAPK by Ang II in VSMCs

The signal transduction pathway leading to the activation of ERK1/2, JNK, and p38 MAPK in VSMCs was investigated. VSMCs were first stimulated with Ang II to determine whether MAP kinases are activated in this system.

Ang II activated MAP kinases in a dose-dependent manner, with a maximal stimulation seen at 10⁻⁷ mol/L (data not shown). This dose was therefore used in subsequent time-course experiments, and ERK1/2 activity was measured with a phospho-specific antibody, which only detects the phosphorylated activated forms. Ang II stimulation induced ERK1/2 activation (Figure 1), which peaked at 15 minutes (5.5-fold) and returned to basal levels within 60 minutes.

JNK activity was measured by using an immune complex kinase assay with GST–c-Jun as substrate. The increase in JNK activity was first detected at 5 minutes after the addition of Ang II. Maximal activity occurred at 30 minutes (5.8-fold) and gradually declined to near basal levels by 60 minutes (Figure 1).

The increase in p38 MAPK activity was examined by using an immune complex kinase assay with ATF-2 as substrate. The time course of p38 MAPK activation by Ang II was quite different from that of JNK. Ang II stimulation of p38 MAPK occurred within 2 minutes and was maximal after 5 minutes (3.4-fold). A rapid deactivation of p38 MAPK then ensued, with a return to basal levels within 15 minutes (Figure 1).

Ang II Activates ERK1/2, JNK, and p38 MAPK Through AT₁ Receptors in VSMCs

To determine which AT receptor subtype is involved in Ang II–induced ERK1/2, JNK, and p38 MAPK activation in VSMCs, the cells were stimulated with Ang II (10⁻⁷ mol/L) after pretreatment with the AT₁ receptor antagonist losartan (10⁻⁶ mol/L) or the Ang II type 2 (AT₂) receptor antagonist PD 123319 (10⁻⁷ mol/L) for 30 minutes. Ang II–induced activation of each MAP kinase was completely inhibited by pretreatment with losartan but not PD 123319 (Figure 2), suggesting that Ang II–induced ERK1/2, JNK, and p38 MAPK activation in VSMCs is mediated through AT₁ receptors.

Ang II–Mediated Activation of ERK1/2: Tyrosine Kinase, PKC, and MEK1/2 Dependent but NAD(P)H Oxidase Independent

Although tyrosine kinase activity appears essential for ERK activation, the tyrosine kinase dependence of Ang II–mediated ERK activation in VSMCs remains unclear. Figure 3 shows that pretreatment of VSMCs with the tyrosine kinase inhibitor genistein (100 µmol/L, 60 minutes) inhibited Ang II (10⁻⁷ mol/L, 15 minutes)–induced ERK1/2 activation. Ang II–induced ERK1/2 activation in VSMCs was also inhibited by preincubation of the cells with the specific protein kinase C (PKC) inhibitor GF109203X (2×10⁻⁶ mol/L, 60 minutes; Figure 3). MEK1/2 lies upstream from ERK1/2 in the signaling cascade, and preincubation of VSMCs with the MEK1/2 inhibitor PD 98059 (30 µmol/L, 60 minutes) completely ablated Ang II–induced ERK1/2 activation (Figure 3).
Ang II stimulation of NAD(P)H oxidase induces ROS generation. However, treatment of VSMCs with either the antioxidant N-acetylcysteine (NAC, 10 mmol/L) or the NAD(P)H oxidase inhibitor DPI (5 μmol/L) did not inhibit Ang II–induced ERK1/2 activation (Figure 3).

Ang II–Mediated Activation of JNK and p38 MAPK: Tyrosine Kinase and PKC Independent but NAD(P)H Oxidase Dependent

In contrast to Ang II–induced ERK1/2 activation, neither JNK (Figure 4) nor p38 MAPK (Figure 5) activation in VSMCs was affected by preincubation with the tyrosine kinase inhibitor, genistein (100 μmol/L, 60 minutes), or the specific PKC inhibitor, GF109203X (2×10^{-6} mol/L, 60 minutes). A positive control for the inhibition of Ang II–induced p38 MAPK activation was provided by pretreatment of VSMCs with the p38 MAPK–selective inhibitor, SB203580 (10 μmol/L, Figure 5). The effects of the antioxidant NAC (10 mmol/L) and the NAD(P)H oxidase inhibitor DPI (5 μmol/L) on the Ang II–induced activation of JNK and p38 MAPK also showed marked differences in their effects.

**Figure 1.** A, Time course of activation of ERK1/2, JNK, and p38 MAPK by Ang II is shown. VSMCs were stimulated with 10^{-7} mol/L Ang II for the indicated periods of time. Cells were harvested, lysed, and used for subsequent analysis. The activity of ERK1/2 was assayed by immunoblots with use of a phosphospecific anti-ERK1/2 antibody, which detected only phosphorylated activated forms (prefix p). The activities of JNK were measured by JNK kinase assay with c-Jun as substrate. The activities of p38 MAPK were measured by p38 MAPK assay with ATF-2 as substrate. Representative blots are shown. B, The intensity of each band on the blot was quantified by densitometric scanning, and the activities of MAP kinases are shown as fold increases of the average from 3 independent experiments compared with unstimulated controls (1.0). *P<0.05.

**Figure 2.** A, Ang II activates ERK1/2, JNK, and p38 MAPK through the AT1 receptor. Ang II receptor antagonists losartan (10^{-6} mol/L, AT1 receptor specific) and PD 123319 (10^{-6} mol/L, AT2 receptor specific) were added to the cultured medium for 30 minutes and exposed to 10^{-7} mol/L Ang II for an additional 15 minutes (ERK), 30 minutes (JNK), and 5 minutes (p38 MAPK). The activity of ERK1/2 was assayed by immunoblots with use of a phospho-specific anti-ERK1/2 antibody, which detected only phosphorylated activated forms. The activities of JNK were measured by JNK kinase assay with c-Jun as substrate. The activities of p38 MAPK were measured by p38 MAPK assay with ATF-2 as substrate. A representative blot is shown for each kinase. B, The intensity of each band on the blot was quantified by densitometric scanning, and the activities of ERK1/2, JNK, and p38 MAPK are shown as fold increases of the average from 3 independent experiments compared with unstimulated controls (1.0). *P<0.05.
on ERK1/2 activation. JNK (Figure 4) and p38 MAPK (Figure 5) activation was substantially inhibited by the action of the ROS-perturbing agents.

ROS Generation in VSMC Membranes
As shown in Figure 6, Ang II induced ROS production in the membrane fractions of VSMCs. To determine the source of Ang II–mediated O$_2^-$ generation in VSMCs, an antibody was used against the p22phox subunit of the phagocyte NAD(P)H oxidase multicomponent enzyme complex. Specificity of the antibody was verified by immunoblot (data not shown) and by the use of either a specific peptide (QVNPIPVTDEVV) or a nonspecific peptide (TPNIEQPVVVDV). Ang II–induced ROS production was inhibited by anti-p22phox but not by the preimmunoglobulin. The inhibitory effect of the antibody could be arrested by preincubation with the specific but not by the nonspecific peptide (Figure 6).

Intracellular Generation of ROS by Ang II
To confirm the hypothesis that generation of ROS is involved in p38 MAPK activation, we identified intracellular generation of ROS in VSMCs with H$_2$DCF-DA and fluorescence microscopy. Exposure of VSMCs to Ang II (10$^{-7}$ mol/L) resulted in a rapid increase in DCF fluorescence within 1 minute after stimulation (Figure 7). Transfection with antisense p22phox oligonucleotides reduced Ang II–induced ROS induction. Matched randomized control oligonucleotides had no effect (Figure 7). As demonstrated in Figures 3 and 5, DPI and NAC inhibited the Ang II–induced p38 MAPK but not ERK activation. Similarly, transfection of VSMCs with p22phox antisense but not matched randomized control oligonucleotides significantly attenuated the subsequent Ang II–mediated p38 MAPK but not ERK activation (Figure 8).

Ang II–Induced Activation of AP-1 DNA Binding
The JNK substrate, c-Jun, forms homodimers or heterodimers with c-Fos to form the AP-1 transcription factor. To determine whether Ang II could activate AP-1 DNA binding in VSMCs, nuclear extracts from Ang II–stimulated VSMCs were incubated with a $^3$P-AP-1 consensus sequence. As shown in Figure 9, Ang II was able to increase the DNA-binding activity of AP-1 after stimulation for 30 minutes, with a peak activation at 2 hours (4-fold). Incubation with an excess of an unlabeled AP-1 consensus sequence served as a
marker for specific binding by competing with the radiolabeled AP-1 probe.

To determine whether Ang II–dependent AP-1 activity was due to NAD(P)H oxidase, cells were pretreated with the NAD(P)H oxidase inhibitor DPI (5 μmol/L, 60 minutes), resulting in a substantial reduction (40%) of Ang II–induced DNA-binding activity of AP-1 (Figure 9B, rightmost lane). The involvement of c-Jun and c-Fos in AP-1 formation was investigated in Ang II–stimulated cells by the addition of antibodies against c-Jun or c-Fos to the binding reaction. This resulted in a shift of the binding complex to a slower migrating species, which was more pronounced for c-Jun–than for c-Fos–containing AP-1 complexes (Figure 9).

**Discussion**

It is now recognized that Ang II acts not only as a vasoactive peptide but also as a growth factor that stimulates proliferative and hypertrophic growth in VSMCs. Among the signaling events likely to be important in Ang II–mediated effects is the generation of O$_2^−$ by activation of NAD(P)H oxidase. Superoxide and its metabolites can function as intracellular second messengers. In view of this, we chose to examine the effects of Ang II and ROS generation on MAPK activation.

ERKs are normally activated by growth factors, hormones, or cytokines. In contrast to ERKs, JNK and p38 MAPK are only weakly stimulated by growth factors and phorbol esters. The stimulation of p38 MAPK by Ang II involves ROS and NAD(P)H oxidase but neither tyrosine kinase nor PKC. VSMCs were treated with genistein (100 μmol/L) and GF109203X (2×10$^{-6}$ mol/L) for 60 minutes, respectively, or treated with the antioxidant NAC (10 mmol/L) for 10 minutes, DPI (5 μmol/L) for 60 minutes, or SB203580 (10 μmol/L) for 30 minutes, followed by stimulation with Ang II (10$^{-7}$ mol/L) for 5 minutes. p38 MAPK activity was measured as described in the Figure 1 legend. A, Representative blot is shown. B, The intensity of each band on the blot was quantified by densitometric scanning, and the activities of p38 MAPK are shown as fold increases of the average from 3 independent experiments compared with unstimulated controls (1.0). *P < 0.05.
stimulates all 3 MAP kinases, with the strongest activation of ERK (5.5-fold) and JNK (5.8-fold) and less for p38 MAPK (3.4-fold). In contrast to our findings, Ushio-Fukai et al. recently reported that Ang II activates ERK and p38 MAPK more strongly than does JNK. The explanation for these differences is still unclear.

Similarly, the time courses of MAPK activation were also different after Ang II stimulation. Our results indicate that Ang II activates JNK and p38 MAPK in VSMCs with a more rapid activation of p38 MAPK, even though the signal pathway of the 2 kinases is very similar.

At present, 2 major subtypes of Ang II receptor, AT₁ and AT₂ receptors, have been identified. We demonstrated that Ang II activated ERK1/2, JNK, and p38 MAPK exclusively via the AT₁ receptor in VSMCs.

In cardiac myocytes, Ang II activates ERKs through the PKC-dependent pathway. In fibroblasts, however, tyrosine kinases, but not PKC, play a critical role in Ang II–induced ERK activation. Previous reports showed that depletion of PKC by pretreatment with PKC-activating phorbol 12-myristate 13-acetate markedly blocked Ang II–induced ERK stimulation in VSMCs. Eguchi et al. reported that Ang II induced Ras and ERK activation in cultured VSMCs. In this system, however, Ang II–induced ERK activation was only partially impaired by pretreatment with phorbol 12-myristate 13-acetate. Hence, differing data exist for the role of PKC and tyrosine kinase in the Ang II–mediated ERK activation in VSMCs.

In the present study and consistent with recent observations, Ang II–induced ERK activation was tyrosine kinase and PKC dependent. MEK lies upstream from ERK in the signaling cascade and has been shown to activate ERK by tyrosine and threonine phosphorylation. PD 98059, a specific MEK1/2 inhibitor, had the same inhibiting effect on ERK activation in VSMCs as has been shown in cardiac myocytes. The reason for the discrepancies between published results is still unclear. However, on the basis of the present results and previous findings, it is most likely that Ang II induces ERK activation by a tyrosine kinase–dependent, PKC-dependent, and MEK-dependent pathway in VSMCs.

Recently, it has been reported that Ang II activates JNK in GN4 rat liver epithelial cells in a Ca²⁺-dependent PKC-independent manner and that the tyrosine kinase inhibitor genistein prevented Ang II–induced JNK activation in these cells. In contrast, in cultured cardiac myocytes, activation of JNK by Ang II was strongly suppressed by downregulation of PKC. In neonatal myocytes, downregulation of diacylglycerol-regulated PKC isoforms inhibited the activation of p38 MAPK by ET-1. With respect to the p38 MAPK pathway, only few data on the activation in VSMCs exist. In the present study, Ang II–induced activation of JNK and p38 MAPK was mediated by a tyrosine kinase–independent and PKC-independent pathway. These results are in agreement with a report of JNK activation in a PKC-independent manner in VSMCs. Oxidative stress in vessels induced by ROS has been implicated in the pathogenesis of cardiovascular disease. The sources of ROS in the vasculature are diverse and include VSMCs. It appears that NAD(P)H oxidase is an important enzymatic origin of O₂⁻ and can be stimu-
lated by Ang II.2 p22phox, one of the electron transfer elements of NAD(P)H oxidase, is also expressed in VSMCs,1 although its role in signal transduction is, to date, only partially understood. Using antisense techniques, Ushio-Fukai et al1 described the role of p22phox in the late ROS release in VSMCs after 4 hours of Ang II stimulation. In a more recent publication, the same group also suggested a role for p22phox in the rapid Ang II–mediated ROS release in VSMCs because it could be inhibited with DPI, a potent inhibitor of flavonoid-containing enzymes, such as NAD(P)H oxidase.9 Using a specific inhibitory antibody as well as antisense techniques, we now provide proof that p22phox is the critical component for rapid Ang II–dependent ROS generation in VSMCs.

In the present study, treatment with the radical scavenger NAC or the inhibitor of NAD(P)H oxidase (DPI) antagonized the stimulatory effects of Ang II on JNK and p38 MAPK but not ERK2/2 activity. These findings could be further confirmed by the use of antisense against p22phox, which prevented p38 MAPK but not ERK activation. Thus, the data further support the involvement of NAD(P)H oxidase in ROS formation that, in turn, appears to be necessary for signal transduction.

Activation of JNK and p38 MAPK would be expected to result in phosphorylation of c-Jun and ATF-2 transcription factors, increasing their trans-activating activity. JNK has been reported to phosphorylate 2 serine residues in the putative activation domain of c-Jun and to increase its transcriptional activity.17 c-Jun forms a homodimer or a heterodimer with c-Fos to form the transcription factor AP-1, and it trans-activates many genes. The transcription of the c-Jun gene itself is also controlled by AP-1.17 JNK and p38 MAPK phosphorylate several transcription factors (c-Jun and ATF-2) that contribute to the stimulation of AP-1 activity.14,17 In the present study, we demonstrated that Ang II rapidly increased the DNA-binding activity of AP-1, containing mainly c-Jun and, to a small extent, c-Fos proteins. This is consistent with our result that Ang II strongly activates JNK. Because it is known that c-Jun is the substrate of only JNK, Ang II–induced JNK activation seems to be especially important for AP-1 activation. The demonstration that DPI reduced the Ang II–mediated DNA-binding activity of AP-1 confirmed our previous findings that NAD(P)H oxidase is involved in the signaling pathway of Ang II.

Taken together, the present study demonstrates that generation of ROS via the activation of NAD(P)H oxidase by Ang II is a link between hormone receptor interaction and the stimulation of JNK and p38 MAPK activity. ROS may serve as integral signaling molecules that exert a concentration-dependent effect on gene expression by MAP kinase activation, contributing to the pathogenesis of atherosclerosis.

References


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