p38 MAP kinase pathway regulates angiotensin II-induced contraction of rat vascular smooth muscle

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Angiotensin II (ANG II), the primary active component of the renin-angiotensin system, plays an important role in vascular function. It was originally identified as a potent vasoconstrictor hormone whose response involved both a direct action on vascular smooth muscle and an indirect effect mediated by the sympathetic nervous system (10). More recently, ANG II has been shown to stimulate protein synthesis and induce cellular hypertrophy in vascular smooth muscle cells (SMC) (8, 22, 23) and to promote vascular cell migration (18).

The cellular mechanisms underlying these diverse actions of ANG II are not clearly understood but are likely to involve the activation of distinct signaling pathways. All of the vascular effects of ANG II are mediated by the G protein-coupled AT1 receptor subtype. Binding of ANG II to the AT1 receptor activates multiple heterotrimeric G proteins that link the receptor to the stimulation of phospholipases C and D and to the inhibition of adenylyl cyclase (11, 25). These early biochemical events ultimately lead to the activation of complex cascades of protein serine/threonine kinases.

Among the serine/threonine kinases that are commonly employed to transduce extracellular signals are the mitogen-activated protein (MAP) kinases. Three subfamilies of MAP kinases have been extensively characterized in mammalian cells: the extracellular signal-regulated kinases (ERKs), the c-Jun NH2-terminal kinases (JNKs)/stress-activated protein kinases (SAPKs), and p38 (16, 56, 57). All these MAP kinases are activated by dual phosphorylation of tyrosine and threonine residues within the regulatory motif Thr-X-Tyr, catalyzed by a specific MAP kinase kinase family member. The ERK isoforms ERK1 and ERK2 are activated predominantly by growth factors, differentiation stimuli, phorbol esters, and Ca2+ (34). Once activated, ERK1 and ERK2 can phosphorylate numerous cytoplasmic and nuclear proteins, including the protein kinases p90rsk and Mnk1/Mnk2, stathmin, and the transcription factor Elk-1 (16, 56). Activation of the ERK pathway is associated with the mitogenic response to growth factors and the differentiation of specific cell lineages (49). In contrast, the JNK and p38 MAP kinase pathways are strongly activated by cellular stresses (heat and osmotic shock, ultraviolet irradiation, inhibition of protein synthesis), endotoxic lipopolysaccharide, inflammatory cytokines, and chemotactic peptides (33). JNK exists in multiple isoforms (8, 22, 23) and to promote vascular cell migration (18).
have been shown to phosphorylate and activate the transcription factors c-Jun, Elk-1, and ATF2 (16, 56). The other stress-activated MAP kinase pathway is comprised of p38 (also known as RK, p40, CSBP, and Mxi2) and the related homologs p38β (also termed p38–2), p38γ (also termed ERK6 and SAPK3), and p38δ (also termed SAPK4) (see Ref. 57 and references therein). Physiological substrates of p38 and p38β include the protein kinases MAP kinase-activated protein (MAPKAP) kinase-2, MAPKAP kinase-3, and Mnk1/Mnk2 as well as the transcription factors CHOP/GADD153 and Elk-1 (16, 56). The related kinases MAPKAP kinase-2 and MAPKAP kinase-3 phosphorylate the small heat shock protein HSP27 in vivo (20, 28, 40, 50). The physiological substrates of p38γ and p38δ remain to be identified. Activation of the JNK and p38 MAP kinase pathways is mainly associated with the response to stress and inflammation (33).

Here, we report that the G protein-coupled receptor agonist ANG II activates p38 MAP kinase in cultured aortic SMC and in the intact aorta, leading to activation of MAPKAP kinase-2 and phosphorylation of HSP27. The enzymatic activation of p38 is mediated by the AT1 receptor and is dependent on the generation of reactive oxygen species (ROS). Most importantly, we demonstrate that the p38 signaling pathway is selectively implicated in the vasoconstrictor action of ANG II on vascular smooth muscle.

METHODS

Materials and antibodies. Phenylephrine was obtained from Winthrop, GF-109203X, Go-6976, herbimycin A, and AG490 were obtained from Biomol. The protein kinase C (PKC) inhibitor CGP-41251 was a gift from Ciba-Geigy. 1,2-Bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid (BAPTA)-AM and LY-294002 were from Calbiochem. Genistein was from LC Services. The Src family kinase inhibitor PP1 was a gift from Pfizer. Wortmannin, N-acetyl- l-cysteine (NAC), glutathione (GSH), diphenylethylen iodide (DPI), and rotenone were from Sigma. SB-203580 was obtained from Calbiochem and dissolved in DMSO to give a 30 mM stock solution. Recombinant GST-ATF2 fusion protein was expressed in *Escherichia coli* and purified as described (52). Recombinant HSP27 was purified from *E. coli* transformed with a plasmid containing the Chinese hamster HSP27 coding sequence. The source of other materials has been described previously (23).

Antiserum HSK-592 was produced in rabbits against a synthetic peptide corresponding to amino acids 351–360 of murine p38 (Quality Controlled Biochemicals) and is specific to p38 isoform. The anti-MAPKAP kinase-2 serum was raised against a GST-MAPKAP kinase-2 fusion protein and specifically immunoprecipitates the p45 and p54 isoforms of the enzyme (28). Antiserum L2R3 was raised in rabbits against the COOH-terminal peptide AGKSEQSGAK of hamster HSP27 (37).

Cell culture. Rat aortic SMC were cultured and synchronized as described previously (23).

Protein kinase assays. The phosphotransferase activity of p38 MAP kinase was measured by a specific immune complex kinase assay using GST-ATF2 as substrate as described previously (52). Briefly, the cells were lysed in Triton X-100 lysis buffer, and 250 μg of proteins were incubated for 2 h at 4°C with 10 μl of p38 antiserum HSK-92 preadsorbed to protein A-Sepharose beads. The immune complexes were washed three times with lysis buffer and once with p38 kinase assay buffer [25 mM HEPES, pH 7.4, 25 mM MgCl2, 2 mM dithiothreitol (DTT), 25 mM β-glycerophosphate, and 100 μM sodium orthovanadate]. The beads were then resuspended in 20 μl of p38 kinase assay buffer containing 1 μg of GST-ATF2, 50 μM ATP, and 5 μCi [γ-32P]ATP. The reaction was initiated with ATP, incubated at 30°C for 30 min, and stopped by addition of 2× Laemmli’s sample buffer. The samples were analyzed by SDS gel electrophoresis, and the band corresponding to GST-ATF2 was excised and counted. The enzymatic activity of MAPKAP kinase-2 was assayed by immune complex kinase assay with the use of recombinant HSP27 as substrate. After stimulation, the cells were washed and extracted in lysis buffer containing 20 mM MOPS, pH 7.0, 10% glycerol, 50 mM sodium fluoride, 5 mM EGTA, 0.5 mM EDTA, 1 mM DTT, 80 mM β-glycerophosphate, 1 mM sodium orthovanadate, 5 mM sodium pyrophosphate, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1% Triton X-100. The extracts were clarified by centrifugation at 17,000 g for 20 min at 4°C and diluted four times in buffer I (20 mM Tris·HCl, pH 7.5, 150 mM NaCl, 1 mM EGTA, 0.1 mM EDTA, 1 mM DTT, 40 mM p-nitrophe- nyl phosphate, 1 μM leupeptin, 0.1 mM PMSF, 0.05% Triton X-100, and 0.3 μg of protein kinase A inhibitor) containing 2.5 μg of recombinant HSP27, 100 μM ATP, and 3 μCi [γ-32P]ATP. The reactions were incubated at 30°C for 30 min and terminated by addition of sample buffer. The phosphorylation of the substrate protein was examined after SDS gel electrophoresis by autoradiography and quantified by densitometry using the National Institutes of Health (NIH) Image software.

For MAPKAP kinase-2 assays in the intact aorta, rat aortic rings (prepared as described in Contractility studies) were stimulated with ANG II for different times and snap frozen by immersion in liquid N2. Frozen aortas were stimulated with ANG II for different times and snap frozen by immersion in liquid N2. Frozen aortas were stimulated with ANG II for different times and snap frozen by immersion in liquid N2. Frozen aortas were stimulated with ANG II for different times and snap frozen by immersion in liquid N2.
30 min with the indicated concentrations of SB-203580 and stimulated for 24 h with ANG II in the continuous presence of the inhibitor.

Contrastility studies. The thoracic aorta was removed from male Fisher 344 rats (300–350 g) killed by breathing 100% CO2; placed in oxygenated Krebs solution at room temperature; cleared of adherent fat, blood, and excess connective tissue; and transversally cut to produce rings ~3 mm wide. The endothelium was removed by gentle, repeated rubbing of the vessel lumen with a metal rod. Each ring was suspended between a metal hook and a thread loop under a tension of 1 g in a 5-ml tissue bath containing oxygenated (95% O2–5% CO2) and warmed (37°C) Krebs solution. The composition of Krebs was (in mM) 117.5 NaCl, 4.7 KCl, 1.2 KH2PO4, 1.18 MgSO4, 2.5 CaCl2, 25.0 NaHCO3, and 5.5 D-glucose. Isometric changes of the vascular tone were measured using a force transducer (model 52–9545, Harvard) coupled to an LKB chart recorder (model 2210 or REC 102).

Contrastility studies were based on the construction of full cumulative concentration-response curves for the agonists phenylephrine, added 1 h after the beginning of tissue incubation, and ANG II, tested at time 3 h. The tissues were washed extensively with fresh Krebs after the maximal effect was reached. For experiments with SB-203580 and DPI, the inhibitor or vehicle was added to the bathing solution 30 min before agonist treatment. Contrastility results are expressed as the mean absolute force of contraction in grams. The concentration-response curves are characterized by the half-maximal effective concentration and the maximal absolute force of contraction. Statistical analysis was by Mann-Whitney test using InStat 2.0 software (GraphPad Software).

Other methods. Dose-response curves were analyzed according to a four-parameter logistic equation using the ALLFIT computer program (17).

RESULTS

ANG II stimulates p38 MAP kinase activity in aortic SMC. To explore the role of p38 MAP kinase in the physiological actions of ANG II on blood vessels, we first tested the ability of the hormone to regulate the activity of p38 in cultured cells. Rat aortic SMC were made quiescent by serum deprivation and then treated with ANG II, and p38 was immunoprecipitated from cell lysates. The phosphotransferase activity of the enzyme was assayed directly using GST-ATF2 as substrate. Addition of ANG II caused a rapid activation of p38, which reached a maximum at 5 min and returned to near basal level after 120 min (Fig. 1A). This time course of p38 activation contrasts with that observed in response to cellular stresses, in which activation of the enzyme is slower and more sustained (J. C. Scimeca and S. Meloche, unpublished observations; Ref. 46). To determine the subtype of ANG II receptors involved in the activation of p38, the cells were pretreated with selective receptor antagonists before ANG II stimulation. Figure 1B shows that incubation with the AT1-selective antagonist losartan completely abolished p38 activation, whereas the AT2 antagonist PD-123319 had no effect. These results demonstrate that ANG II activates p38 MAP kinase through the G protein-coupled AT1 receptor in vascular SMC.

Lack of involvement of classic AT1 receptor signaling pathways in the activation of p38 MAP kinase by ANG II. In contrast to the ERK pathway, very little is known about the early signaling events that trigger activation of the p38 MAP kinase pathway. We used a pharmacological approach to characterize the signaling path-
ways coupling the AT₁ receptor to activation of p38 in vascular SMC. The best documented signaling pathway of the AT₁ receptor is the stimulation of phospholipase C, which generates the second messengers diacylglycerol and inositol 1,4,5-trisphosphate (11, 25). Diacylglycerol is a physiological activator of conventional and novel PKC isoforms, whereas inositol 1,4,5-trisphosphate binds to specific receptor channels to release Ca²⁺ from intracellular stores. We therefore investigated the role of PKC and Ca²⁺ in the stimulatory effect of ANG II on p38 enzymatic activity. As shown in Fig. 2A, treatment of cells with CGP-41251 and GF-109203X, compounds that inhibit conventional and novel PKC isoforms, or with Go-6976, a more selective inhibitor of conventional Ca²⁺-dependent PKC isoforms, had no effect on ANG II-dependent activation of p38. The role of Ca²⁺ was analyzed by incubating the cells with the membrane-permeable Ca²⁺ chelator BAPTA-AM. Chelation of intracellular Ca²⁺ did not inhibit the activation of p38 by ANG II but, in contrast, potentiated the basal and hormone-stimulated activity of the enzyme. To better define the role of Ca²⁺ in the regulation of p38, we examined the effect of BAPTA-AM on the time course of activation of p38. Interestingly, treatment with BAPTA-AM not only enhanced the activity of p38 in resting cells but also prevented the rapid inactivation of the kinase in ANG II-stimulated cells (Fig. 3). These results suggest that p38 is negatively regulated by a Ca²⁺-dependent protein phosphatase that is constitutively present in aortic SMC. In this regard, we have recently shown that Ca²⁺ chelation completely abolishes the induction of MAP kinase phosphatase-1 expression in aortic SMC (52), thereby suggesting that a member of the dual-specificity phosphatase family may be responsible for the inactivation of p38 in these cells.

In common with other G protein-coupled receptor agonists, ANG II stimulates tyrosine phosphorylation of multiple substrates in target cells, including vascular SMC (38). Recent observations made in various cellular systems have implicated protein tyrosine kinases in the activation of the ERK MAP kinase pathway by G protein-coupled receptors (55). We therefore asked whether activation of a cellular tyrosine kinase was required for ANG II-dependent activation of p38. Figure 2B shows that treatment of aortic SMC with the chemically and mechanically distinct broad spectrum tyrosine kinase inhibitors genistein and herbimycin A did not prevent the activation of p38 by the hormone. Similarly, treatment of cells with the Src family-selective tyrosine kinase inhibitor PP1 or the Jak2 inhibitor AG490 did not interfere with p38 activation (Fig. 2C).

**Fig. 2.** Effect of signaling inhibitors on ANG II-stimulated p38 MAP kinase activity in aortic SMC. Quiescent rat aortic SMC were pretreated for 30 min with the indicated signaling inhibitors, with the exception of herbimycin A, which was present for 18 h. The cells were then stimulated in the absence (cont) or presence of 100 nM ANG II for 5 min. The enzymatic activity of p38 was determined as described in Fig. 1.

The concentrations of the drug inhibitors were as follows: A: 1 μM CGP-41251, 1 μM GF-109203X, and 1 μM Go-6976; B: 30 μM genistein (Gen) and 0.5 μM herbimycin A (HA); C: 10 μM PP1 and 50 μM AG490; D: 100 nM wortmannin (Wort) and 50 μg/ml LY-294002 (LY). Similar results were obtained in 3 independent experiments.
In a recent study, it was reported that tyrosine phosphorylation of p38 in response to the G protein-coupled receptor agonist N-formyl-Met-Leu-Phe is partially blocked by the phosphatidylinositol 3-kinase inhibitor wortmannin in neutrophils (32). Because it was recently shown that ANG II stimulates phosphatidylinositol 3-kinase activity in vascular SMC (E. Giasson and S. Meloche, unpublished observations; Ref. 51), we tested the possibility that p38 is a downstream target of this signaling pathway. However, treatment of cells with wortmannin or LY-294002, two structurally different inhibitors of phosphatidylinositol 3-kinase, did not inhibit ANG II-dependent activation of p38 (Fig. 2D). Taken together, these data indicate that ANG II stimulates p38 enzymatic activity by a pathway distinct from the classic AT1 receptor signaling pathways.

Generation of ROS is necessary for ANG II-dependent activation of p38 in aortic SMC. Accumulating evidence indicates that ROS may function as intracellular second messengers in receptor signaling pathways (19, 35). Specifically, treatment of various cell types with oxidizing agents has been shown to activate the MAP kinase family members ERK1/ERK2 (5, 26), JNK (14, 26), and p38 (14, 26, 27). In light of these observations, we next sought to determine whether the activation of p38 by ANG II might be dependent on the production of ROS. To address this question, we first incubated aortic SMC with the antioxidants NAC and GSH (3, 41) before ANG II stimulation. Figure 4A shows that both NAC and GSH strongly attenuated ANG II-dependent activation of p38. Previous studies have demonstrated the existence of a membrane-associated NADH/NADPH oxidase system in vascular SMC (24, 43). The activity of this oxidase increases in response to ANG II stimulation (24). To determine whether the vascular NADH/NADPH oxidase might be involved in the activation of p38 by ANG II, we tested the effect of DPI, a potent inhibitor of flavin-containing enzymes (44). Incubation of aortic SMC with DPI markedly inhibited ANG II-stimulated p38 activity, whereas the mitochon-
rial NADH dehydrogenase inhibitor rotenone had no effect (Fig. 4B). To directly test the hypothesis that ROS can activate p38, the cells were exposed to the oxidant H₂O₂. Addition of H₂O₂ potently stimulated the enzymatic activity of p38, resulting in sixfold activation at 15 min (Fig. 4C). These results are consistent with recent findings by Ushio-Fukai et al. (53) and strongly suggest that ROS act as second messengers for ANG II in mediating the activation of p38 in vascular SMC.

**ANG II stimulates MAPKAP kinase-2 activity and HSP27 phosphorylation in aortic SMC.** We next wanted to determine whether the stimulation of p38 by ANG II results in the activation of MAPKAP kinase-2, a known physiological substrate of the enzyme (20, 50). Aortic SMC were stimulated with ANG II, and the activity of MAPKAP kinase-2 was determined by immune complex kinase assay using recombinant HSP27 as substrate. Addition of ANG II caused a 17-fold increase in MAPKAP kinase-2 activity at 5 min (Fig. 5A). This effect of ANG II was completely abolished by preincubating the cells with the pyridinyl imidazole compound SB-203580, a highly specific inhibitor of p38 and p38δ enzymes (29, 39). The effect of SB-203580 was dose dependent, with an IC₅₀ value of 0.10 ± 0.03 µM and a maximal inhibitory effect observed at ~10 µM (Fig. 5A). In contrast, incubation of cells with PD-98059, a selective inhibitor of the ERK pathway, did not interfere with MAPKAP kinase-2 activation (data not shown).

Activation of MAPKAP kinase-2 leads to phosphorylation of HSP27 in diverse cell types (20, 28, 50). To examine the effect of ANG II on the phosphorylation of HSP27, aortic SMC were metabolically labeled with ³²P_i and stimulated with ANG II, and HSP27 was immunoprecipitated from cell lysates before analysis by SDS gel electrophoresis. Treatment with ANG II significantly increased the phosphorylation of HSP27, and this effect was completely suppressed by preincubating the cells with SB-203580 (Fig. 5B). These results demonstrate that ANG II activates a p38 MAP kinase cascade that leads to phosphorylation of HSP27 in vascular SMC.

**Involvement of the p38 MAP kinase pathway in ANG II-induced vascular contraction.** To evaluate the biological significance of p38 activation by ANG II, we tested the effect of SB-203580 on the hypertrophic and vasoconstrictor action of the hormone. The hypertrophic effect of ANG II was evaluated by measuring the rate of protein synthesis in cultured rat aortic SMC. For these experiments, quiescent cells were preincubated with increasing concentrations of SB-203580 before stimulation with ANG II for 24 h in the continuous presence of the inhibitor. Figure 6 shows that SB-203580 had little effect on ANG II-induced protein synthesis up to a concentration of 10 µM, where it partially inhibited the response of the hormone. The IC₅₀ value of SB-203580 for inhibition of protein synthesis was ~100-fold higher than that observed for inhibition of MAPKAP kinase-2 (Fig. 5A). This finding is not consistent with a major involvement of p38 in the regulation of protein synthesis by ANG II.

The role of the p38 MAP kinase pathway in vascular smooth muscle contraction was investigated using fresh rat aortic rings. To extrapolate the results obtained in cultured aortic SMC to the intact aorta, we first examined the ability of ANG II to activate the p38 pathway in intact aortic rings. To this end, aortic rings were stimulated with ANG II for different times and then frozen in liquid N₂ before assay for MAPKAP kinase-2 activity. As shown in Fig. 7, ANG II caused a robust activation of MAPKAP kinase-2 in the intact aorta, with kinetics comparable to those seen in cultured aortic SMC. Preincubation of aortas with 10 µM SB-203580 nearly abolished ANG II-dependent activation of the enzyme. As expected, ANG II induced a strong contraction of the rat aorta, with a half-maximal...
effect observed at 6.6 ± 0.9 nM (Fig. 8B). Incubation with SB-203580 significantly shifted the concentration-response curve of ANG II to the right (IC$_{50}$ = 15.5 ± 4.7 nM, $P < 0.02$) and reduced maximal ANG II-induced contraction. The effect of SB-203580 was dose dependent, with 50% inhibition of ANG II-induced contraction observed at 0.17 ± 0.09 μM (Fig. 8B). By contrast, SB-203580 had no effect on the apparent potency or maximal response of the α-adrenergic agonist phenylephrine (Fig. 8A). These results provide strong evidence that a p38 MAP kinase modulates the vasoconstrictor action of ANG II in vascular smooth muscle.

Fig. 6. Effect of SB-203580 on ANG II-stimulated protein synthesis in aortic SMC. Quiescent rat aortic SMC were pretreated for 30 min with the indicated concentrations (Conc) of SB-203580 and then stimulated in the absence (cont) or presence of 100 nM ANG II for 24 h in the continuous presence of the inhibitor. The rate of protein synthesis was measured by [3H]leucine incorporation. Each value represents the mean ± SE of triplicate determinations. Similar results were obtained in 4 independent experiments.

Fig. 7. ANG II stimulates the activity of MAPKAP kinase-2 in the intact rat aorta. Rat aortic rings in tissue organ baths were preincubated for 30 min with vehicle alone or with 10 μM SB-203580 and then stimulated with 100 nM ANG II for the indicated times. Tissue extracts were prepared and subjected to immunoprecipitation with MAPKAP kinase-2 antiserum. Phosphotransferase activity was assayed using recombinant HSP27 as substrate. The values represent the means ± SE of data from two aortic rings isolated from different animals. Similar results were obtained in 3 independent experiments.

Fig. 8. Effect of SB-203580 on ANG II-induced contraction of rat aorta. Rat aortic rings in tissue organ baths were preincubated for 30 min with vehicle alone or with 10 μM SB-203580 before addition of agonist. Full cumulative concentration-response curves were constructed in the continuous presence of the inhibitor. Each value represents the mean ± SE of 10 determinations in tissues derived from 5 rats equally represented in each experimental group. A: concentration-response curve of phenylephrine. B: concentration-response curve of ANG II. Inset: dose-response curve of SB-203580 for the inhibition of ANG II-induced contraction of rat aorta. See text for statistical analysis.

Because the stimulation of p38 activity by ANG II is dependent on the generation of ROS (Fig. 4) (53), we also tested the effect of DPI on ANG II-induced contraction of the rat aorta. Preincubation with 20 μM DPI significantly depressed maximal ANG II-induced contraction ($P < 0.03$) and shifted the concentration-response curve of the hormone rightward (Fig. 9).

DISCUSSION

ANG II is a pleiotropic hormone that exerts a wide spectrum of actions on the cardiovascular system through activation of multiple signaling pathways. In this paper, we report that ANG II activates the p38 MAP kinase pathway in rat aortic smooth muscle and that this pathway is involved in the vasoconstrictor action of the hormone. ANG II, working through the AT$_1$ receptor subtype, stimulated the enzymatic activ-
and tumor necrosis factor-

lipopolysaccharide-induced synthesis of interleukin-1 

pound, which was initially developed as an inhibitor of 

and p38 

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kinase isoforms. SB-203580 is a pyridinyl imidazole 

logical roles and physiological substrates of p38 MAP 

itors has been crucial for the identification of the bio-

icity of p38, which then promoted the activation of MAP-

KAP kinase-2 and the phosphorylation of HSP27. Most 

importantly, incubation with the p38 MAP kinase in-

hibitor SB-203580 reduced the contractile effect of 

ANG II on fresh rat aortic rings.

The development of small cell-permeant drug inhib-

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logical roles and physiological substrates of p38 MAP 

kinase isoforms. SB-203580 is a pyridinyl imidazole 

derivative that inhibits the kinase activity of p38 (39) 

and p38β (29) with a remarkable selectivity. This com-

pound, which was initially developed as an inhibitor of 
lipopolysaccharide-induced synthesis of interleukin-1 

and tumor necrosis factor-α, has been used in a variety 
of cellular systems to establish a role for p38 isoforms 
in regulating the expression of cytokines and other inflam-

matory-related molecules (see Ref. 16 and refer-

ences therein). In vivo studies demonstrated that SB-

203580 potently inhibits inflammatory cytokines pro-
duction and exerts therapeutic activity in animal 
model s of arthritis, bone resorption, and endotoxin 

shock (6). The p38 MAP kinase pathway has also been 

implicated in the regulation of actin filament dynamics 
in response to adverse stimuli such as heat shock or 

oxidative stress (27), in the regulation of cardiac-spe-
cific gene expression (59), and in the modulation of 
voltage-dependent calcium channels (58). Our observa-
tion that SB-203580 antagonizes ANG II-induced con-
traction in the rat aorta provides the first evidence for 
a role of the p38 MAP kinase pathway in vascular 
smooth muscle contraction. This role of the p38 path-
way appears to be receptor specific, because contrac-
tion induced by the α-adrenergic agonist phenyleph-
rine was not affected by SB-203580. Given that SB-

203580 inhibits the activity of both p38 and p38β, we 
cannot draw a firm conclusion on the relative contrib-
ution of these p38 MAP kinase isoforms to vascular 
smooth muscle contraction at this stage. However, the 

fact that ANG II stimulates the activity of p38 in 
cultured vascular SMC supports the hypothesis that 
p38 is involved in that response. Importantly, these 
findings suggest that SB-203580 and related com-

pounds may show therapeutic usefulness in conditions 
other than chronic inflammatory diseases.

The regulatory pathways that lead to activation of 
p38 MAP kinase are still poorly defined in mammalian 
cells. p38 is phosphorylated and activated by the dual-

specificity kinases MKK3 and MKK6 (57). In this 

study, we used a pharmacological approach to identify 
the early signaling events that are responsible for 
coupling the AT1 receptor to the activation of p38.
None of the conventional intracellular signals com-

monly activated by Gαq- or Gαi-coupled receptors were 
found to be involved in the stimulation of p38 activity.
However, the following observations provide strong 
evidence for a role of ROS as second messengers in 

the p38 activation pathway. First, ANG II-dependent p38 
activation is inhibited by pretreatment with cells with the 
antioxidants NAC and GSH. NAC can directly reac-
t with free radicals but also increases the intracellular 

concentration of GSH (3). Second, the activation of p38 
by ANG II is markedly attenuated by the NADH/
NADPH oxidase inhibitor DPI. Previous studies have 
shown that DPI inhibits ANG II-stimulated production 
of ROS in vascular SMC (24) and in intact rat aortic 
ring segments (47). Also, during the course of this 
study, Ushio-Fukai et al. (53) reported that ANG II 
stimulates phosphorylation of p38 in vascular SMC 
and that treatment with DPI or expression of catalase 
partially inhibits ANG II-dependent phosphorylation 
of the kinase. Third, addition of the exogenous oxidant 
H2O2 mimics the activation of p38 by ANG II. As for 
many other signaling pathways, the direct cellular 
targets of ROS in the p38 MAP kinase pathway remain 

to be identified.

There is now considerable evidence that low concen-

trations of ROS can function as classic second messen-
ger molecules (19, 35). ROS are produced by every cell 
type not only as by-products of electron transfer reac-
tions but also in response to stimulation with a variety 
of cytokines and growth factors such as tumor necrosis 

factor-α, interleukin-1, platelet-derived growth factor, 

epidermal growth factor, and ANG II (19, 35). The 
mechanism responsible for the agonist-dependent gen-

eration of ROS in nonphagocytic cells remains to be 

clarified. However, recent work suggests that a mem-
brane-associated NADH/NADPH oxidase system simi-
lar to the neutrophil NADPH oxidase may be the pri-
mary source of ROS in vascular tissue (24, 43, 45). 
The presence of p22phox, a subunit of cytochrome Ox55
has been demonstrated in vascular SMC (21). As men-
tioned above, ANG II increases production of ROS via 
an NADH/NADPH-dependent membrane-bound oxida-

dase system in cultured aortic SMC and in the intact 
aorta (24, 47). Importantly, treatment with DPI (24), a
pharmacological inhibitor of the flavoprotein component of NADH/NADPH oxidase, or inhibition of p22phox expression by an antisense approach (54) significantly decreases ANG II-stimulated ROS production in vascular SMC. The increase of ROS levels has been associated with the induction/activation of transcription factors (13), the activation of MAP kinase isoforms (5, 14, 26, 27), and the inhibition of tyrosine phosphatases (30). Interestingly, it has been reported that ROS exert contractile effects on SMC of vascular origin (4, 48). In the present study, we found that DPI significantly reduces ANG II-induced contraction of the aorta. The hypertensive response induced by ANG II infusion in the rat was also found to be associated with increased vascular superoxide production, thereby highlighting the physiopathological importance of NADH/NADPH oxidase-derived ROS in the regulation of vascular tone and arterial pressure (47). Together, these observations are consistent with the idea that activation of the p38 pathway may link the ANG II-dependent production of ROS to vascular contraction.

The mechanism by which activation of the p38 MAP kinase pathway modulates the contractile response to ANG II remains to be established. One possibility is that HSP27 directly regulates the cycling of myosin cross bridges along actin filaments through its ability to interact with actin. HSP27 behaves as an actin cap-binding protein in vitro and is able to inhibit actin polymerization in vinculin-rich fraction of turkey gizzard smooth muscle (42). In vivo, the protein preferentially localizes with membrane ruffles and lamellipodia, which are active sites of actin polymerization, and modulates actin filament dynamics (36, 37). The actin regulatory functions of HSP27 are dependent on phosphorylation of the protein (7, 27, 37). Thus it is possible that agonist-dependent phosphorylation of HSP27 causes a conformational change in the protein that results in the dissociation of HSP27 from actin filaments and the release of an inhibitory constraint on the contractile apparatus. In support of this hypothesis, Bitar et al. (9) reported that incubation of permeabilized rectusigmoid SMC with monoclonal antibodies to HSP27 blocks the sustained contraction induced by bombesin and PKC. A second possibility is that activated MAPKAP kinase-2 directly phosphorylates the 20-kDa light chain subunit of myosin II, which is the on switch of actin-activated myosin ATPase activity (2). It was recently reported that MAPKAP kinase-2 is able to phosphorylate the regulatory light chain of myosin II in vitro on the same site (Ser 19) as myosin light chain kinase (31). However, the physiological relevance of this phosphorylation in vivo remains to be demonstrated. Finally, a third possibility is that p38 MAP kinase sensitizes the contractile apparatus by phosphorylating a thin filament-associated protein such as caldesmon. Caldesmon is an 87-kDa protein that binds actin and myosin and inhibits actomyosin ATPase activity (2). Caldesmon is a substrate for several protein kinases in vitro, including the ERK MAP kinases (1, 12). Phosphorylation of caldesmon by ERK1/ERK2 reduces its affinity for actin, leading to loss of inhibition of the actomyosin ATPase activity (2). These various hypotheses are currently being tested in our laboratory.

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REFERENCES

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