Angiotensin II Stimulates Two Myelin Basic Protein/Microtubule–Associated Protein 2 Kinases in Cultured Vascular Smooth Muscle Cells

Terutaka Tsuda, Yasuhiro Kawahara, Yoshihiro Ishida, Masanobu Koide, Kozui Shii, and Mitsuhito Yokoyama

In cultured vascular smooth muscle cells, angiotensin II (Ang II) stimulated a cytosolic protein kinase activity toward myelin basic protein (MBP) in a time- and dose-dependent manner. Phorbol 12-myristate 13-acetate (PMA) and phorbol 12,13-dibutyrate also increased the MBP kinase activity. Downregulation of protein kinase C by prolonged treatment of the cells with phorbol 12,13-dibutyrate markedly attenuated the Ang II– and PMA-induced MBP kinase activation. The Ang II– and PMA-stimulated MBP kinase activities were resolved almost equally into two distinct fractions on Mono-Q HR5/5 column chromatography (kinase 1 and kinase 2). The kinase assay in polyacrylamide gel revealed that apparent molecular masses of kinase 1 and kinase 2 were 40 and 45 kD, respectively. Microtubule-associated protein 2 also served as a substrate for both the kinases. Immunoblot analysis with an antiphosphotyrosine antibody suggested that both the kinases were tyrosine-phosphorylated during the action of Ang II. Phosphoamino acid analysis revealed that Ang II and PMA induced phosphorylation of both the kinases on serine/threonine as well as tyrosine residues. Phosphopeptide mapping patterns of kinase 1 and kinase 2 isolated from Ang II-stimulated cells were almost identical with those from PMA-stimulated cells. These results indicate that in vascular smooth muscle cells Ang II activates two species of MBP/microtubule-associated protein 2 kinases mainly through the protein kinase C signaling pathway and suggest that tyrosine and serine/threonine phosphorylation may be involved in this process. (Circulation Research 1992;71:620–630)

Key Words • angiotensin II • myelin basic protein/microtubule–associated protein 2 kinase • vascular smooth muscle cells • protein kinase C • tyrosine phosphorylation

Hypertrophy of vascular smooth muscle cells (VSMCs) is one of the fundamental features of hypertension.1–3 This structural change plays an important role in the increase in vascular resistance that causes chronic hypertension.4 Angiotensin II (Ang II), a potent vasoconstrictor peptide, plays crucial roles in the pathogenesis of some forms of hypertension. Since several lines of evidence suggest that Ang II acts as a growth promoter for VSMCs, this vasoconstrictor peptide is considered to be implicated directly in VSMC hypertrophy of hypertension.5,6 In cultured VSMCs, Ang II causes a rapid phospholipase C–mediated hydrolysis of inositol phospholipids and the subsequent generation of diacylglycerol and inositol triphosphate,7–10 which serve as second messengers for protein kinase C activation and intracellular Ca2+ mobilization, respectively.11,12 Ang II has been shown to stimulate ribosomal protein S6 kinase activity13 and protein synthesis14 in VSMCs. It has also been demonstrated that Ang II stimulates the phosphorylation of nuclear membrane proteins, lamins,15 and the expression of the proto-oncogenes c-fos and c-myc,16–18 one of the earliest genetic programs associated with cell growth. These Ang II–induced events appear to be regulated through the protein kinase C and/or Ca2+ signaling pathways and may be involved in the induction of hypertrophy of VSMCs. However, the molecular mechanisms by which the protein kinase C and Ca2+ signaling pathways regulate these distal events have not been elucidated.

Mitogen-activated myelin basic protein (MBP)/microtubule-associated protein 2 (MAP2) kinase is a serine/threonine–specific protein kinase whose activation is commonly induced by a variety of growth factors including epidermal growth factor (EGF), platelet-derived growth factor (PDGF), insulin, insulin-like growth factor I, fibroblast growth factor, and nerve growth factor.19–25 It has been demonstrated that MBP/MAP2 kinase is phosphorylated on both tyrosine and serine/
threonine residues in response to these mitogens and that both types of phosphorylation are required for the activation of this kinase. MBP/MAP2 kinase that is thus activated phosphorylates and reactivates the phos- phatase-inactivated S6 kinase II purified from Xenopus eggs. S6 kinase II has been shown to phosphorylate nuclear lamin C in addition to ribosomal protein S6. It has recently been demonstrated that MBP/ MAP2 kinase also phosphorylates the products of the c-jun, c-fos, and c-myc proto-oncogenes. Therefore, MBP/ MAP2 kinase is considered to play an important role in mediating signals from the growth factor receptors to the ribosomes and nucleus. It has also been demon- strated that protein kinase C-activating phorbol esters and synthetic diacylglycerol stimulate tyrosine phosphorylation and activity of MBP/ MAP2 kinase in various cell types. However, it has not yet been clarified as to whether MBP/ MAP2 kinase is involved in the signaling pathways of phospholipase C-linked hor- mones including Ang II.

In a preceding report, we have shown that Ang II induces tyrosine phosphorylation of several proteins in VSMCs. Among them, two proteins have apparent molecular masses of 40 and 45 kD, which are similar to the molecular mass of MBP/ MAP2 kinase. In the present study, we examined the ability of Ang II to activate MBP/ MAP2 kinase in VSMCs and found that Ang II activates two species of MBP/ MAP2 kinases mainly through the protein kinase C signaling pathway. We also provide evidence indicating that these two MBP/ MAP2 kinases are identical with the 40- and 45-kD proteins that are tyrosine-phosphorylated during the action of Ang II in VSMCs.

Materials and Methods

Materials

Ang II, phorbol 12-myristate 13-acetate (PMA), phorbol 12,13-dibutyrate (PDBu), 4α-phorbol 12,13- didecanoate (4α-PDD), MBP, histone H1, histone H2B, casein, protamine, Walsh peptide, staurosporine, and 1-tosylamide 2-phenylethyl chloromethyl ketone (TPCK)—treated trypsin were purchased from Sigma Chemical Co., St. Louis, Mo. Ionomycin was from Calbiochem Corp., La Jolla, Calif. Recombinant human PDGF-BB and mouse submaxillary gland EGF were from Collaborative Research, Inc., Bedford, Mass. [γ-32P]ATP (3,000 Ci/mm mol) and carrier-free [32P]orthophosphate were from Amersham Japan, Tokyo. MAP2 was purified from bovine brain as described. Rat liver 40S ribosomal subunits were prepared as described. The purities of MBP and MAP2 preparations were assessed by sodium dodecyl sulfate (SDS)—poly- acrylamide gel electrophoresis (PAGE) and were approximately 50% and 90%, respectively. Mouse monoclonal anti-phosphotyrosine antibody (immunoglobulin [Ig] M) was developed by immunizing with v-abl—expressing bacteria as described. Growth media of hybridoma cells were purified by phosphotyrosine affinity column chromatography. This antibody specifically rec- ognizes phosphotyrosine-containing proteins. Mouse monoclonal anti-MAP2 kinase antibody (IgG) was pur- chased from Zymed Laboratories Inc., San Francisco, Calif. Goat anti-mouse IgM and IgG conjugated with peroxidase were from Cappel, West Chester, Pa. Nitro- cellulose membrane filters (0.45 μm) were from Schlei- cher & Schuell, Dassel, FRG. Millex-GV filters and polyvinylidene difluoride membranes (Immobilon-P, 0.45 μm) were from Millipore Corp., Bedford, Mass. The Centricron 10 microconcentrator was from W.R. Grace & Co., Baltimore, Md. DEAE-cellulose was from Whatman, Maidstone, England. Mono Q HES/5 and Superose 12 HR10/30 columns, phenyl-Sepharose, and amypholytes were from Pharmacia, Uppsala, Sweden. Other materials and chemicals were obtained from commercial sources.

Cell Cultures

VSMCs were isolated from rat thoracic aorta by enzymatic dissociation as described previously. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. They were passaged twice a week with harvesting by trypsin/EDTA and seeding at a 1:4 ratio in 75-cm² flasks. For experiments, cells between passage levels 9 and 18 were seeded into 100-mm dishes (2×10⁵ cells/cm²), fed every other day, and used at confluence (4–6 days).

Preparation of Cell Extracts

VSMCs were cultured in serum-free DMEM for 48 hours and washed twice with serum-free DMEM containing 10 mM HEPES, pH 7.4. The cells were then stimulated with various stimuli in the same medium for the indicated periods of time. The reactions were termi- nated by chilling the dishes on ice and washing twice with ice-cold phosphate-buffered saline. All further steps were performed at 4°C. The cells were scraped from the dishes into a total volume of 0.5 ml per dish of ice-cold buffer A containing 20 mM Tris-Cl, pH 7.5, 2 mM EGTA, 1 mM sodium orthovanadate, 10 mM β-glycerophosphate, 1 mM dithiothreitol (DTT), 1 μM (p-amidinophenyl)methanesulfonyl fluoride (APMSF), and 100 kallikrein-inactivating units/ml aprotinin, soni- cated twice for 10 seconds (Micro Ultrasonic Cell Disrupter [set at half maximal intensity], Kontes, Vine- land, N.J.), and then centrifuged at 100,000g for 20 minutes (model 50 Ti rotor, Beckman Instruments, Inc., Fullerton, Calif.). Supernatants were assayed for MBP kinase activity or used as starting materials for MBP kinase purification.

Assay for MBP Kinase Activity

Unless specifically indicated, assays were performed at 30°C for 5 minutes in a final volume of 50 μl containing 50 mM β-glycerophosphate, pH 7.5, 1 mM DTT, 1.5 mM EGTA, 10 mM magnesium acetate, 40 μM [γ-32P]ATP (2.5×10⁵ cpm/nmol), 0.25 mg/ml MBP, and 10 μl of the cytosolic extracts or enzyme fractions. The reaction was terminated by the addition of Laemmli's sample buffer. The samples were boiled for 5 minutes and then electrophoresed in 12% SDS-poly- acrylamide gel; this procedure was followed by autoradiography. For quantification, the bands corresponding to MBP were excised from the gels, and the radioactivity was counted using a liquid scintillation counter. During the purification procedures, aliquots of each column fraction (10 μl) were assayed in the same reaction mixture at 30°C for 5 minutes. The reaction
was terminated by the addition of 1 ml ice-cold 20% trichloroacetic acid and 60 mM sodium pyrophosphate, and precipitates were collected on a nitrocellulose membrane filter. The filter was washed four times with the same solution, and the radioactivity was counted using a liquid scintillation counter. Measurements of MBP phosphorylation assayed in this manner were identical to values obtained after separation of MBP by SDS-PAGE.

**Purification of MBP Kinase**

All chromatographic separations were carried out at 4°C. Cell extracts prepared from twenty 100-mm dishes of either vehicle- or Ang II- or PMA-treated cells were applied to a DEAE-cellulose column (0.9×1.6 cm) pre-equilibrated with buffer A. The column was washed with 5 ml buffer A and eluted with a 30-ml linear gradient of 0–500 mM NaCl in the same buffer at a flow rate of 0.5 ml/min. Fractions of 1.2 ml each were collected. Fractions containing MBP kinase activity were collected, diluted with 3 vol buffer A, passed through a 0.22-μm Millex-GV filter, and applied to a Mono Q HR5/5 column pre-equilibrated with buffer A. After the column was washed with 10 ml buffer A, the elution was performed with a 60-ml linear gradient of 0–400 mM NaCl in the same buffer at a flow rate of 0.5 ml/min. Fractions of 0.625 ml each were collected into tubes containing 62.5 μl of 10 mg/ml bovine serum albumin (BSA) to stabilize MBP kinase activity. Fractions containing MBP kinase activity were collected, concentrated with the Centricon 10 microconcentrator, and applied to a Superose 12 HR10/30 column pre-equilibrated with buffer B containing 20 mM Tris/Cl, pH 7.5, 2 mM EGTA, 1 mM sodium orthovanadate, 50 mM β-glycerophosphate, 1 mM DTT, 100 mM NaCl, 1 μM APMSF, and 100 kallikrein-inactivating units/ml apro- tinin. The elution was performed with buffer B at a flow rate of 0.5 ml/min, and fractions of 0.5 ml each were collected into tubes containing 50 μl of 10 mg/ml BSA.

**Kinase Assay in Polyacrylamide Gel Containing MBP**

Detection of MBP kinase activity in polyacrylamide gel was performed according to the method of Kameshita and Fujisawa with slight modifications. Briefly, the kinase fractions were subjected to SDS-PAGE using a separation gel (11% polyacrylamide gel) containing 1 mg/ml MBP. After washing the gel with 50 mM Tris/Cl, pH 8.0, and 20% 2-propanol, and then with 50 mM Tris/Cl, pH 8.0, and 5 mM 2-mercaptoethanol (buffer C) to remove SDS, the enzymes were denatured by treating the gel with buffer C containing 6 M guanidine HCl and then renatured with buffer C containing 0.04% Tween 20. The gel was incubated at 22°C for 1 hour with 40 mM HEPES, pH 8.0, 2 mM DTT, 0.1 mM EGTA, 5 mM MgCl₂, and 25 μM [γ-32P]ATP (1×10⁵ cpm/nmol) for kinase reactions. The gel was extensively washed with 5% trichloroacetic acid containing 1% sodium pyrophosphate, stained with Coommassie brilliant blue R-250, dried, and subjected to autoradiography on Kodak X-Omat AR film.

**Immunoblot Analysis With Anti-Phosphotyrosine Antibody**

Immunoblot analysis with anti-phosphotyrosine antibody was performed as described previously. The cytosolic extracts or enzyme fractions were subjected to SDS-PAGE (8–16% gradient gel) using the buffer system of Laemmli. The separated proteins were electrophoretically transferred to nitrocellulose membranes as described by Towbin et al. Nitrocellulose blots were soaked for 1 hour in 50 mM Tris/Cl, pH 7.5, 200 mM NaCl, and 5% BSA and then incubated with the mouse monoclonal anti-phosphotyrosine antibody in 50 mM Tris/Cl, pH 7.5, 150 mM NaCl, 0.05% Tween 20, 1% BSA, and 20% calf serum for 2 hours, followed by peroxidase-labeled goat anti-mouse IgG in the same buffer for 1 hour. Blots were extensively washed with 50 mM Tris/Cl, pH 7.5, 200 mM NaCl, and 0.05% Tween 20 after each incubation. Peroxidase-labeled proteins were visualized by incubation with peroxidase color development reagents containing the enzyme substrate 3,3′-diaminobenzidine.

**Phosphoamino Acid Analysis and Tryptic Phosphopeptide Mapping**

VSMCs were labeled with 2 mCi carrier-free [32P]orthophosphate in 4 ml of 20 mM HEPES, pH 7.4, 130 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1.5 mM CaCl₂, and 10 mM glucose for 4 hours. Agonists were added to the buffer for the final labeling periods. Cell extracts were prepared as described above except that the cells were homogenized by several passages through a 1-ml syringe with a 26-gauge needle. 32P-labeled MBP kinases were isolated by column chromatographies based on the method of Hoshi et al; this procedure was followed by two-dimensional gel electrophoresis. Briefly, the extracts were applied to a DEAE-cellulose column (0.48×1.1 cm) pre-equilibrated with buffer A. The column was washed with 0.8 ml buffer A containing 20 mM NaCl and eluted with 0.8 ml buffer A containing 250 mM NaCl. The eluates were then applied to a phenyl-Sepharose column (0.48×1.1 cm) pre-equilibrated with buffer A containing 2 M NaCl. After the column was washed with 0.8 ml buffer A containing 20% ethylene glycol, the elution was performed with 0.8 ml buffer A containing 60% ethylene glycol. The eluates were then dialyzed against buffer A for 5 hours, lyophilized, and dissolved in 50 μl of 8 M urea, 2% Nonidet P-40, 2% amphotolys (one part at pH 3.5–10 and four parts at pH 6–8), and 5% 2-mercaptoethanol. The samples were subjected to two-dimensional gel electrophoresis by the method of O’Farrell with slight modifications using 2% amphotolys (one part at pH 3.5–10 and four parts at pH 6–8) in the first dimension and 12% polyacrylamide gel in the second dimension. For phosphoamino acid analysis, separated proteins were electrophoretically transferred to polyvinylidene difluoride membranes. 32P-labeled MBP kinases were visualized by immunoblotting with the mouse monoclonal anti-MAP2 kinase antibody by the same method as described for anti-phosphotyrosine immunoblot except that peroxidase-labeled goat anti-mouse IgG was used as a second antibody. The immunoreactive spots were excised and subjected to hydrolysis with 6N HCl for 3 hours at 110°C by the method of Hildebrandt and Fried followed by one-dimensional high voltage electrophoresis at pH 3.5. For tryptic phosphopeptide mapping, two-dimensional gel was dried and subjected to autoradiography. The radioactive spots corresponding to MBP kinases were excised from the gel.
labeled MBP kinases were extracted and subjected to two-dimensional tryptic phosphopeptide mapping as described except that the proteins were digested with 100 μg TPCK-treated trypsin.

**Protein Determination**

Cell protein was determined by the method of Bradford with BSA as a standard.

**Statistical Analysis**

Where applicable, results are expressed as mean±SEM. Differences between means were evaluated by t test where appropriate. A value of p<0.05 was taken to be significant.

**Results**

**Stimulation of MBP Kinase Activity by Ang II**

Consistent with the previous observations described in other cell types, treatment of VSMCs with EGF or PDGF markedly increased cytosolic MBP kinase activity (Figure 1). Under the identical conditions, Ang II also increased MBP kinase activity to the same extent as that increased by these growth factors. Figure 2 shows the time course of Ang II–induced MBP kinase activation. The Ang II–stimulated MBP kinase activity was detectable at as early as 30 seconds, peaked at 2 minutes, and was sustained above the basal level for at least 30 minutes after stimulation. The concentrations of Ang II required for half maximal and maximal stimulation of MBP kinase activity were approximately 0.1 and 100 nM, respectively (Figure 3).

**Effects of Protein Kinase C–Activating Phorbol Esters and Downregulation of Protein Kinase C on MBP Kinase Activity**

As shown in Figure 4, the protein kinase C–activating phorbol ester, PMA, increased MBP kinase activity in the cytosolic fraction of VSMCs. The maximal level of PMA-stimulated MBP kinase activity was the same as that stimulated by Ang II. Another active phorbol ester, PDBu, also induced this reaction, whereas 4α-PDD, which does not activate protein kinase C, failed to induce the reaction (data not shown). We have previously shown that treatment of VSMCs with PDBu for 24 hours markedly downregulates protein kinase C and inhibits Ang II–as well as PMA-induced phosphorylation of the 76-kd protein kinase C substrate. As shown in Figure 4, downregulation of protein kinase C attenuated the Ang II– and PMA-induced MBP kinase activation by 61% and 79%, respectively.

**Partial Purification of Ang II–Stimulated MBP Kinase**

When extract supernatants of VSMCs that had been treated with Ang II were subjected to DEAE-cellulose
Characterization of Ang II–Stimulated Kinase 1 and Kinase 2

Kinase 1 and kinase 2 eluted from the gel filtration column were characterized. As shown in Table 1, MAP2 also served as a substrate for both the kinases, but histone H1, histone H2B, casein, protamine, or 40S ribosomal subunits did not. Kinase 1 and kinase 2 maximally incorporated 0.95±0.10 and 0.89±0.09 mol phosphate, respectively, into 1 mol MBP and 5.8±0.5 and 5.9±0.5 mol phosphate, respectively, into 1 mol MAP2. After either kinase 1 or kinase 2 maximally phosphorylated MBP, the other kinase did not phosphorylate this substrate further (data not shown), suggesting that the two kinases phosphorylate MBP at the same site. The $K_m$ values for MBP and MAP2 were determined by double-reciprocal plots (data not shown). The $K_m$ values of kinase 1 and kinase 2 for MBP were 32±5 and 30±5 $\mu$M, respectively, and those for MAP2 were 4.7±0.6 and 2.4±0.2 $\mu$M, respectively. The $K_m$ values of kinase 1 and kinase 2 for ATP were 97±12 and 85±10 $\mu$M, respectively. GTP (up to 1 mM) did not reduce $\gamma$-32P labeling of MBP when added to the standard assay containing 40 $\mu$M [\gamma-$\gamma$-32P]ATP, suggesting that it was a poor phosphoryl donor for these enzymes (data not shown). Both kinase 1 and kinase 2 were not inhibited by the heat-stable inhibitor of cAMP-dependent protein kinase (Walsh peptide) or staurosporine (data not shown). As shown in Table 2, either Mg$^{2+}$ or Mn$^{2+}$ is required for the activities of both kinase 1 and kinase 2, whereas Ca$^{2+}$ and Zn$^{2+}$ did not support the activities of both the kinases. In addition, Ca$^{2+}$ and Zn$^{2+}$ inhibited both kinase activities supported by Mg$^{2+}$.

Immunoblot Analysis of Ang II–Stimulated Kinase 1 and Kinase 2 With Anti-Phosphotyrosine Antibody

When the partially purified preparations of kinase 1 and kinase 2 were analyzed by immunoblotting with the anti-phosphotyrosine antibody, proteins with apparent molecular masses of 40 and 45 kD were recognized by this antibody in the kinase 1 and kinase 2 preparations, respectively (Figure 8). The electrophoretic mobilities of the immunoreactive 40- and 45-kD proteins were identical with those of kinase 1 and kinase 2, respectively, detected by the kinase assay in polyacrylamide gel (data not shown). In a preceding report, we have shown that Ang II stimulates tyrosine phosphorylation of several proteins, including two proteins with apparent molecular masses of 40 and 45 kD in VSMCs.36 Figure 8 also shows that the mobilities on SDS-PAGE of the tyrosine-phosphorylated 40- and 45-kD proteins in the partially purified kinase preparations were identical with those of the 40- and 45-kD proteins, respectively, which were tyrosine-phosphorylated during the action of Ang II in VSMCs. Furthermore, the elution profiles of kinase 1 and kinase 2 were identical with those of the tyrosine-phosphorylated 40- and 45-kD proteins, respectively, on Mono Q and Superose 12 column chromatographies (Figures 5B, 6B, and 6D). These observations strongly suggest that kinase 1 and kinase 2 are identical with the 40- and 45-kD proteins, respectively, which are tyrosine-phosphorylated during the action of Ang II in VSMCs.

Phosphoamino Acid Analysis of Ang II– and PMA-Stimulated Kinase 1 and Kinase 2

To gain further evidence that kinase 1 and kinase 2 are tyrosine-phosphorylated and to examine whether serine/threonine residues are also phosphorylated during the action of Ang II, kinase 1 and kinase 2 were
isolated from $^{32}$P-labeled VSMCs and subjected to phosphoamino acid analysis. Ang II and PMA increased phosphorylation levels of each kinase to a similar extent, as estimated by measuring the radioactivities of isolated kinase 1 and kinase 2 (kinase 1, control, 67±19 cpm; Ang II, 490±51 cpm; PMA, 474±64 cpm; and kinase 2, control, 76±21 cpm; Ang II, 370±50 cpm; PMA, 387±46 cpm). Consistent with the results obtained by anti-phosphotyrosine immunoblotting, Ang II treatment of VSMCs increased tyrosine phosphorylation of kinase 1 and kinase 2 as detected by phosphoamino acid analysis (Figure 9). In addition, Ang II also increased serine and threonine phosphorylation of kinase 1 and kinase 2. Figure 9 also shows that PMA increased phosphorylation levels of kinase 1 and kinase 2 on serine, threonine, and tyrosine residues.

**Tryptic Phosphopeptide Mapping of Ang II– and PMA-Stimulated Kinase 1 and Kinase 2**

Two-dimensional mappings of the tryptic digests of kinase 1 and kinase 2 isolated from $^{32}$P-labeled VSMCs are shown in Figure 10. Kinase 1 from Ang II–stimulated cells generated a phosphopeptide mapping almost identical with that generated by kinase 1 from PMA-stimulated cells. The phosphopeptide mapping pattern of kinase 2 isolated from Ang II–stimulated cells was almost identical with that of kinase 2 isolated from PMA-stimulated cells.

**Discussion**

In the present study, we demonstrated that Ang II stimulated MBP kinase activity in VSMCs and that this kinase activity was resolved into two distinct peaks on Mono Q column chromatography. We further purified these two peaks separately by gel filtration chromatography and characterized them. According to chromatographic behavior, substrate specificity, effects of protein kinase modulators, cation dependence, and apparent molecular masses, both of these two kinases are identical with or closely related to the enzymes identified as MAP or MAP2 kinase, mitogen-activated protein kinase, MBP kinases, or extracellular signal-regulated kinases (ERKs) in various cell types stimulated by a variety of growth factors and other extracellular stimuli. Initial reports from Sturgill's laboratory have suggested that MAP2 kinase activity in insulin-stimulated Swiss 3T3 cells is derived from a single polypeptide with an apparent molecular mass of approximately 40 kDa. However, evidence is now accumulating that there are multiple members of the MBP/MAP2 kinase family in mammalian cells. Krebs and colleagues have described that MBP kinase activity in EGF-stimulated Swiss 3T3 cells is separated into two distinct peaks with apparent molecular masses of 41 kDa (MBP kinase 1) and 44 kDa (MBP kinase 2) on Mono Q column chromatography. Sakai and colleagues have shown that MAP2 kinase activity par-
tially purified from NGF- or EGF-stimulated PC12 cells or EGF-stimulated fibroblastic 3Y1 cells resides in the two closely related polypeptides with apparent molecular masses of approximately 40 kD. Finally, recent cloning studies by Boulton and colleagues have revealed that there are at least three members (ERK1, ERK2, and ERK3) of the MBP/MAP2 kinase family. The molecular masses of ERK1, ERK2, and ERK3 predicted from their cDNAs are 43, 41.2, and 62.6 kD, respectively. They have also elucidated the enzyme profiles of ERK1 and ERK2 on Mono Q column chromatography. Based on chromatographic behavior on Mono Q column chromatography and apparent molecular masses, it is likely that kinase 1 and kinase 2 described in this article correspond to ERK2 and ERK1, respectively. During the purification procedures, MBP kinase activity was recovered almost totally into kinase 1 and kinase 2 fractions, and these fractions did not contain 62.6-kD MBP kinase activity, as demonstrated by the kinase assay in polyacrylamide gel, suggesting that MBP/MAP2 kinase corresponding to ERK3 may not be expressed abundantly in VSMCs. We also compared biochemical properties of kinase 1 and kinase 2 to test the possibility that these two species of MBP/MAP2 kinases may play different roles in VSMC functions. However, we could not detect any differences in biochemical properties between these two enzymes in vitro. Moreover, EGF and PDGF also induced the activation of both the kinases (data not shown). Thus, it is not clear at present whether these two kinases play different roles in VSMCs.

It has been demonstrated that protein kinase C-activating phorbol esters and synthetic diacylglycerol stimulate MBP/MAP2 kinase activity in various cell types. NGF has been shown to induce the activation of MBP/MAP2 kinase in a manner partly dependent on, but largely independent of, protein kinase C in PC12 cells. Activation of T-cell antigen receptors by anti-CD3 and anti-Ti antibodies has been shown to stimulate MBP/MAP2 kinase activity in part via a protein kinase C-dependent mechanism. In the present study, we demonstrated in VSMCs that PMA and PDBu stimulated the MBP kinase activity and that Ang II-induced MBP kinase activation was markedly inhibited by downregulation of protein kinase C. Furthermore, Ang II and PMA generated almost identical phosphoamino acid analysis and tryptic phosphopeptide mapping patterns of kinase 1 and kinase 2. These results suggest that Ang II induces the MBP/MAP2 kinase activation mainly through the protein kinase C signaling pathway. The Ca2+ ionophore ionomycin also induced an increase in the MBP kinase activity to nearly the same extent as Ang II and PMA (data not shown). However, Ang II-induced MBP kinase activation was inhibited only by 25% by prior treatment of VSMCs with EGTA (data not shown), which depletes intracellular as well as extracellular Ca2+ and completely abolishes the Ang II-induced phosphorylation of vimentin, an entirely Ca2+-dependent event. These observations suggest that the Ca2+ signaling pathway may not play a major role in the Ang II-induced MBP/MAP2 kinase activation in VSMCs.

In a preceding report, we have demonstrated that in VSMCs Ang II induces tyrosine phosphorylation of several proteins including two proteins with apparent molecular masses of 40 and 45 kD. We showed in the
FIGURE 7. Kinase assay in polyacrylamide gel containing myelin basic protein. Peak fractions of kinase 1 (lane 1) and kinase 2 (lane 2) eluted from the Superose 12 HR10/30 column were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis using a separation gel (11% polyacrylamide gel) containing 1 mg/ml myelin basic protein. After denaturation and renaturation of the enzymes, kinase reaction was performed in the gel as described under "Materials and Methods." The result shown is representative of three independent experiments.

The present study that kinase 1 and kinase 2 were coeluted during the purification procedures and comigrated on SDS-PAGE with the 40- and 45-kd proteins, respectively, which were tyrosine-phosphorylated during the action of Ang II in VSMCs. These results strongly suggest that the two species of MBP/MAP2 kinases are identical with the 40- and 45-kd proteins, which are tyrosine-phosphorylated during the action of Ang II in VSMCs. Consistent with these observations, tyrosine phosphorylation of kinase 1 and kinase 2 actually occurred after Ang II stimulation, as detected by phosphoamino acid analysis. In addition, phosphoamino acid analysis revealed that the two species of MBP/MAP2 kinases were phosphorylated not only on tyrosine but also on serine/threonine residues during the action of Ang II. Since both tyrosine and serine/threonine phosphorylation are essential for growth factor–induced activation of MBP/MAP2 kinase,\textsuperscript{16,27} it is highly possible that phosphorylation of MBP/MAP2 kinases on tyrosine is also an important event during vascular smooth muscle cell growth.

TABLE 1. Substrate Specificity of Angiotensin II–Stimulated Kinase 1 and Kinase 2

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Kinase 1: pmol min(^{-1}) (\mu)l(^{-1})</th>
<th>Kinase 1: %</th>
<th>Kinase 2: pmol min(^{-1}) (\mu)l(^{-1})</th>
<th>Kinase 2: %</th>
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</thead>
<tbody>
<tr>
<td>MBP</td>
<td>1.52±0.13</td>
<td>100</td>
<td>1.78±0.13</td>
<td>100</td>
</tr>
<tr>
<td>MAP2</td>
<td>1.75±0.18</td>
<td>115</td>
<td>1.96±0.19</td>
<td>110</td>
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<tr>
<td>Histone H1</td>
<td>0.03±0.01</td>
<td>2</td>
<td>0.05±0.02</td>
<td>3</td>
</tr>
<tr>
<td>Histone H2B</td>
<td>0.02±0.01</td>
<td>1</td>
<td>0.02±0.01</td>
<td>1</td>
</tr>
<tr>
<td>Casein</td>
<td>0.03±0.01</td>
<td>2</td>
<td>0.03±0.01</td>
<td>2</td>
</tr>
<tr>
<td>Protamine</td>
<td>0.03±0.01</td>
<td>2</td>
<td>0.01±0.01</td>
<td>1</td>
</tr>
<tr>
<td>40S ribosomal subunits</td>
<td>0.03±0.01</td>
<td>2</td>
<td>0.04±0.01</td>
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</table>

MBP, myelin basic protein; MAP2, microtubule-associated protein 2. Values are mean±SEM (n=3).

Kinase 1 and kinase 2 partially purified from angiotensin II–stimulated vascular smooth muscle cells were assayed for kinase activities toward various protein substrates under the standard conditions except that 0.4 mg/ml of each substrate was used instead of 0.25 mg/ml MBP. Percentages indicate values obtained when the incorporated radioactivity into MBP was regarded as 100%.

FIGURE 8. Immunoblot analysis of angiotensin II–stimulated kinase 1 and kinase 2 with anti-phosphotyrosine antibody. Lane 1, cytosolic extract of control vascular smooth muscle cells; lane 2, cytosolic extract of angiotensin II–treated vascular smooth muscle cells; lane 3, kinase 1; lane 4, kinase 2. The cytosolic extracts of vascular smooth muscle cells treated with 100 nM angiotensin II or its vehicle for 2 minutes and the peak fractions of kinase 1 and kinase 2 eluted from the Superose 12 HR10/30 column were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The separated proteins were transferred to nitrocellulose membranes and immunoblotted with the anti-phosphotyrosine antibody. Arrows indicate the positions of the 40- and 45-kd proteins. The result shown is representative of three independent experiments.

TABLE 2. Effects of Divalent Cations on the Activities of Angiotensin II–Stimulated Kinase 1 and Kinase 2

<table>
<thead>
<tr>
<th>Additions</th>
<th>Kinase 1: pmol min(^{-1}) (\mu)l(^{-1})</th>
<th>Kinase 1: %</th>
<th>Kinase 2: pmol min(^{-1}) (\mu)l(^{-1})</th>
<th>Kinase 2: %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Magnesium acetate</td>
<td>1.15±0.09</td>
<td>100</td>
<td>1.23±0.17</td>
<td>100</td>
</tr>
<tr>
<td>None</td>
<td>0.06±0.01</td>
<td>5</td>
<td>0.07±0.01</td>
<td>6</td>
</tr>
<tr>
<td>MgCl(_2)</td>
<td>1.27±0.12</td>
<td>110</td>
<td>1.50±0.26</td>
<td>122</td>
</tr>
<tr>
<td>MnCl(_2)</td>
<td>0.65±0.11</td>
<td>57</td>
<td>0.99±0.07</td>
<td>80</td>
</tr>
<tr>
<td>CaCl(_2)</td>
<td>0.05±0.01</td>
<td>4</td>
<td>0.08±0.01</td>
<td>7</td>
</tr>
<tr>
<td>ZnCl(_2)</td>
<td>0.06±0.01</td>
<td>5</td>
<td>0.04±0.01</td>
<td>3</td>
</tr>
<tr>
<td>MgCl(_2)+CaCl(_2)</td>
<td>0.46±0.05</td>
<td>40</td>
<td>0.51±0.09</td>
<td>41</td>
</tr>
<tr>
<td>MgCl(_2)+ZnCl(_2)</td>
<td>0.06±0.01</td>
<td>5</td>
<td>0.06±0.01</td>
<td>5</td>
</tr>
</tbody>
</table>

Values are mean±SEM (n=3).

Kinase 1 and kinase 2 partially purified from angiotensin II–stimulated vascular smooth muscle cells were assayed for myelin basic protein kinase activity in the absence or presence of the indicated divalent cations. The concentrations of the divalent cations were 10 mM. Percentages indicate values obtained when the incorporated radioactivities into myelin basic protein in the standard assay condition using 10 mM magnesium acetate were regarded as 100%.
sine and serine/threonine residues is also required for Ang II–induced activation of these enzymes.

The in vivo functions of MBP/MAP2 kinase remain to be firmly established, but accumulating evidence suggests that MBP/MAP2 kinase acts as an intermediate that mediates signals from growth factor receptors to the ribosomes and nucleus. MBP/MAP2 kinase has been shown to phosphorylate and activate ribosomal protein S6 kinase II purified from Xenopus eggs. Since Ang II induces S6 kinase activation and protein synthesis in VSMCs, it is possible that MBP/MAP2 kinases are involved in the signaling pathway from Ang II receptors to ribosomes. We have demonstrated in VSMCs that Ang II induces the phosphorylation of nuclear membrane proteins, lamins A, B, and C in a protein kinase C–dependent manner. However, the Ang II–induced phosphorylation pattern of lamins differs from that of lamins phosphorylated directly by protein kinase C in vitro, suggesting that Ang II–induced phosphorylation of lamins is catalyzed by another protein kinase(s) that may be directly or indirectly activated by protein kinase C. A recent observation has revealed that S6 kinase II phosphorylates nuclear lamin C, suggesting that MBP/MAP2 kinases may also be involved in the signaling pathway from Ang II receptors to the nucleus. In addition to S6 kinase II, several potential substrates for MBP/MAP2 kinase have recently been proposed. They include the products of the c-jun, c-fos, and c-myc proto-oncogenes, EGF receptor, and Raf-1, a serine/threonine kinase encoded by the cellular homologue of the viral transforming gene v-raf, which are all considered to play important roles in the regulation of cell growth. To elucidate the precise roles of MBP/MAP2 kinases in the growth-promoting activity of Ang II for VSMCs, it is essential to identify the physiological substrates for MBP/MAP2 kinases in VSMCs.

**Figure 9.** Phosphoamino acid analysis of kinase 1 and kinase 2. 32P-labeled vascular smooth muscle cells were stimulated with 100 nM angiotensin II for 2 minutes or 100 nM phorbol 12-myristate 13-acetate for 5 minutes. Lanes 1–3, kinase 1; lanes 4–6, kinase 2; lanes 1 and 4, control; lanes 2 and 5, angiotensin II; lanes 3 and 6, phorbol 12-myristate 13-acetate. Kinase 1 and kinase 2 were isolated and subjected to phosphoamino acid analysis as described under “Materials and Methods.” The positions of phosphoserine (P-Ser), phosphothreonine (P-Thr), phosphotyrosine (P-Tyr), and origin (Ori) are indicated at the right. The result shown is representative of three independent experiments.

**Figure 10.** Tryptic phosphopeptide mapping of kinase 1 and kinase 2. 32P-labeled vascular smooth muscle cells were stimulated with 100 nM angiotensin II for 2 minutes or 100 nM phorbol 12-myristate 13-acetate for 5 minutes. Kinase 1 and kinase 2 were isolated and subjected to tryptic phosphopeptide mapping as described under “Materials and Methods.” Panel A: Kinase 1 from angiotensin II–stimulated cells. Panel B: Kinase 1 from phorbol 12-myristate 13-acetate–stimulated cells. Panel C: Kinase 2 from angiotensin II–stimulated cells. Panel D: Kinase 2 from phorbol 12-myristate 13-acetate–stimulated cells. +, Anode; −, cathode. The result shown is representative of three independent experiments.
Acknowledgments

We are grateful to Yumi Shiraishi and Yoshie Agehara for their skillful technical assistance.

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Circ Res. 1992;71:620-630
doi: 10.1161/01.RES.71.3.620

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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