p38 Mitogen-activated Protein Kinase Is a Critical Component of the Redox-sensitive Signaling Pathways Activated by Angiotensin II

ROLE IN VASCULAR SMOOTH MUSCLE CELL HYPERTROPHY*

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Angiotensin II induces an oxidant stress-dependent hypertrophy in cultured vascular smooth muscle cells. To investigate the growth-related molecular targets of H$_2$O$_2$, we examined the redox sensitivity of agonist-stimulated activation of the mitogen-activated protein kinase (MAPK) family. We show here that angiotensin II elicits a rapid increase in intracellular H$_2$O$_2$ and a rapid and robust phosphorylation of both p42/p44 MAPK (16-fold) and p38 MAPK (15-fold). However, exogenous H$_2$O$_2$ activates only p38 MAPK (14-fold), and diphenylene iodonium, an NADH/NADPH oxidase inhibitor, attenuates angiotensin II-stimulated phosphorylation of p38 MAPK, but not p42/p44 MAPK. Furthermore, in cells stably transfected with human catalase, angiotensin II-induced intracellular H$_2$O$_2$ generation is almost completely blocked, resulting in inhibition of phosphorylation of p38 MAPK, but not p42/p44 MAPK, and a subsequent partial decrease in angiotensin II-induced hypertrophy. Specific inhibition of either the p38 MAPK pathway with SB203580 (4-((4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole) or the p42/p44 MAPK pathway with PD98059 (2-(2′-amino-3′-methoxyphenyl)oxanaphthalen-4-one) also partially, but significantly, attenuates angiotensin II-induced hypertrophy; however, simultaneous blockade of both pathways has an additive inhibitory effect, indicating that the hypertrophic response to angiotensin II requires parallel, independent activation of both MAPK pathways. These results provide the first evidence that p38 MAPK is a critical component of the oxidant stress (H$_2$O$_2$)-sensitive signaling pathways activated by angiotensin II in vascular smooth muscle cells and indicate that it plays a crucial role in vascular hypertrophy.

Vascular smooth muscle cell (VSMC)$^3$ proliferation and hypertrophy are hallmarks of vascular diseases, including hypertension and atherosclerosis. Recently, it has become apparent that growth of VSMCs has an essential redox-sensitive component. Rao and Berk (1) showed that reactive oxygen species generated by the combination of xanthine with xanthine oxidase stimulates proliferation of VSMCs and that this effect is mainly due to H$_2$O$_2$. Antioxidants have been shown to inhibit VSMC proliferation (2) or cause apoptosis, suggesting that some level of oxidant stress is required for normal cell growth (3). Antioxidants also attenuate the response to growth factors and hypertrophic agents (3, 4), indicating that endogenous agonists require production of reactive oxygen species for a full growth response. In VSMCs, the peptide hormone, angiotensin II (Ang II) induces cellular hypertrophy by acting through G protein-coupled AT$_1$ receptors (5–7). We have reported previously that Ang II-induced hypertrophy is mediated by reactive oxygen species such as superoxide (O$_2^-$) and H$_2$O$_2$, which are derived, at least in part, from a membrane-associated NADH/ NADPH oxidase (7, 8). However, the redox-sensitive signaling pathways involved in Ang II-induced hypertrophy have not been defined.

One potential target for reactive oxygen species may be the mitogen-activated protein kinase (MAPK) family. MAPKs are serine/threonine kinases that transduce signals from the cell membrane to the nucleus in response to classical growth factors and G protein-coupled receptor agonists, as well as cellular stress (9–11). Four groups of MAPKs have been identified in mammalian cells: the extracellular signal-regulated kinases 1 and 2 (ERK1/2, also termed p42/p44 MAPK), the c-Jun NH$_2$-terminal kinases (JNK, also termed stress-activated protein kinase, SAPK), p38 MAPK (also termed CSBP) and Big MAPK 1 (BMK1, also termed ERK5) (10–12). Although the MAPK families are structurally related, they are generally activated by distinct extracellular stimuli and phosphorylate different molecular substrates (9–11). ERK1/2 are stimulated by growth factors and mitogenic stimuli and play pivotal roles in cell growth and differentiation (10, 11). ERK1/2 stimulation initiates phosphorylation events leading to activation of transcription factors such as Elk-1 and c-Fos, and stimulation of p90$^{rsk}$ ribosomal S6 kinase (10). In contrast, p38 MAPK and JNK are primarily activated by cellular stresses, including heat and osmotic shock, UV irradiation, proinflammatory cytokines, and hypoxia/reoxygenation in other systems (13, 14). JNKs phosphorylate and activate transcription factors such as c-Jun, ATF-2, and Elk-1 (15–18), whereas p38 MAPK phosphorylates, and activates, ribosomal S6 kinase. All of these signaling pathways have been implicated in cell growth and proliferation. Therefore, we determined the role of p38 MAPK in angiotensin II-stimulated cell growth and proliferation in VSMCs.

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§ The abbreviations used are: VSMCs, vascular smooth muscle cells; Ang II, angiotensin II; O$_2^-$, superoxide; MAPK, mitogen-activated protein kinase; ERK1/2, extracellular signal-regulated kinase 1 and 2; JNK, c-Jun NH$_2$-terminal kinases; SAPK, stress-activated protein kinase; p38 MAPK, 38-kDa MAPK; BMK1, Big MAPK-1; p90$^{rsk}$, 90-kDa ribosomal S6 kinase; ATF, activating transcription factor; CHOP, C/EBP-homologous protein; MEF2C, myocyte enhancer binding factor-2C; MAPKAP kinase 2/3, MAPK-activated protein kinase 2 or 3; HSP, heat shock protein; MEK, MAPK/ERK kinase; HRP, horseradish peroxidase; DCF-DA, 2′,7′-dichlorofluorescein diacetate; DPI, diphenylene iodonium; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole; PD98059, 2-(2′-amino-3′-methoxyphenyl)oxanaphthalen-4-one; MAPK KK, MAPK kinase kinase; MKK3/6, MAPK kinase 3 or 6; PAK-1, p21-activated kinase-1; p70$^{srk}$, 70-kDa ribosomal S6 kinase; PAGE, polyacrylamide gel electrophoresis.
rylates ATF-2 and C/EBP-homologous protein (CHOP) (19, 20).
In addition, p38MAPK phosphorylates and activates MAPKAP kinase 2/3 and phosphorylates the heat shock proteins HSP27/27 (21, 22), which enhances its protective properties.

Interestingly, recent reports have provided evidence that p38MAPK phosphorylates the transcription factor MEF2C, which has been shown to be required for proper growth and development of cardiac muscle (23) and to increase transcription of the c-Jun gene (24). Despite their apparent growth-related downstream targets, studies of activation of the p38MAPK pathway have focused mainly on its response to a variety of physiochemical stresses and inflammatory cytokines (13, 14). However, a recent study implicated a role of p38MAPK in cell growth such as cardiac hypertrophy and T-cell proliferation (25, 26).

ERK1/2 are activated by growth factors via the conserved Ras/Raf/MEK pathway, while p38MAPK is regulated by stress-related signals via different upstream kinases (13, 14). Several reports demonstrate that p38MAPK is activated by H2O2 in other systems (27, 28); however, the sensitivity of ERK1/2 to oxidative stress is controversial and varies with the cell type and the stimulus (12, 27, 29, 30). In VSMCs, it has been shown that Ang II activates ERK1/2 via redox-insensitive mechanisms (29), and its activation is necessary, but not sufficient, for Ang II-induced vascular hypertrophy (31). In contrast, the effects of Ang II on p38MAPK have not been studied. Thus, our previous data establishing a role for O2− and H2O2 in VSMC hypertrophy raises the possibility that a redox-sensitive MAPK such as p38MAPK may represent the necessary additional pathway linking the Ang II-induced increase in intracellular oxidative stress to hypertrophy.

In this study, we compared the ability of Ang II to activate p38MAPK and ERK1/2, assessed their relative redox sensitivity, and examined their respective roles in hypertrophy. We found that p38MAPK is a major redox-sensitive MAPK that is preferentially activated by Ang II through intracellular generation of H2O2, but that both ERK1/2 activation and p38MAPK activation were required for hypertrophic effects of Ang II. These observations suggest that p38MAPK is a critical component of the redox-sensitive signaling pathways involved in Ang II-induced hypertrophy in VSMCs.

**EXPERIMENTAL PROCEDURES**

**Materials**—Goat anti-rabbit IgG-horseradish peroxidase (HRP) conjugate was purchased from Bio-Rad. The enhanced chemiluminescence (ECL) Western blotting detection system, nitrocellulose membranes (Hybond-ECL, 0.45 μm) and [tyrosyl-3,5-3H]Ang II (5-I-isoleucine) (47 Ci/mmol) were obtained from Amersham Pharmacia Biotech. Lipofectin and Opti-MEMI reduced serum medium were from Life Technologies, Inc. G418 sulfate (Geneticin) and TRI reagent were from Mediatech, Inc. G418 sulfate (Geneticin) and TRI reagent were from Mediatech, Inc.

**Detection of Catalase Expression in Transfected VSMCs—**Stable Transfection of Catalase Expression Plasmid in VSMCs—VSMCs were permanently transfected with pCI-neo alone or pCI-neo/catalase plasmid as described previously for pcDNA3/p22phox antisense transfection (8). Transfected cells were maintained in selection medium until they were plated into 35- or 100-mm dishes for experiments.

**RNA Isolation and Northern Blot Analysis—**Total RNA was extracted from cells as described previously (33). Aliquots of RNA samples were separated by electrophoresis in 1.0% agarose gels containing 6.6% formaldehyde. RNA was transferred onto a nylon membrane and immobilized by UV cross-linking (Stratalinker, Stratagene, La Jolla, CA). The probe, catalase cDNA derived from XbaI/SalI digest of pCI-neo/catalase, was labeled with [α-32P]dCTP using a random primer labeling kit (Prime-It II). After UV cross-linking, membranes were prehybridized at 68 °C for 2 h in QuikHyb solution (Stratagene). The hybridization was performed for 2 h at 68 °C with [32P]-labeled probe in the same solution. Membranes were briefly rinsed and washed twice in 1 × SSC + 0.1% SDS at 50 °C. Staining of the 28 S rRNA band by ethidium bromide, after transfer to the membrane, was used for normalization.

**Detection of p38MAPK, ERK1/2, and JNK Phosphorylation by Immunoblotting—**Immunoprecipitation and p38MAPK Activity Assay—p38MAPK activity in immunoprecipitates was measured using the p38MAPK assay kit (New England Biolabs) according to the manufacturer’s instructions. This protocol measures p38MAPK-induced phosphorylation of recombinant ATF-2, as assessed by Western blotting using 12% SDS-PAGE and phospho-specific ATF-2 antibody (1:1,000).

**Detection of Catalase Protein Expression in Transfected VSMCs—**Stable Transfection of Catalase Expression Plasmid in VSMCs—VSMCs were permanently transfected with pCI-neo alone or pCI-neo/catalase plasmid in 100-mm dishes were washed three times with 5 ml of ice-cold PBS and placed on ice. Cells were lysed with 500 μl of ice-cold lysis buffer, pH 7.4 (in mm) 50 HEPES, 5 EDTA, 50 NaCl, 1% Triton X-100, protease inhibitors (10 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin), and the hybridization was performed for 2 h with PBS containing 6% non-fat dry milk and 0.1% Tween 20. The blots were incubated for 1 h with primary antibodies (rabbit polyclonal phospho-specific p38, ERK1/2, and JNK antibodies that detect MAPK only when activated by phosphorylation on TXY, at 1:2,000, 1:40,000, and 1:1,000, respectively) in PBS containing 1% non-fat dry milk and 0.1% Tween 20. After incubation with antibodies (HRP-conjugated goat anti-rabbit antibody, 1:1,000) for 1 h in PBS containing 1% non-fat dry milk and 0.1% Tween 20, phosphorylated forms of proteins were detected by ECL chemiluminescence. It has been shown that phosphorylation of MAPks is associated with the activation of MAPks (27, 34, 35); therefore phosphorylation was routinely taken as a measure of MAPK enzymatic activity. In some experiments, p38MAPK activity was verified directly in VSMC p38MAPK immunoprecipitates.

**Immunoprecipitation and p38MAPK Activity Assay—VSMC lysates were prepared as described above for MAPK phosphorylation assays.** For immunoprecipitation, 400-μg cell lysates were incubated with rabbit anti-p38MAPK antibody (1:50 dilution) overnight at 4 °C, and then incubated with 20 μl of protein A-Sepharose beads for 1.5 h at 4 °C with gentle rocking. The beads were washed four times with 500 μl of lysis buffer and two times with 500 μl of kinase buffer (in mm) 25 Tris, pH 7.5, 5 β-glycerophosphate, 2 dithiothreitol, 0.1 Na2VO3, 10 MgCl2). p38MAPK activity in immunoprecipitates was measured using the p38MAPK assay kit (New England Biolabs) according to the manufacturer’s instructions. This protocol measures p38MAPK-induced phosphorylation of recombinant ATF-2, as assessed by Western blotting using 12% SDS-PAGE and phospho-specific ATF-2 antibody (1:1,000).

**Detection of Catalase Protein Expression in Transfected VSMCs by Immunoblotting—**Confluent VSMCs were transfected with pCI-neo alone or pCI-neo/catalase plasmid in 100-mm dishes were washed three times with 5 ml of ice-cold PBS. Cells from duplicate 100-mm dishes were sonicated, combined in 10 ml of PBS, and were centrifuged at 9,000 × g for 10 min. Pellets were re-suspended in 1 ml of ice-cold lysis buffer, pH 7.4 (in mm) 50 HEPES, 5 EDTA, 50 NaCl, containing protease inhibitors (10 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin) and phosphatase inhibitors ([in mm] 50 sodium fluoride, 1 sodium orthovanadate, 10 sodium pyrophosphate). After sonication for 30 s on ice, protein was quantified by the Bradford assay. Proteins were separated on 9% polyacrylamide gels.
using SDS-PAGE and transferred to Hybond-ECL nitrocellulose membranes at 100 V for 1 h. Membranes were blocked for 1 h with PBS containing 5% non-fat dry milk and 0.1% Tween 20 and were incubated for 1 h with primary anti-human erythrocyte catalase antibody (1:500) in PBS containing 1% non-fat dry milk and 0.1% Tween 20, washed three times with PBS containing 1% non-fat dry milk and 0.1% Tween 20, and then incubated with HRP-conjugated goat anti-rabbit secondary antibody (1:1,000) for 1 h. Expression of catalase protein was detected by ECL chemiluminescence.

Measurements of Intracellular H$_2$O$_2$ Levels—Cells were plated at low density, grown for 48 h in DMEM containing 10% calf serum, and grown for an additional 24 h in DMEM containing 0.1% calf serum. Cells were stimulated with 100 nM Ang II for 1 min, and the H$_2$O$_2$-sensitive fluorophore DCF-DA (5 μM) (36) was used to measure intracellular H$_2$O$_2$ by laser confocal scanning microscopy (MRC-1000, Bio-Rad) as described previously (8). Although DCF-DA is oxidized by both H$_2$O$_2$ and other peroxides, the complete inhibition of fluorescence in Ang II-stimulated cells by addition of catalase (350 units/ml) (data not shown) and by catalase overexpression (Fig. 4C) indicates that the

FIG. 1. Effects of H$_2$O$_2$ on p38MAPK and ERK1/2 phosphorylation in VSMCs. Growth-arrested VSMCs stimulated with H$_2$O$_2$ were harvested, and aliquots (25 μg) of the 1% Triton X-soluble fraction of VSMCs lysates were separated by 10% SDS-PAGE. Size-fractionated proteins were transferred to nitrocellulose membranes. Membranes were then probed with rabbit polyclonal phospho-specific anti-p38MAPK and -ERK1/2 antibodies, which only detect phosphorylated, activated forms. Specific binding was detected with HRP-conjugated anti-rabbit IgG and visualized by chemiluminescence. A, time course for p38MAPK and ERK1/2 phosphorylation by H$_2$O$_2$. Cells were stimulated with 200 μM H$_2$O$_2$ for the indicated times. B, dose response of p38MAPK phosphorylation by H$_2$O$_2$. Growth-arrested VSMCs were stimulated with various concentrations of H$_2$O$_2$ (50–200 μM) for 15 min. In A and B, top panels are representative immunoblots of p38MAPK (A) and ERK1/2 (B). Bottom panels represent averaged data quantified by densitometry of immunoblots using NIH image 1.61, expressed as -fold increase in phosphorylation, in which the phosphorylation observed in cells at time 0 (for A) or in unstimulated cells (for B) was defined as 1.0 (control). Values are the mean ± S.E. for three independent experiments. *, p < 0.05 versus control.

Fig. 2. Effects of Ang II on p38MAPK and ERK1/2 phosphorylation in VSMCs. Preparation of cell lysates, Western blotting, and detection of phosphorylation were performed as described in the legend to Fig. 1. A, time course of p38MAPK and ERK1/2 phosphorylation by Ang II. Growth-arrested VSMCs were stimulated with 100 nM Ang II for the indicated times. B, dose response of p38MAPK phosphorylation by Ang II. Growth-arrested VSMCs were stimulated with various concentrations of Ang II (0.1–1000 nM) for 5 min. In A and B, top panels are representative immunoblots of Ang II-induced phosphorylation of p38MAPK (A) and ERK1/2 (B). Bottom panels represent averaged data quantified by densitometry of immunoblots using NIH image 1.61, expressed as -fold increase in phosphorylation, in which the phosphorylation observed in cells at time 0 (for A) or in unstimulated cells (for B) was defined as 1.0 (control). Values are the mean ± S.E. for three independent experiments. *, p < 0.05 versus control.
fluorescence signal evoked by Ang II was predominantly derived from
H₂O₂.

[^H]Leucine Incorporation—To measure hypertrophy of VSMCs, cells were
quiesced for 48–72 h in DMEM containing 0.1% calf serum. Twenty-four
hours before harvest, cells were incubated with [^H]leucine (0.5
μCi/ml) in the presence or absence of 100 nM Ang II for 24 h. [^H]Leucine
incorporation was measured as described previously (7).

Ang II Receptor Binding—The Ang II receptor binding assay was
performed as described previously (37). B_satur (maximum number of
binding sites) was determined by single point saturation binding.

Statistical Analysis—Results are expressed as mean ± S.E. Sta-
tistical significance was assessed by Student’s paired two-tailed t test or
analysis of variance on untransformed data, followed by comparison of
group averages by contrast analysis, using the SuperANOVA statistical
program (Abacus Concepts, Berkeley, CA). A p value of <0.05 was
considered to be statistically significant.

RESULTS

Effect of Exogenous H₂O₂ on MAPK Activation—H₂O₂ has
been shown to activate p38MAPK and JNK in other cell types,
but its effects on ERK1/2 are controversial (12, 13, 27, 29, 30,
38). To assess the effect of H₂O₂ on MAPKs in VSMCs, we
exposed cells to 200 μM H₂O₂ and measured activation of indi-
vidual MAPKs. H₂O₂ induced a rapid activation of p38MAPK,
with a peak occurring 15 min after H₂O₂ addition (14 ± 1-fold
increase) (Fig. 1A). p38MAPK activation was still detectable at
30 min. Measurement of p38MAPK activity in p38MAPK im-
munoprecipitates showed similar results (see Fig. 7). H₂O₂-
induced p38MAPK phosphorylation was dose-dependent, with
a threshold of 50 μM and a maximal effect occurring at 200 μM
(Fig. 1B). These concentrations are similar to those previously
reported for H₂O₂-stimulated proliferation of VSMCs (1). In
contrast, ERK1/2 phosphorylation was inhibited by H₂O₂, at
2–5 min, was slightly but not significantly increased to above
basal levels by 10 min, and then declined again to below the
control levels. H₂O₂ had a small effect on JNK activation (max-
imum 2-fold increase at 15 min) (data not shown). These data
suggest that p38MAPK is the major MAPK activated by H₂O₂
in VSMCs.

Ang II Activates p38MAPK in VSMCs—Because p38MAPK
is a redox-sensitive kinase (Fig. 1) and Ang II increases intra-
cellular oxidant stress in VSMCs (7), we examined whether
Ang II activates p38MAPK. As shown in Fig. 2A, Ang II caused

Fig. 3. Effects of diphenylene iodonium, an inhibitor of the
NADH/NADPH oxidase, on p38MAPK and ERK1/2 phosphoryla-
tion by Ang II in VSMCs. Growth-arrested VSMCs were treated with
or without 10 μM DPI for 30 min prior to treatment with (+) or without
(−) 100 nM Ang II for 5 min. Equal aliquots of protein (25
μg) from whole cell homogenates were used to measure
p38MAPK and ERK1/2 phosphorylation, as described in the legend to
Fig. 1. This immunoblot is representative of identical results obtained
from 2 separate experiments.

Fig. 4. Effect of overexpression of catalase on intracellular H₂O₂ pro-
duction by Ang II in VSMCs. A and B, Northern and Western blot analysis of
catalase mRNA (A) and protein (B) levels in VSMCs stably transfected with expres-
sion vector pCI-neo containing human catalase cDNA. Representative blots of
catalase mRNA (A) and protein (B) levels in a vector-transfected clone (pCI-neo)
and a catalase-transfected clone with highest mRNA expression (pCI-neo/Cat).
Top in A, the size of the catalase mRNA band is 2.3 kilobases. Bottom in A, the 28
S ribosomal RNA band stained with ethidium bromide. B, proteins (28 μg)
from whole cell homogenates were separated by 9% SDS-PAGE and transferred
to nitrocellulose membranes. Membranes were then probed with rabbit anti-human
catalase antibody. Specific binding was detected with HRP-conjugated anti-rab-
bit IgG and visualized by chemilumines-
cence. The molecular mass standards are
indicated on the right of membrane. C, increase in intracellular H₂O₂ levels in
vector-transfected cells and catalase-
overexpressing cells stimulated with 100
nM Ang II for 1 min, as measured by con-
focal microfluorometry. C, panels a and b:
vector-transfected cells stimulated with
vehicle or Ang II, respectively. C, panels c
and d: catalase-overexpressing cells stimu-
lated with vehicle or Ang II, respectively.
Fluorescence was visualized at a × 20 magnification, using a laser intensity
of 30, an iris setting of 3.5, and a gain of
1,200.
a rapid, robust activation of p38MAPK. p38MAPK phosphorylation peaked at 5 min (15 ± 2-fold increase) and then gradually decreased, remaining above base line for at least 30 min. The ability of Ang II to stimulate p38MAPK activity was verified in p38MAPK immunoprecipitates (see Fig. 7). Ang II-induced p38MAPK phosphorylation was dose-dependent, with a threshold at 0.1 nM and a maximal effect occurring at 100 nM (Fig. 2B). As reported by others (41), Ang II also induced a rapid, robust activation of ERK1/2 with a similar time course for p38 activation (16 ± 3-fold increase at 5 min) (Fig. 2A). Ang II had a small stimulatory effect on JNK with a peak at 5 min (2-fold increase) (data not shown).

To determine whether the Ang II-stimulated p38MAPK activation is redox-sensitive, we examined the effect of DPI, an inhibitor of flavin-containing oxidative enzymes, on p38MAPK phosphorylation. We have previously shown that DPI inhibits Ang II-stimulated production of reactive oxygen species that are derived from the NADH/NADPH oxidase in VSMCs (7). As shown in Fig. 3, DPI (10 μM) inhibited Ang II-induced p38MAPK phosphorylation by 72 ± 3%. In contrast, DPI had no effect on Ang II-stimulated ERK1/2 phosphorylation. These data suggest that Ang II-induced p38MAPK, but not ERK1/2, activation is mediated by reactive oxygen species.

Role of Intracellular H₂O₂ in Ang II Stimulation of p38MAPK—Because exogenous H₂O₂ is a strong stimulant for p38MAPK, we postulated that this reactive oxygen species might be rapidly produced by Ang II and mediate subsequent redox-sensitive signaling events such as p38MAPK activation. Therefore, we measured intracellular H₂O₂ ([H₂O₂]) levels produced by Ang II, as measured by oxidation of the peroxide-sensitive fluorophore DCF-DA. As expected, [H₂O₂]i was rapidly increased by Ang II stimulation within 1 min (143 ± 3% control at 1 min, n = 15 (five fields for each experiment)), a time course consistent with a potential role for this molecule in downstream signaling.

To assess the role of increased [H₂O₂]i in Ang II-induced p38MAPK activation, we stably overexpressed catalase in VSMCs. We initially isolated 39 clones of Geneticin-resistant catalase-transfected cells; however, only two of these significantly overexpressed catalase mRNA and protein. We selected and amplified the highest expressor for further study (Fig. 4, A and B). As shown in Fig. 4C, the Ang II-induced increase in [H₂O₂]i at 1 min was dramatically decreased in catalase-overexpressing cells. In these cells, Ang II-stimulated p38MAPK phosphorylation and activity were also markedly inhibited (79 ± 3 and 89 ± 4%, respectively) (Fig. 5B). This effect was not due to differences in AT₁ receptor expression in catalase-overexpressing cells, since vector- and catalase-transfected cells were matched for receptor number (Bmax: 1034 fmol/mg of protein and 1237 fmol/mg of protein in vector- and catalase-transfected cells, respectively). Furthermore, Ang II-induced ERK1/2 activation was unaffected (Fig. 5A). These results strongly suggest that intracellular H₂O₂ generation mediates p38MAPK, but not ERK1/2, activation by Ang II.

Role of p38MAPK in Ang II-induced Hypertrophy—We have shown previously that reactive oxygen species play an important role in Ang II-induced hypertrophy (7, 8). This was further confirmed by the present observation that Ang II-stimulated hypertrophy was partially, but significantly, inhibited in catalase-overexpressing cells (Fig. 6). To determine whether this inhibition was related to the H₂O₂-dependent activation of p38MAPK by Ang II, we used a specific p38MAPK inhibitor SB203580, a pyridyl imidazole that inhibits activation of the immediate substrate of p38MAPK, MAPKAP kinase-2 (39). As shown in Fig. 7, SB203580 (10 μM) nearly abolished both H₂O₂- and Ang II-induced activation of p38MAPK. Importantly, SB203580 also dose-dependently inhibited Ang II-stimulated [3H]leucine incorporation (Fig. 8A). The incomplete inhibition of [3H]leucine incorporation in VSMCs suggests that p38MAPK activation is necessary, but not sufficient, for hypertrophy. Previous studies have shown that ERK1/2 also participates in Ang II-induced hypertrophy (31). Therefore, we hypothesized that activation of both p38MAPK and ERK1/2 pathways may be necessary for a full hypertrophic response. Inhibition of the ERK1/2 pathway by PD98059, a compound that specifically inhibits the ERK1/2 kinase MEK without affecting p38MAPK or JNK/SAPK (40), also partially attenuated Ang II-induced hypertrophy when used at concentrations that have been shown to inhibit ERK1/2 activation (Fig. 5B) (40). When both the p38MAPK inhibitor and the MEK (ERK1/2) inhibitor were combined at maximally effective concentrations, the inhibitory effect on Ang II-induced hypertrophy was additive (p < 0.01) (Fig. 8C). These results suggest that both p38MAPK and ERK1/2 pathways independently contribute to Ang II-induced hypertrophy.

**DISCUSSION**

Recently, it has become apparent that reactive oxygen species are important signaling entities in Ang II stimulation of VSMC growth (7, 8). Here we provide direct evidence that Ang II elicits a rapid increase in intracellular H₂O₂ within 1 min in cultured VSMCs, suggesting that it may mediate subsequent early growth-related signaling events. One potential target of oxidative stress may be the MAPK family of proteins, kinases that play a critical role in transmitting cell surface signals to the nucleus. We show here that p38MAPK is the major MAPK activated by exogenous H₂O₂ in VSMCs. Although Ang II ro-
Here we show that Ang II, a peptide hormone that binds to the AT1 receptor, activates p38MAPK, which is traditionally associated with endotoxins, inflammatory cytokines, and environmental stresses in other systems (13, 14). We show that Ang II, a peptide hormone that binds to the G protein-coupled AT1 receptor, activates p38MAPK in addition to ERK1/2 in VSMCs. This is consistent with the recent observation that p38MAPK can be activated by thrombin in human platelets (42, 43) and by chemotactic peptide in human neutrophils (44). More recently, Yamauchi et al. (45) reported that stimulation of m1 or m2 muscarinic receptors or the β-adrenergic receptor transfects into HEK293 cells induces p38MAPK activation. Although in each case p38MAPK activation is mediated by G protein-coupled receptors, the upstream signaling mechanisms responsible for p38MAPK activation by such agonists are unclear, but are likely to be quite different from those responsible for ERK1/2 activation. ERK1/2 activation results from sequential stimulation of Ras-associated protein kinase C-ζ, Raf-1, or MEK kinase and MEK1/2 in VSMCs (41). In contrast, in other cell types, p38MAPK activation is a consequence of Rac/Cdc42-mediated PAK-1 activation, followed by MAPKKK activation and MKK3/6 stimulation (46). In this study, we found that ERK1/2 is not activated by exogenous H2O2 and that Ang II activates ERK1/2 through redox-sensitive mechanisms in VSMCs (Figs. 1A, 3, and 5), which is consistent with previous reports (12, 29). On the other hand, p38MAPK was robustly activated by both exogenously applied H2O2 and agonist-induced intracellularly produced H2O2. Indeed, when the Ang II-induced increase in intracellular H2O2 generation was blocked by overexpression of catalase (Fig. 4C), activation of p38MAPK, but not ERK1/2, was markedly inhibited (Fig. 5). These data suggest that intracellular H2O2 produced by Ang II plays an important role as a potential signal transducer that couples the AT1 receptor to p38MAPK, but not to ERK1/2. Thus, reactive oxygen species may be proximal signaling molecules that discriminate between the two pathways activated by Ang II in VSMCs.

It has been reported that exogenous H2O2 stimulates p38MAPK activity in other cell types (27, 28), but its functional role is incompletely understood. We have shown previously that intracellular H2O2 generation plays an important role in Ang II-induced hypertrophy (7, 8), but the molecular target of H2O2 has not been identified. Our present data suggest that it may be the redox-sensitive p38MAPK pathway that is involved in the H2O2-mediated growth response in VSMCs, since (a) the NADH/NADPH oxidase inhibitor DPI, which also inhibits Ang II-induced hypertrophy (7), attenuates p38MAPK activation by Ang II (Fig. 3); (b) in catalase-overexpressing cells with diminished Ang II-induced H2O2 production and subsequent p38MAPK activation (Figs. 4C and 5), Ang II-induced hypertrophy is inhibited (Fig. 6); and (c) specific inhibition of p38MAPK by the pyridinyl imidazole derivative SB203580 (Fig. 7) (39) leads to a significant decrease in Ang II-induced hypertrophy (Fig. 8A). Thus, the p38MAPK pathway may provide a functional link between hormone-stimulated production of reactive oxygen species and vascular hypertrophy.

The present study emphasizes the potentially critically important role of p38MAPK in redox-sensitive growth-related signaling pathways. Consistent with our results, recent reports suggested a role for p38MAPK in cardiac hypertrophy and T-cell proliferation (25, 26). However, p38MAPK has also been proposed to be involved in apoptosis (13). Indeed, although a growing body of evidence supports the concept that reactive oxygen species are pro-proliferative in VSMCs, some conflicting results have been reported. Fiorani et al. (47) showed that although H2O2 increased DNA synthesis, this increase was followed by cell death. Li et al. (48) reported that when VSMCs were subjected to excess oxidant stress by exposure to glucose/glucose oxidase or diethylmaleate, the resulting H2O2-induced apoptosis via the formation of hydroxyl radicals. Whether H2O2 induces cell growth or apoptosis may be determined by the difference in the magnitude of the oxidant stress. While a certain level of oxidant stress appears to be growth-promoting, more severe stress may lead to cell death. Our studies suggest that the H2O2 produced in response to Ang II and the resulting

FIG. 6. Effect of overexpression of catalase on Ang II-induced hypertrophy in VSMCs. Growth-arrested VSMCs transfected with vector alone (pCl-neo) or cells overexpressing catalase (pCl-neo/Cat) were stimulated with 100 nM Ang II for 24 h. Bars represent the percent increase in [3H]leucine incorporation stimulated by Ang II over that in unstimulated cells. Values are the mean ± S.E. for four independent experiments performed in triplicate. *, p < 0.01 for increase in [3H]leucine incorporation by Ang II in catalase-overexpressing cells versus vector-transfected cells.

FIG. 7. Effect of SB203580 on H2O2- and Ang II-induced activation of p38MAPK in VSMCs. VSMCs were incubated with SB203580 at the indicated concentrations for 30 min and then exposed to H2O2 (200 μM) for 15 min (A) or Ang II (100 nM) for 5 min (B). p38MAPK activity was assessed by phosphorylation of ATF-2 in p38MAPK immunoprecipitates. Each membrane is representative of results obtained from three independent experiments.
activation of p38MAPK is of a magnitude consistent with activation of the growth program.

Oxidative stress alone appears to be insufficient to support the full hypertrophic response to Ang II. This is demonstrated by the incomplete inhibition of hypertrophy in catalase-overexpressing cells. The use of cells transfected with catalase permits targeting catalase intracellularly, overcoming the potential incomplete effectiveness of exogenous addition of catalase, where catalase must exert its effect by hydrolyzing H₂O₂ as it diffuses out of the cell. The efficacy of catalase overexpression in VSMCs was confirmed by the dramatic inhibition of Ang II-induced H₂O₂ accumulation (Fig. 4C). Since inhibition of the hypertrophic response was partial under these conditions, it is likely that other redox-insensitive pathways are involved. This notion is further supported by our previous and present observations that inhibition of the NADPH oxidase (7, 8) or the redox-sensitive p38MAPK pathway (Fig. 8A) incompletely attenuated Ang II-induced hypertrophy. Since previous studies have shown that activation of the ERK1/2 pathway is necessary, but not sufficient, for Ang II-induced hypertrophy (31), we hypothesized that activation of both the p38MAPK and ERK1/2 pathways may be necessary for a full hypertrophic response to Ang II. Indeed, the p38MAPK inhibitor, SB203580, and the MEK (ERK1/2) inhibitor, PD98059, exerted additive inhibitory effects on Ang II-stimulated hypertrophy, providing strong evidence that both MAPK pathways independently contribute to Ang II-induced hypertrophy. One possible explanation for this requirement for dual activation of distinct MAPK pathways is that they activate different kinases (e.g. p38MAPK stimulates p70 S6K while ERK1/2 activates p90 rsk), known to be downstream of the ERK1/2. p70 S6K phosphorylates ribosomal protein S6, whose activation is correlated with protein synthesis at the level of initiation (53, 54). Importantly, it has recently been found that p70 S6K is a substrate for p38MAPK (49). Thus, it appears that ERK1/2 and p38MAPK pathways may converge at the level of ribosomal protein S6 to regulate vascular hypertrophy.

In summary, we demonstrate here that p38MAPK is a highly redox-sensitive MAPK that is activated by exogenous H₂O₂ and Ang II in VSMCs. The Ang II-induced activation of p38MAPK is mediated by intracellular H₂O₂ generation. This pathway may be a link between hormone-stimulated production of reactive oxygen species and vascular hypertrophy. In itself, however, p38MAPK is not sufficient for hypertrophy, but rather appears to require parallel, independent activation of the redox-insensitive ERK1/2 pathway. Thus, the present study provides the first evidence that p38MAPK is a critical component of the redox-sensitive signaling pathways activated by Ang II in VSMCs and indicates that they play a crucial role in vascular hypertrophy. The downstream targets of p38MAPK that participate in the hypertrophic response, as well as their interaction with known ERK1/2 substrates, remain to be clarified.

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