p38 MAP Kinase Regulates Vascular Smooth Muscle Cell Collagen Synthesis by Angiotensin II in SHR But Not in WKY

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Abstract—Vascular remodeling in hypertension is associated with cell growth and increased deposition of extracellular matrix components, particularly collagen. Mechanisms underlying these processes are unclear, but MAP kinases, particularly ERK1/2 and p38 MAP kinase, may be important. We studied the role of ERK1/2 and p38 MAP kinase in vascular smooth muscle cell (VSMC) collagen synthesis and growth mediated by angiotensin (Ang) II in spontaneously hypertensive rats (SHR). Cultured mesenteric VSMC from Wistar-Kyoto rats and SHR were used. Phosphorylation of ERK1/2 and p38 MAP kinase were assessed by Western blots with phosphospecific antibodies. Ang II–stimulated DNA and collagen synthesis were determined by measuring incorporation of ³H-thymidine and ³H-proline, respectively. mRNA expression of procollagen I and III was determined by reverse transcription–polymerase chain reaction. Ang II increased ERK1/2 and p38 MAP kinase phosphorylation. Responses were augmented in SHR. Effects were inhibited by irbesartan, a selective AT₁ antagonist, but not by PD123319, a selective AT₂ blocker. Ang II stimulated ³H-thymidine and ³H-proline incorporation. These actions were enhanced 2- to 3-fold in SHR. PD98059, selective inhibitor of the ERK1/2 pathway, attenuated Ang II–induced growth and collagen effects and normalized responses in SHR. SB212190, a selective p38 MAP kinase inhibitor, did not alter Ang II–elicited DNA synthesis but reduced collagen production and mRNA expression of procollagen I and III in SHR. These data demonstrate that (1) Ang II–mediated activation of p38 and ERK1/2 is increased in SHR, (2) augmented growth responses are generated by ERK1/2-dependent, p38 MAP kinase-independent pathways, and (3) p38 MAP kinase influences Ang II–induced collagen production in SHR but not in Wistar-Kyoto rats. These results indicate differential roles of ERK1/2 and p38 MAP kinase in AT₁-stimulated VSMC growth and collagen production, which may contribute to vascular remodeling in hypertension. (Hypertension. 2001; 37[part 2]:574-580.)

Key Words: kinase ■ renin-angiotensin system ■ hypertrophy ■ arteries ■ hypertension, arterial

Hypertension is associated with vascular structural changes (vascular remodeling) characterized by increased media-to-lumen ratio.¹ The pathophysiological processes contributing to vascular remodeling include, among others, vascular smooth muscle cell (VSMC) growth (hypertplasia and/or hypertrophy) and increased synthesis and rearrangement of extracellular matrix proteins, such as collagen types I and III.²–⁴ Vascular remodeling has an important angiotensin (Ang) II–dependent component because interruption of the renin-angiotensin system with ACE inhibitors or AT₁ receptor antagonists corrects vascular structure and endothelial dysfunction in small arteries of hypertensive patients and hypertensive rat models⁵–⁷ and decreases coronary artery periarteriolar collagen deposition in hypertensive patients given long-term treatment with ACE inhibitors.⁸

The molecular and cellular mechanisms underlying Ang II–dependent processes in vascular remodeling have not been fully elucidated, but activation of MAP kinases, particularly ERK1/2 and p38 MAP kinase, may be important. We previously demonstrated that increased ERK1/2 signaling by Ang II is associated with VSMC hypertrophy and hyperplasia and that inhibition of MEK1/2, the kinase that activates ERK1/2, normalizes exaggerated responses in VSMCs from SHR.³⁻⁹ Ang II–stimulated ERK1/2 activation also plays a role in hypertension-associated aortic and renal vascular fibrosis by stimulating collagen type I formation.¹⁰⁻¹³ The role of p38 MAP kinase in these processes is unclear. The p38 MAP kinase pathway typically mediates apoptosis and inflammatory reactions in response to cellular stresses.¹⁴ However, there is increasing evidence that p38 MAP kinase is also activated by G protein–coupled receptors, such as AT₁, and that this kinase plays an important role in the induction of hypertrophic responses in susceptible cells.¹⁴⁻¹⁶ Studies with neonatal cardiomyocytes demonstrated that activation of p38 MAP kinase–dependent pathways is associated with hypertrophy,¹⁷ and in myocardial ischemia, cardiac hypertrophy,
and atherosclerosis, upregulation of p38 MAP kinase is, at least in part, responsible for the induction of early-response genes and cell growth.\textsuperscript{14,18,19} P38 MAP kinase may also influence cardiac hypertrophy by stimulating collagen synthesis and fibrogenesis. Although p38 MAP kinase appears to be important in growth signaling in cardiac cells, little is known about these events in the vasculature, and it is unclear whether p38 MAP kinase influences cellular processes associated with Ang II–dependent vascular remodeling in hypertension.

The objective of this study was to determine whether Ang II activates p38 MAP kinase in VSMCs and to evaluate whether this MAP kinase pathway influences cellular growth and production of extracellular matrix proteins in VSMCs from spontaneously hypertensive rats (SHR). We also evaluated the role of ERK1/2 in these processes. Our data indicate that Ang II increases p38 MAP kinase activity in SHR. These effects are associated with enhanced growth responses, augmented synthesis of collagen, and increased expression of procollagen mRNA. PD98059, a selective inhibitor of the ERK1/2 pathway,\textsuperscript{20} decreased DNA and protein synthesis, and SB202190, a selective p38 MAP kinase inhibitor,\textsuperscript{21} abrogated collagen synthesis in SHR but not in Wistar-Kyoto rats (WKY). Moreover, SB202190 decreased Ang II–induced expression of procollagen in SHR. Results from this study suggest that Ang II increases activity of multiple MAP kinases, which may play differential roles in VSMC function in hypertension. Whereas ERK1/2 appears to be important in vascular hyperplasia and hypertrophy, p38 MAP kinase is an important regulator of collagen synthesis in SHR. These events could contribute to altered VSMC growth and increased deposition of extracellular matrix proteins, important processes in vascular remodeling in hypertension.

**Methods**

**Animal Experiments**

The study was approved by the Animal Ethics Committee of the Clinical Research Institute of Montreal. Seventeen-week-old male WKY (n = 20) and SHR (n = 20) (Taconic Farms Inc) were studied. Systolic blood pressure (SBP) was recorded in conscious rats by the tail-cuff method. SBP was significantly higher (\(P < 0.001\)) in SHR (190 ± 2.1 mm Hg) than in WKY (112 ± 1.0 mm Hg).

**Cell Culture**

Rats were killed by decapitation. VSMC derived from mesenteric arteries were isolated and characterized as described in detail previously.\textsuperscript{22} Early passaged cells (passages 4 to 7) were used. Cells were rendered quiescent by serum deprivation for 36 hours before experimentation.

**Western Blotting of MAP Kinases**

Quiescent cells were stimulated with Ang II for various times. In some experiments, cells were pretreated for 20 to 30 minutes with 10\(^{-3}\) mol/L irbesartan (selective AT\(_1\) receptor antagonist) or PD123319 (selective AT\(_2\) receptor blocker). This concentration was selected because we previously demonstrated that AT\(_1\) effects in VSMCs are completely blocked by 10\(^{-3}\) mol/L irbesartan and that AT\(_2\) effects in PC12W cells (which exclusively express AT\(_2\) receptors) are completely inhibited by 10\(^{-3}\) mol/L PD123319. Cells were prepared for immunoblotting according to our previously described methods.\textsuperscript{3,9} Equal amounts of proteins were loaded on a 10% SDS-polyacrylamide gel and transferred to PVDF membrane (Boehringer Mannheim) for 1 hour at 100 V. Membranes were blocked with blocking buffer and incubated for 24 hours at 4°C with a phosphospecific ERK1/2 antibody (Calbiochem) (diluted 1:1000) or phosphospecific p38 MAP kinase antibody (Calbiochem) (diluted 1:1000). Membranes were washed, incubated with a goat anti-rabbit horseradish peroxide–conjugated antibody (Bio-Rad Laboratories), diluted 1:2000 for 1 hour, and washed extensively. Membranes were incubated with Blotting Substrate (POD) (Boehinger Mannheim), exposed to film, and developed. Band intensity was measured by computer analysis with the ImageQuant program.

**Determination of DNA and Collagen Synthesis**

DNA synthesis was evaluated by measuring incorporation of \(^{3}H\)-thymidine into DNA, and collagen synthesis was determined by measuring \(^{3}H\)-proline incorporation. Quiescent cells were stimulated for 36 hours with increasing concentrations of Ang II. To assess the role of ERK1/2–dependent and p38 MAP kinase–dependent pathways, cells were cotreated with PD98059 or SB202190 (10\(^{-3}\) mol/L). \(^{3}H\)-thymidine incorporation was measured as previously described.\textsuperscript{3,9} \(^{3}H\)-thymidine (5 \(\mu\)Ci/mL) was added to Ang II–stimulated cells and incubated for 4 hours. \(^{3}H\)-proline incorporation was measured according to the protocol of Dubey et al.\textsuperscript{22} \(^{3}H\)-proline (1 \(\mu\)Ci/mL) was added at the same time as Ang II addition.

**Reverse Transcription–Polymerase Chain Reaction Analysis of Procollagen I and III mRNA**

Total RNA was extracted from cells with TRizol (Gibco BRL, Life Technologies) and treated with RNase-free DNase. Contamination of sample RNA by genomic DNA was excluded by directly subjecting sample RNA to polymerase chain reaction (PCR) amplification without a reverse transcription (RT) step. mRNA (0.5 \(\mu\)g) was reverse-transcribed in a final volume of 20 \(\mu\)L with M-MLV RT and 1 \(\mu\)g/mL oligo(dT) primer. Single-stranded cDNA (4 \(\mu\)L) was used for PCR to amplify a 405-bp fragment of proI collagens (procollagen I) cDNA with the complementary antisense primer GTTTACAGGAACACGAGCAGG, the sense primer CGATGGAT-TCCAGTTGTA, at an annealing temperature of 56°C. For a 447-bp fragment of proI(III) collagens (procollagen III) cDNA, the antisense primer was CACCTCTGAACTGTGTAAGTG, and the sense primer was CCACCGTAACCTCAAGGTG, and annealing temperature used was 58°C. GAPDH was used as housekeeping gene. PCR was performed for 22 cycles with Taq polymerase (Gibco-BRL).

**Quantification of PCR Products**

PCR products (10 \(\mu\)L per lane) were subjected to electrophoresis with 1.5% agarose gel containing ethidium bromide. The gel was dried, subjected to ultraviolet light, and photographed. The band intensities were measured with an ImageQuant software package (Molecular Dynamics). Signals of the expressed cDNAs were expressed relative to the intensity of GAPDH cDNA in each coamplified sample.

**Data Analysis**

Each experiment was repeated at least 3 times. Results are presented as mean ± SEM and compared by ANOVA or Student’s \(t\) test where appropriate. Concentration-response curves were fitted by nonlinear regression, and the concentration (in mol/L) giving 50% response (EC\(_{50}\)) was determined and pD\(_2\) calculated as \(-\log EC_{50}\). A value of \(P<0.05\) was considered significant.

**Results**

**Ang II–Induced Activation of ERK1/2 and p38 MAP Kinase**

Ang II stimulated phosphorylation of p38 MAP kinase and ERK1/2 in VSMCs from WKY and SHR. Ang II increased p38 MAP kinase activity 2- to 3-fold in SHR cells, with peak responses obtained at 5 minutes (Figure 1). p38 MAP kinase phosphorylation was only modestly increased by Ang II in
VSMCs from WKY. Irbesartan but not PD123319 abrogated Ang II–induced activation of MAP kinases, indicating the exclusive role of AT1 receptors in these processes (Table). In agreement with our previous findings, Ang II induced a 4- to 5-fold increase in ERK1/2 activity in SHR compared with WKY (1.5-fold increase). These responses were maximal at 5 minutes.

**Effects of PD98059 and SB202190 on Ang II–Induced DNA, Protein, and Collagen Synthesis**

Ang II dose-dependently increased synthesis of DNA with significantly greater effects in cells from SHR (Emax=309±21% of control, pD2=8.3±0.06) than from WKY (Emax=218±13% of control, pD2=8.1±0.05) (Figures 2 and 3). Collagen synthesis was also increased by Ang II in a dose-dependent fashion, with enhanced responsiveness in SHR (Emax=249±12% of control, pD2=8.32±0.07) compared with WKY (Emax=187±11% of control, pD2=8.07±0.06). To evaluate the role of p38 MAP kinase and ERK1/2 in Ang II–mediated actions, cells were exposed to SB202190 (selective p38 MAP kinase inhibitor) or PD98059 (selective MEK1/2 inhibitor). ERK1/2 inhibition decreased Ang II–induced 3H-thymidine and 3H-proline incorporation and normalized responses in SHR (Figures 2 and 3). SB202190 had no effect on DNA synthesis in either group, whereas it significantly attenuated collagen synthesis in the SHR group (Figures 2 and 3).

To determine whether Ang II–induced growth effects in SHR are generalized phenomena associated with G protein–coupled receptor activation, we also investigated effects of endothelin-1 (ET-1), a potent vasoactive agonist that has been implicated in the pathogenesis of hypertension. ET-1 induced a small increase in 3H-thymidine incorporation in WKY cells (Emax=166±26% of control), which was not significantly different in cells from SHR (Emax=171±11%). 3H-proline incorporation was only slightly increased by ET-1, with similar responsiveness in cells from WKY (Emax=126±0.5) and SHR (Emax=124±2).

**Effects of PD98059 and SB202190 on Ang II–Induced Procollagen mRNA Expression**

To evaluate in greater detail the role of MAP kinases on collagen synthesis by VSMCs, expression of procollagen I and III mRNA was determined by measuring the ratio of optical density of collagen I and III over GAPDH under control conditions and after 40 hours of Ang II stimulation, in the absence or presence of PD98059 or SB202190 (Figure 4). Ang II induced a 2-fold increase in procollagen I mRNA expression (P<0.01 versus basal and WKY counterpart) in SHR cells. Expression of procollagen III mRNA was also increased by Ang II, but responses did not reach significance (P=0.057 versus basal and WKY counterpart). However, at 44 hours, Ang II induced a significant increase in procollagen III mRNA expression (1.8-fold) in SHR cells. Procollagen mRNA expression was not significantly altered by Ang II in cells from WKY, at least for the time period studied. Neither
PD98059 nor SB202190 altered procollagen mRNA expression in WKY. In SHR, SB202190 significantly decreased Ang II–stimulated expression of procollagen I and III, whereas PD98059 had no effect. SB202190 and PD98059 alone increased expression of procollagen I mRNA.

**Discussion**

The major findings of our study demonstrate that (1) in addition to ERK1/2, Ang II stimulates the phosphorylation of p38 MAP kinase in VSMCs, (2) Ang II–induced activation of ERK1/2 and p38 MAP kinase are enhanced and sustained in cells from SHR, (3) increased Ang II–stimulated synthesis of DNA and collagen in SHR is mediated through ERK1/2-dependent, p38 MAP kinase-independent pathways, and (4) enhanced collagen synthesis and mRNA expression of procollagen I and III by Ang II involve p38 MAP kinase in SHR. Our data suggest that Ang II regulates multiple MAP kinases in VSMCs and that in SHR activation of ERK1/2 contributes mainly to hyperplasia and hypertrophy, whereas p38 MAP kinase appears to be involved in collagen production. These events may contribute to increased VSMC growth and extracellular matrix deposition, which play important pathophysiological roles in vascular remodeling in hypertension.

Increasing evidence suggests that Ang II influences vascular structural changes in small arteries in hypertension. In addition to the well-known growth-promoting actions of Ang II, this vasoactive agent induces vascular, cardiac, and renal fibrosis. In cultured cardiac fibroblasts, Ang II stimulates collagen protein synthesis, and in vivo, Ang II increases collagen I mRNA expression in rat hearts. ACE inhibition
reduced collagen deposition in large and small arteries of SHR. In the L-NAME model of hypertension, AT₁ receptor antagonism prevented activation of collagen I gene in the renal and aortic vasculature and decreased development of vascular fibrosis. Tharaux et al demonstrated that Ang II produces an AT₁-receptor–mediated increase of procollagen I gene activity and an overexpression of mRNA procollagen I in freshly isolated renal and aortic vascular tissue from transgenic mice harboring the luciferase reporter gene under the control of the collagen I promoter. In our study, Ang II induced a dose-dependent increase in ³H-proline incorporation, indicating a stimulatory effect of Ang II on collagen synthesis, particularly in cells from SHR. Evaluation of mRNA expression of procollagen by RT-PCR confirmed that Ang II–induced collagen production is increased in SHR. These Ang II–elicited responses were associated with increased synthesis of DNA and protein, indicating that in SHR, Ang II stimulates collagen production as well as hyperplasia and hypertrophy. This does not appear to be a generalized phenomena associated with ligand binding to G protein–coupled receptors because ET-1, which, like Ang II, signals through G protein–coupled receptors, induced only a modest increase in DNA and collagen synthesis, and responses were not significantly increased in cells from SHR.

Activation of collagen I gene expression by Ang II in aortic tissue from mice requires activation of the ERK1/2 and transforming growth factor-β1 signaling pathways. These processes were not dependent on p38 MAP kinase or nuclear factor-κB. In our study, specific inhibition of MEK1/2 attenuated Ang II–induced synthesis of DNA and collagen in WKY and normalized responses in SHR. SB202190, the selective inhibitor of p38 MAP kinase, did not alter DNA synthesis.
synthesis in either group, indicating that p38 MAP kinase does not play a major role in Ang II–induced cell growth. However, inhibition of the p38 MAP kinase pathway decreased Ang II–stimulated collagen synthesis in cells from SHR, with no effect in cells from WKY. In support of these findings, Ang II–induced expression of procollagen I and III was reduced by SB202190 in cells from SHR but not in cells from WKY. Although PD98059 decreased 3H-proline incorporation, it did not alter mRNA expression of procollagens, suggesting that ERK1/2 effects on collagen production are probably posttranscriptional. These data suggest that in pathological conditions associated with vascular fibrosis, such as hypertension, p38 MAP kinase activation may be an important mediator of increased collagen synthesis. Interestingly, PD98059 and SB202190 alone increased expression of procollagen I mRNA in SHR cells. Although the exact reasons for these effects are unclear, it may be possible that inhibition of one pathway, for example, ERK1/2, leads to upregulation of the other pathway, for example, p38 MAP kinase, which in turn influences procollagen mRNA expression. Further details regarding these associations await clarification.

p38 MAP kinase, of which there are at least 4 isoforms (p38α, p38β, p38γ, and p38δ), are typically activated by inflammatory stimuli and cellular stresses, which induce apoptosis. However, p38 MAP kinase has also been associated with cardiovascular hypertrophy. The divergent effects of this MAP kinase may be mediated by different isoforms. We demonstrate that Ang II activates p38 MAPK and that augmented responses in SHR are associated with processes that stimulate collagen synthesis. A possible mechanism underlying Ang II–induced p38 MAP kinase actions in SHR may be due to oxidative stress–dependent pathways. p38 MAP kinase is a redox-sensitive kinase and in hypertension, Ang II–induced generation of vascular reactive oxygen species is increased. In WKY, Ang II elicited a modest increase in p38 MAP kinase activity, which did not influence collagen synthesis or mRNA expression of procollagens. These findings are in keeping with previously reported data, which failed to demonstrate a p38 MAP kinase–dependent pathway in collagen gene activation by Ang II. Irbesartan but not PD123319 inhibited Ang II–induced phosphorylation of p38 MAP kinase, indicating that in VSMCs, Ang II activation of this MAP kinase is mediated by the AT1 receptor subtype.

Mechanisms underlying increased Ang II–induced hyperresponsiveness in SHR have not been fully elucidated but are probably due to postreceptor phenomena and not to differences at the receptor level. This is supported by our previous findings that Ang receptor density and AT1 receptor mRNA and protein expression are not different in VSMCs from age-matched adult SHR and WKY. It may be possible that dysregulation of upstream modulators, such as Src, PI3 kinase, or other kinases, could contribute to altered growth signaling by Ang II in cells from genetically hypertensive rats. In support of this are findings that Ang II–induced activation of Src is increased in SHR and that Src-dependent regulation of p38 MAP kinase and ERK1/2 are altered in VSMCs from SHR.

Conclusions
The results from our study suggest that Ang II increases the activity of multiple MAP kinases, which may play differential roles in VSMC function in hypertension. Whereas ERK1/2 influences vascular hyperplasia and hypertrophy, p38 MAP kinase is an important regulator of collagen synthesis in SHR. These findings contribute to altered VSMC growth and increased deposition of extracellular matrix proteins, important processes in vascular remodeling in genetic hypertension.

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