Vol. 188, No. 1, 1992 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 15, 1992 Pages 257-264

Angiotensin II Stimulates the pp44 and pp42 Mitogen-activated Protein Kinases in Cultured Rat Aortic Smooth Muscle Cells [1]

J.L.Duff*, B.C.Berk¶[2], and M.A.Corson¶

*Department of Biochemistry and ¶Department of Medicine, Division of Cardiology, Emory University School of Medicine, Atlanta, GA 30322

Received August 27, 1992

Vasoconstrictors such as angiotensin II (ang II) stimulate vascular smooth muscle cell growth and share many signal transduction mechanisms with growth factors. Recently, growth factors have been shown to stimulate mitogen-activated protein (MAP) kinases, a family of serine/threonine protein kinases which phosphorylate $pp90^{rsk}$, a cytosolic kinase that phosphorylates ribosomal S6 protein. We examined the effect of ang II on MAP kinase activity and phosphorylation. Ang II stimulated MAP kinase activity by 4-fold after 5 min exposure and also increased tyrosine phosphorylation of 42 kDa (74 $\pm$41%) and 44 kDa (263 $\pm$ 85%) proteins, shown to be $pp42^{mapk}$ and $pp44^{mapk}$ by Western blot analysis using a MAP kinase antibody. These results suggest that ang II-stimulated protein synthesis is mediated by a MAP kinase dependent pathway.

Vasoconstrictors such as ang II play an important role in the pathogenesis of hypertension and restenosis following angioplasty. Comparing the similarities in the signal transduction events initiated by ang II to those activated by growth factors has provided insight into the regulation of vascular growth by vasoconstrictor hormones. In cultured VSMC, ang II stimulates a hypertrophic growth response characterized by increases in cell size and protein content without an increase in cell number (1-3). Recently it has been shown that signal events activated by growth factor receptors coupled to both tyrosine kinases and G-proteins converge at the level of serine/threonine kinases (4). Among the best candidates for such serine/threonine kinase integrators in VSMC are the mitogen activated protein (MAP) kinases (5) and Raf-1 kinase (6).

MAP kinases, also referred to as extracellular signal-regulated kinases (ERKs) (7), are activated by threonine and tyrosine phosphorylation in response to a variety of extracellular stimuli, including insulin (8), epidermal growth factor (9), phorbol

[1]This work was supported by NIH grant HL-44721 (BCB).

[2] To whom correspondence should be addressed at Division of Cardiology, Emory University School of Medicine, P.O. Drawer LL, Atlanta, GA 30322.

0006-291X/92 $4.00

esters (10), and platelet derived growth factor (11). It has recently been found that activated MAP kinases phosphorylate and activate pp90^{rsk} (12), a cytosolic kinase that phosphorylates ribosomal S6 protein, which correlates with increased protein synthesis. Ang II has been shown to stimulate the phosphorylation of ribosomal S6 protein (13) and protein tyrosine phosphorylation (14) in VSMC. Based on these findings we hypothesized that ang II may activate MAP kinases in VSMC. We show here that the enzymatic activity of two putative MAP kinases is stimulated by ang II in rat aortic smooth muscle cells (RASMC). The time course for this activity correlates well with the tyrosine phosphorylation of 42 and 44 kDa proteins. These proteins appear to be two related MAP kinases, pp44^{mapk} (ERK1) and pp42^{mapk} (ERK2), based on Western blot analysis of RASMC.

METHODS

Cell culture

RASMC were isolated from 250-300 g male Sprague Dawley rats and maintained in 10% calf serum (CS)/DMEM as described (1). Passage 8 to 13 RASMC at 70-80% confluence were growth arrested by incubation in 0.4% CS/DMEM for 48 hr prior to use.

Protein kinase assay

RASMC were incubated in DMEM/10 mM HEPES (pH 7.4) containing ang II or vehicle. After washing, the cells were lysed by adding 1 ml ice-cold lysis buffer (20 mM HEPES, pH 7.4, 80 mM β-glycerophosphate, 200 μM Na_3VO_4, 2 mM dithiothreitol (DTT), 10 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), and 2 mM EDTA), followed by immediate freezing on ethanol/dry ice. The cells were then thawed on ice, scraped, and sonicated. The homogenate was centrifuged at 14,000 x g at 4°C for 30 min. Supernatants were immediately assayed for kinase activity and then stored at -80°C for protein assay. MAP kinase activity was assayed by the incorporation of [^{32}P] into myelin basic protein (MBP, Sigma) or a synthetic MBP(4-14) peptide (Upstate Biotechnology Inc.). The reaction mixture contained: 5 mM β-glycerophosphate, 20 mM HEPES pH 7.4, 10 mM $MgCl_2$, 2 mM DTT, 0.02 % Triton X-100, 100 μM Na_3VO_4, 50 μM ATP, 5 μCi [γ-^{32}P]ATP, 1 mg/ml MBP (or 0.5 mg/ml MBP(4-14) peptide), and 22 μl of the protein sample (~20 μg) in a total volume of 50 μl. Samples were incubated at 30°C for 20 min. Incorporation of [^{32}P] was measured as described by Erikson et al. (15).

[^{32}P]-Orthophosphate Labeling and Immunoprecipitation of [^{32}P]-Labeled Proteins

RASMC were radiolabeled by incubation in phosphate-free DMEM containing 0.33 mCi/ml [^{32}P] orthophosphate for 4 hrs. After stimulation the cells were washed and lysed in 10 mM HEPES, pH 7.4, 50 mM $Na_4P_2O_7$, 50 mM NaF, 50 mM NaCl, 5 mM EDTA, 5 mM EGTA, 100 μM Na_3VO_4, 0.01% Triton X-100, 0.5 mM PMSF, and 10 μg/ml leupeptin. Cytosolic extracts were prepared as described above, and immunoprecipitation performed per manufacturer's protocol with an agarose conjugated antiphosphotyrosine antibody (Oncogene Science, Inc.). Proteins were size fractionated on 10% SDS-PAGE, stained, dried, and subjected to autoradiography. Quantitative densitometry was performed using a LaCie scanner interfaced with a personal computer. Each band was scanned in two dimensions and the density corrected for background present in the lane.

Western Blot Analysis

After treatment RASMC were lysed in the immunoprecipitation buffer and cytosolic extracts prepared as before. After 10% SDS-PAGE, proteins were electrophoretically transferred overnight to nitrocellulose membranes and Western blot analysis, using MAP kinase antibody (Biodesign International) at 1:2000, was performed. Following incubation with alkaline phosphatase-conjugated goat anti-mouse IgG at 1:2000 (Fisher), the blots were developed by adding the alkaline phosphatase substrate BCIP/NBT (Kirkegaard and Perry) in the presence of 0.1 M Tris pH 9.0 for 10-30 min.

RESULTS

Time course for ang II stimulation of MAP kinase activity

To investigate the time course for activation of MAP kinase by ang II, cell lysates from RASMC were incubated with 200 nM ang II for various times and subjected to kinase assays. While optimizing the activity assay we found that the activity was stimulated by Triton X-100 and sodium orthovanadate, providing further evidence that the measured activity was due to MAP kinases. The inclusion of calcium chelators EGTA and EDTA in the assay buffer confirmed that the kinase activity detected was not regulated by calcium. Ang II stimulated [^{32}P] incorporation into MBP and MBP 4-14 peptide maximally at 5 min, with a subsequent return to control values within 20 min (Figure 1).

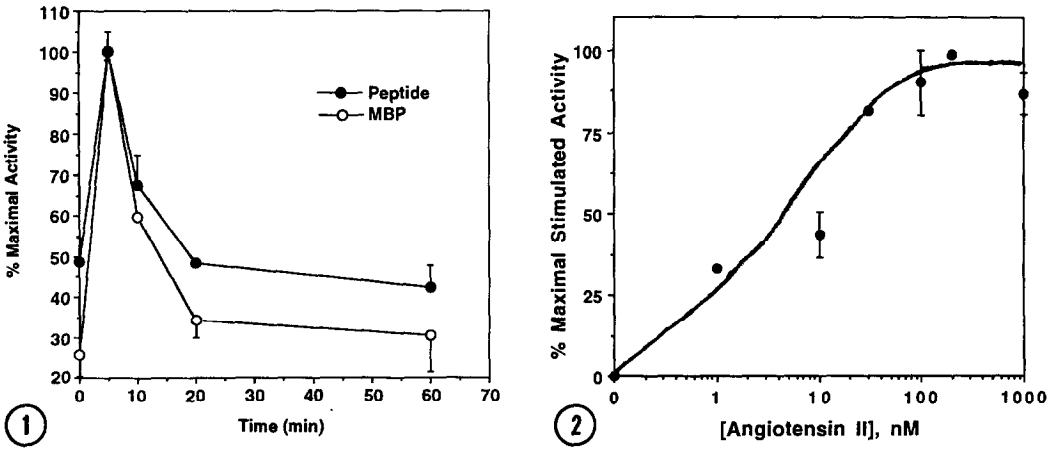

FIGURE 1. Time course for ang II stimulation of MAP kinase activity. Growth arrested RASMC were stimulated with ang II (200 nM) for the indicated time periods. MAP kinase activity toward MBP and MBP 4-14 peptide was determined as described in Materials and Methods. Results are representative of one of four separate experiments. Background phosphorylation in the absence of substrate was subtracted from total incorporated [^{32}P].

FIGURE 2. Dose response for ang II stimulation of MAP kinase activity. Growth arrested RASMC were stimulated with varying concentrations of ang II for 5 min. MAP kinase activity toward MBP was determined. Results depict the mean of three separate experiments. Background phosphorylation in the absence of substrate was subtracted from total incorporated [^{32}P].

Dose response for ang II stimulation of MAP kinase activity

To study the concentration dependence for ang II stimulation of MAP kinase activity, RASMC were treated with varying concentrations of ang II for 5 min and phosphotransferase activity was determined. MAP kinase activity was dependent on the concentration of ang II (Figure 2); stimulated activity was initially detectable at 1 nM, half-maximal at approximately 4.5 nM and maximal at 100 nM.

Ang II stimulates phosphorylation of both pp42^{mapk} and pp44^{mapk}

Because there are at least four MAP kinase gene products (7,16), we sought to identify the putative MAP kinase proteins found in RASMC. A murine anti-peptide antibody generated in response to amino acids 325-345 of ERK2 (pp42^{mapk}) was used to detect MAP kinases in cell lysates from growth arrested RASMC. This antibody identified 42 and 44 kDa bands present in approximately equal amounts (Figure 3a). When cells were stimulated with 100 nM ang II for 5 min, an apparent decrease in electrophoretic mobility of both bands was observed (Figure 3a, lane 2). Similar shifts in electrophoretic mobility in response to agonist stimulation have been observed in pp90^{rsk} (12), Raf-1 kinase (17), pp42^{mapk} (18), and pp44^{mapk} (12) in several cell types. These shifts are believed to be due to protein phosphorylation. The time course for the shift in mobility of the 42 and 44 kDa bands (Figure 3b) matched the time course for ang II stimulation of MAP kinase activity (Figure 1); both bands were maximally shifted at 5 min, ~50% shifted at 10 min, and back to control at 60 min. In addition, a 51 kDa band was detected in 2/3 experiments using the anti-ERK2 antibody. This protein showed no change in electrophoretic mobility in response to ang II.

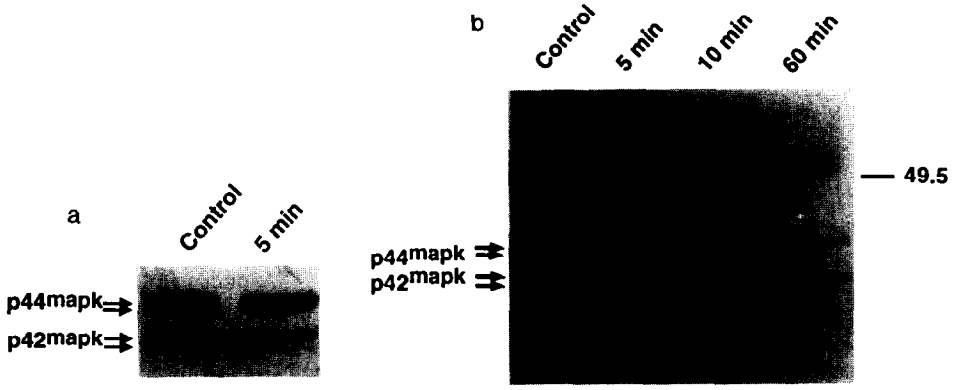

FIGURE 3. **Ang II stimulates pp42^{mapk} and pp44^{mapk} phosphorylation.** Growth arrested RASMC were stimulated with 200 nM ang II for the indicated times and Western blot analysis was performed. Membranes were probed with a murine monoclonal antibody generated in response to amino acids 325-345 of ERK2 (16). **a)** Lane 1, control; lane 2, ang II (5 min). **b)** Lane 1, control; lane 2, 5 min; lane 3, 10 min; lane 4, 60 min. Molecular weight standards are shown on the right. Arrows indicate the position of pp44^{mapk} and pp42^{mapk}. Results are representative of three experiments.

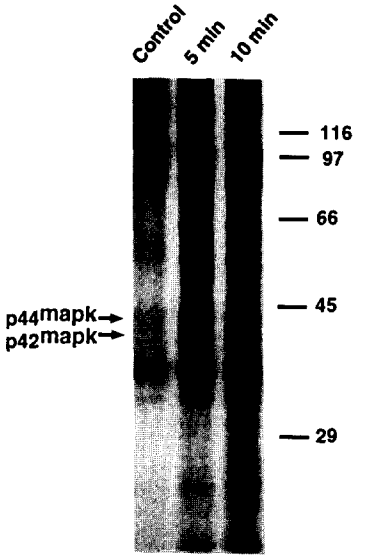

FIGURE 4. Ang II stimulates tyrosine phosphorylation of 42 and 44 kDa proteins. Growth arrested RASMC were labeled with [^{32}P] and stimulated with 200 nM ang II for 5 min. Following immunoprecipitation with an anti-phosphotyrosine antibody, proteins were resolved on 10% acrylamide gels and detected by autoradiography. Molecular weight standards are shown on the right. The arrows indicate the position of pp44^{mapk} and pp42^{mapk}. Results are representative of three experiments.

Ang II stimulates tyrosine phosphorylation of 42 and 44 kDa proteins

Growth arrested, [^{32}P]-labeled RASMC were stimulated with 200 nM ang II, and proteins immunoprecipitated with a monoclonal anti-phosphotyrosine antibody. Control immunoprecipitates contained very little phosphotyrosine (Figure 4, lane 1).Following exposure to 200 nM ang II for 5 min, tyrosine phosphorylation increased in several proteins, including 42 and 44 kDa species. These proteins were putatively identified as pp42^{mapk} and pp44^{mapk} by comigration on Western blots (not shown). Quantitative analysis revealed a 263 $\pm$ 85 % increase in the density of the 44 kDa band and a 74 $\pm$ 41 % increase in the 42 kDa band after 5 min of ang II treatment (N=3). After treatment with ang II for 10 min, the 44 kDa band density was increased 97 $\pm$ 31% over baseline, while the 42 kDa band density was increased 40 $\pm$ 29% (N=2). In contrast to these time dependent changes in tyrosine phosphorylation of the 44 and 42 kDa species, the signal density of an 88 kDa protein, apparently unaffected by ang II, varied by <8% over the time points studied within each of the 3 experiments.

DISCUSSION

The present study demonstrates that ang II, a potent vasoconstrictor, stimulates tyrosine phosphorylation and enzymatic activation of MAP kinases in cultured RASMC. When growth arrested RASMC were exposed to ang II, there was a time- and concentration-dependent increase in MAP kinase phosphotransferase activity

toward MBP. Western blot analysis showed that two MAP kinases are present in cultured RASMC, pp42^{mapk} and pp44^{mapk}, and both are phosphorylated in response to ang II. This is the first report of the stimulation of VSMC MAP kinase tyrosine phosphorylation and activation by a vasoconstrictor. Ang II stimulation of MAP kinases represents yet another signal transduction mechanism shared by vasoconstrictors and growth factors in these cells (19).

It has previously been shown that simultaneous tyrosine and threonine phosphorylation are required for MAP kinase phosphotransferase activity in fibroblasts (20). Ang II treatment caused shifts in the electrophoretic mobility of pp42^{mapk} and pp44^{mapk} with the same time dependence as stimulation of MAP kinase activity. Such shifts in mobility are thought to be caused by increased MAP kinase phosphorylation (7,12,18). Nearly 100% of both pp42^{mapk} and pp44^{mapk} shifted in mobility in response to ang II treatment for 5 min (Figure 3a), suggesting that stoichiometric phosphorylation occurs in response to ang II. Western blot analysis of RASMC lysates also revealed a 51 kDa protein which became dephosphorylated in response to ang II (Figure 3b). This protein may be an ERK-related protein first described by Kyriakis et al. (21), pp54 MAP-2 kinase, which does not phosphorylate MBP or the S6 protein kinase II.

Recent studies have found that both pp42^{mapk} and pp44^{mapk} are similarly activated in response to a variety of extracellular signals, and this activation correlates with increased tyrosine phosphorylation (7,22). Using an anti-phosphotyrosine antibody, we observed increases in phosphotyrosine content of the 42 and 44 kDa proteins in response to ang II treatment. These proteins were identified as pp42^{mapk} and pp44^{mapk} by Western blot analysis. The pp44^{mapk} had more phosphotyrosine as compared to pp42^{mapk}. This difference may be due to differential phosphorylation of MAP kinases in VSMC in response to ang II. However, based on the Western blot analysis (Figures 3), this difference is most likely an artifact due to low affinity of the anti-phosphotyrosine antibody for pp42^{mapk}. Kozma et al. (23) found that tyrosine phosphorylation of pp42^{mapk} could only be detected with certain antisera. They proposed that some antibodies have a low affinity for pp42^{mapk} because the site of tyrosine phosphorylation in pp42^{mapk} is in a highly acidic environment. Of interest, a previous Western blot analysis of ang II-stimulated phosphotyrosine proteins in VSMC revealed a greater increase in the phosphotyrosine content of a 40 kDa protein as compared to a 45 kDa protein (14). Additional studies utilizing isoform-specific anti-MAP kinase antibodies will be needed to determine whether the VSMC MAP kinase isoforms differ quantitatively in their phosphorylation state, and whether this difference correlates with functional activity.

The concentration-dependence for ang II-induced activation of MAP kinases in RASMC is similar to that observed for other early signal transduction events (24,25). The EC_{50} for MAP kinase activity was approximately 4.5 nM which corresponds precisely to the EC_{50} for protein synthesis we have previously observed in these cells, as indicated by incorporation of [^{3}H]leucine (1). Maximal stimulation of MAP kinase

activity, however, required a 10-fold higher concentration of ang II (~100 nM) than that needed to cause maximal Ca^{2+} mobilization (25). This suggests that negative regulatory components, such as phosphatases, are simultaneously activated with MAP kinase in the signal transduction pathway.

The present study provides insight into the mechanisms by which ang II stimulates protein synthesis in VSMC. Ang II has been shown to stimulate phosphorylation of the 40S ribosomal protein S6 (13), which is known to be phosphorylated by $pp90^{rsk}$ and $pp70^{S6K}$ kinases (26). Recently, Chen and Blenis (12) found that activated MAP kinases phosphorylate and activate $pp90^{rsk}$. In contrast, $pp70^{S6K}$ does not appear to be a MAP kinase substrate (27), and $pp90^{rsk}$ and $pp70^{S6K}$ appear to be differentially regulated (27,28). In light of these findings, our data indicate that the $pp90^{rsk}$/MAP kinase pathway plays an important role in ang II-stimulated protein synthesis and hypertrophy.

REFERENCES

1. Berk, B.C., Vekshtein, V., Gordon, H.M., and Tsuda, T. (1989) Hypertension **13**: 305-314.
2. Geisterfer, A.A.T., Peach, M.J., and Owens, G.K. (1988) Circ. Res. **62**: 749-756.
3. Krug, L.M., and Berk, B.C. (1992) Hypertension **20**: 144-150.
4. Sturgill, T.W., and Wu, J. (1991) Biochim. Biophys. Acta. **1092**: 350-357.
5. Cobb, M.H., Boulton, T.G., and Robbins, D.J. (1991) Cell Reg. **2**: 965-978.
6. Morrison, D.K. (1990) Cancer Cells **2**: 377-382.
7. Boulton, T.G., Nye, S.H., Robbins, D.J., Ip, N.Y., Radziejewska, E., Morgenbesser, S.D., DePinho, R.A., Panayotatos, N., Cobb, M.H., and Yancopoulos., G.D. (1991b) Cell **65**: 663-675.
8. Sturgill, T.W., Ray, B.L., Erikson, E., and Maller, J.L. (1988) Nature **334**: 715-718.
9. Ahn, N.G., Seger, R., Bratlien, R.L., Diltz, C.D., Tonks, N.K., and Krebs., E.G. (1991) J. Biol. Chem. **266**: 4220-4227.
10. Tobe, K., Kadowaki, T., Tamemoto, H., Ueki, K., Hara, K., Koshio, O., Momomura, K., Gotoh, Y., Nishida, E., Akanuma, Y., Yazaki, Y., and Kasuga., M. (1991) J. Biol. Chem. **266**: 24793-24803.
11. L'Allemain, G., Sturgill, T.W., and Weber, M.J. (1991b) Mol. Cell Biol. **11**: 1002-1008.
12. Chen, R.-H., Sarnecki, C., and Blenis, J. (1992) Mol. Cell Biol. **12**: 915-927.
13. Scott-Burden, T., Resink, T.J., Baur, U., Burgin, M., and Buhler, F.R. (1988) Biochem. Biophys. Res. Commun. **151**: 583-589.
14. Tsuda, T., Kawahara, Y., Shii, K., Koide, M., Ishida, Y., and Yokoyama, M. (1992) FEBS lett. **285**: 44-48.
15. Erikson, A.K., Payne, D.M., Martino, P.A., Rossomando, A.J., Shabanowitz, J., Weber, M.J., Hunt, D.F., and Sturgill, T.W. (1990) J. Biol. Chem. **265**: 19728-19735.
16. Boulton, T.G., Yancopoulos, G.D., Gregory, J.S., Slaughter, C., Moomaw, C., Hsu, J., and Cobb, M.H. (1990) Science **244**: 64-67.
17. Kovacina, K.S., Yonezawa, K., Brautigan, D.L., Tonks, N.K., Rapp, U.R., and Roth, R.A. (1990) J. Biol. Chem. **265**: 12115-12118.
18. Winston, L.A., and Bertics, P.J. (1992) J. Biol. Chem. **267**: 4747-4751.
19. Berk, B.C., Alexander, R.W., Brock, T.A., Gimbrone, M.A., Jr., and Webb, R.C. (1986) Science **232**: 87-90.

20. Anderson, N.G., Maller, J.L., Tonks, N.K., and Sturgill., T.W. (1990) Nature **343**: 651-653.
21. Kyriakis, J.M., and Avruch, J. (1990) J. Biol. Chem. **265**: 17355-17363.
22. L'Allemain, G., Pouyssegur, J., and Weber, M.J. (1991a) Cell Reg. **2**: 675-684.
23. Kozma, L.M., Rossomando, A.J., and Weber, M.J. (1992) In Methods Enzymology (T. Hunter and B. M. Sefton, Eds.), 201, pp. 28-43. Academic Press, Inc., San Diego.
24. Griendling, K.K., Rittenhouse, S.E., Brock, T.A., Ekstein, L.S., M. A. Gimbrone, J., and Alexander, R.W. (1986) J. Biol. Chem. **261**: 5901-5906.
25. Brock, T.A., Alexander, R.W., Ekstein, L.S., Atkinson, W.J., and M. A. Gimbrone, J. (1985) Hypertension **7**: I-105-I-109.
26. Erikson, R.L. (1991) J. Biol. Chem. **266**: 6007-6010.
27. Ballou, L.M., Luther, H., and Thomas, G. (1991) Nature **349**: 348-350.
28. Chung, J., Kuo, C.J., Crabtree, G.R., and Blenis, J. (1992) Cell **69**: 1227-1236.