



# Involvement of MAP kinase activators in angiotensin II-induced activation of MAP kinases in cultured vascular smooth muscle cells

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# Involvement of MAP kinase activators in angiotensin II-induced activation of MAP kinases in cultured vascular smooth muscle cells

培養血管平滑筋細胞のアンギオテンシン II による MAP キナーゼ活性化における MAP キナーゼ活性化因子の関与

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In cultured vascular smooth muscle cells (VSMC) angiotensin II (ang II) induces tyrosine and serine/threonine phosphorylation and activation of two mitogen-activated protein (MAP) kinases. When extracts of ang II-stimulated VSMC were fractionated by Mono Q anion-exchange column chromatography, three peaks of the activities which in vitro activate inactive MAP kinases were detected. These MAP kinase activator activities were not detected in extracts of unstimulated VSMC. In vitro activation of MAP kinases by the MAP kinase activators was accompanied by tyrosine and serine/threonine phosphorylation of MAP kinases. These results suggest that the MAP kinase activators are involved in the ang II-induced phosphorylation and activation of MAP kinases in VSMC.

Angiotensin II; Mitogen-activated protein kinase; MAP kinase activator; Protein kinase C; Vascular smooth muscle cell

## 1. INTRODUCTION

In cultured vascular smooth muscle cells (VSMC), angiotensin II (ang II) stimulates ribosomal protein S6 kinase activity [1], protein synthesis [2], phosphorylation of nuclear membrane lamins [3] and expression of the protooncogenes, *c-fos* and *c-myc* [4,5]. These ang II-induced events may be involved in the induction of hypertrophy of VSMC, which is one of the fundamental pathogenetic features of hypertension [6], however, the molecular mechanisms by which ang II regulates these ribosomal and nuclear events have not been elucidated.

Mitogen-activated protein (MAP) kinases are members of a family of serine/threonine-specific protein kinases whose activation is commonly induced by a variety of growth factors, such as insulin, epidermal growth factor (EGF) and platelet-derived growth factor [7–9]. These growth factors activate MAP kinases via phosphorylation on both tyrosine and serine/threonine residues [10,11]. MAP kinase thus activated phosphorylates and reactivates the phosphatase-inactivated S6 kinase II [12]. S6 kinase II has been shown to phosphorylate nuclear lamin C [13] in addition to ribosomal protein

S6. Moreover, it has recently been demonstrated that MAP kinases phosphorylate the products of the protooncogenes, *c-jun*, *c-fos* and *c-myc* [14–16]. In a preceding report, we have shown that ang II induces tyrosine and serine/threonine phosphorylation and activation of two MAP kinases in cultured VSMC [17]. Thus it is likely that MAP kinases play important roles as intermediates in the signalling pathways from the ang II receptors, as well as the growth factor receptors, to the ribosomes and nucleus.

Recently, the MAP kinase activators or MAP kinase kinases, which in vitro stimulate tyrosine and serine/threonine phosphorylation and activation of MAP kinases, were found in EGF-stimulated fibroblasts and nerve growth factor (NGF)-stimulated PC12 cells [11,18]. In the present study, we examined whether similar MAP kinase activators are also involved in the activation mechanism of MAP kinases by ang II in cultured VSMC.

## 2. MATERIALS AND METHODS

### 2.1. Materials

VSMC were isolated from rat thoracic aorta by enzymatic dissociation, as described [19]. ang II, phorbol 12-myristate 13-acetate (PMA) and myelin basic protein (MBP) were purchased from Sigma (St. Louis, MO, USA). [ $\gamma$ - $^{32}$ P]ATP (3,000 Ci/mmol) was from Amersham Japan (Tokyo, Japan). Mouse monoclonal anti-MAP kinase antibody and goat anti-mouse IgG conjugated with peroxidase were from Zymed Laboratories (San Francisco, CA, USA) and Cappel (West Chester, PA, USA), respectively. Other materials and chemicals were obtained from commercial sources.

### 2.2. Preparation of inactive MAP kinases

Unstimulated VSMC ( $20 \times 100$ -mm dishes) were scraped into buffer A containing 20 mM Tris-Cl, pH 7.5, 2 mM EGTA, 1 mM sodium

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*Abbreviations:* VSMC, vascular smooth muscle cells; ang II, angiotensin II; MAP kinase, mitogen-activated protein kinase; EGF, epidermal growth factor; NGF, nerve growth factor; PMA, phorbol 12-myristate 13-acetate; MBP, myelin basic protein; EGTA, [ethylene bis(oxyethylenenitrilo)]-tetraacetic acid; DTT, dithiothreitol; APMSF, (*p*-amidinophenyl)-methanesulfonyl fluoride; SDS, sodium dodecylsulfate; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride.

orthovanadate, 10 mM  $\beta$ -glycerophosphate, 1 mM dithiothreitol (DTT), 1  $\mu$ M APMSF and 100 kallikrein-inactivating U/ml of aprotinin, sonicated and centrifuged as described [17]. Supernatants were applied to a DEAE-cellulose column (0.9  $\times$  1.6 cm) pre-equilibrated with buffer A. The column was washed with 5 ml of buffer A containing 20 mM NaCl and the elution was performed with 5 ml of buffer A containing 250 mM NaCl. The eluate was diluted with 9 vols. of buffer A, passed through a 0.22- $\mu$ m 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 of 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. Each fraction (10  $\mu$ l) was analyzed by immunoblotting with anti-MAP kinase antibody and fractions containing immunoreactive MAP kinases were used as the inactive MAP kinase preparation.

### 2.3. Partial purification of MAP kinase activators

Extract supernatants prepared from ang II-stimulated VSMC (20  $\times$  100-mm dishes) [17] were passed through a 0.22- $\mu$ m Millex-GV filter and applied to a Mono Q HR5/5 column pre-equilibrated with buffer A. The column was washed and the elution was performed as described above. Each fraction was assayed for MAP kinase activator activity. Active fractions were 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  $\beta$ -glycerophosphate, 1 mM DTT, 100 mM NaCl, 1  $\mu$ M APMSF and 100 kallikrein-inactivating U/ml of aprotinin. 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. Each fraction was assayed for MAP kinase activator activity. Active fractions were collected and used as the MAP kinase activator.

### 2.4. Assay for MAP kinase activator activity

Column fractions (10  $\mu$ l) were incubated with 10  $\mu$ l of the inactive MAP kinase preparation at 30°C for 15 min in a final volume of 50  $\mu$ l containing 50 mM  $\beta$ -glycerophosphate, pH 7.5, 1 mM DTT, 1.5 mM EGTA, 10 mM magnesium acetate, 40  $\mu$ M [ $\gamma$ - $^{32}$ P]ATP ( $2.5 \times 10^5$  cpm/nmol). After the addition of 10  $\mu$ l of MBP (0.25 mg/ml), the incubation was continued for a further 15 min. The reaction was terminated by the addition of 1 ml of ice-cold 20% trichloroacetic acid and 60 mM sodium pyrophosphate, and the 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.

### 2.5. Phosphorylation of MAP kinases during the incubation with MAP kinase activator

The inactive MAP kinase preparation (10  $\mu$ l) was incubated at 30°C for 15 min with the MAP kinase activator (10  $\mu$ l). The reaction mixture was the same as that used for the MAP kinase activator assay except that the specific activity of [ $\gamma$ - $^{32}$ P]ATP was increased ( $7.5 \times 10^5$  cpm/nmol). The reaction was terminated by the addition of 25  $\mu$ l of SDS sample buffer [20]. The samples were subjected to SDS-PAGE (8–16% gel) followed by autoradiography. For phosphoamino acid analysis, separated proteins were electrophoretically transferred to a PVDF membrane followed by immunoblotting with the anti-MAP kinase antibody. Immunoreactive bands were excised and subjected to phosphoamino acid analysis according to the method of Hildebrandt and Fried [21] with slight modifications [17].

### 2.6. Other methods

The kinase assay in polyacrylamide gels containing MBP was performed according to the method of Kameshita and Fujisawa [22] with slight modifications [17]. Immunoblot analysis with the anti-MAP kinase antibody was performed as described previously [17].

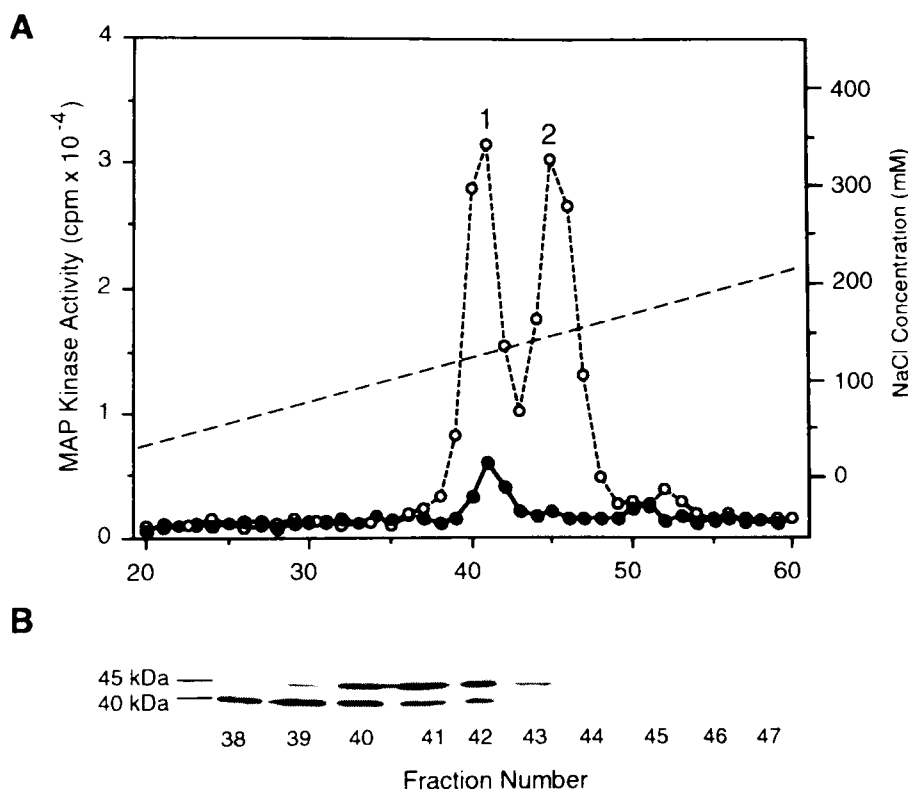


Fig. 1. Mono Q HR5/5 column chromatography of ang II-stimulated or unstimulated MAP kinase activity and anti-MAP kinase immunoblot of Mono Q fractions. (A) Cytosolic extracts of VSMC treated for 2 min with 100 nM ang II (○) or its vehicle (●) were subjected to DEAE-cellulose column chromatography followed by Mono Q HR5/5 column chromatography. Aliquots of each fraction were assayed for MAP kinase activity. (---) NaCl concentration; (1) Kinase 1, (2) Kinase 2. (B) Indicated fractions derived from the unstimulated VSMC were subjected to SDS-PAGE followed by immunoblotting with the anti-MAP kinase antibody.

### 3. RESULTS

We have previously described that in VSMC ang II stimulates activation of two MAP kinases, 40-kDa *Kinase 1* and 45-kDa *Kinase 2* [17]. These MAP kinases can be separated by Mono Q HR5/5 anion-exchange column chromatography; first and second peaks correspond to Kinase 1 and Kinase 2, respectively (Fig. 1A) [17]. When the extracts of unstimulated VSMC were fractionated by the same Mono Q column, MAP kinase activity was hardly detected (Fig. 1A). Immunoblotting of each fraction of unstimulated VSMC extracts with the anti-MAP kinase antibody revealed that inactive Kinase 1 and Kinase 2 were eluted almost together in the fractions from number 38 to 43. These fractions were collected and used as the inactive MAP kinase preparation in the following experiments.

The inactive MAP kinase preparation was incubated with each Mono Q fraction of ang II-stimulated VSMC extracts, and MAP kinase activity was assayed. As shown in Fig. 2, three peaks of the activities which activate the inactive MAP kinase(s) appeared just before two peaks of endogenous MAP kinases. These MAP kinase activator activities were not detected in Mono Q fractions derived from unstimulated VSMC (data not shown). Almost identical elution profiles of three peaks of MAP kinase activator activities and two peaks of MAP kinase activities were obtained when extracts of PMA-stimulated VSMC were fractionated by the Mono Q column (data not shown). Each peak of MAP kinase activator activity was further purified by gel filtration chromatography using a Superose 12 HR10/30 column. Each MAP kinase activator activity was eluted at almost the same position of molecular mass around 60 kDa. In the following *in vitro* experi-

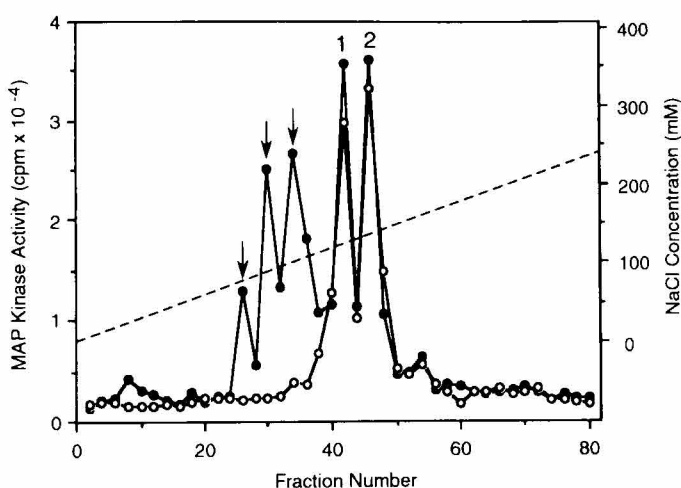


Fig. 2. Mono Q HR5/5 column chromatography of ang II-stimulated MAP kinase activator activity. Cell extracts prepared from ang II-stimulated VSMC were applied to a Mono Q HR5/5 column. Each fraction was incubated with (●) or without (○) the inactive MAP kinase preparation and MAP kinase activity was assayed. Arrows indicate the MAP kinase activators, (1) Kinase 1, (2) Kinase 2.

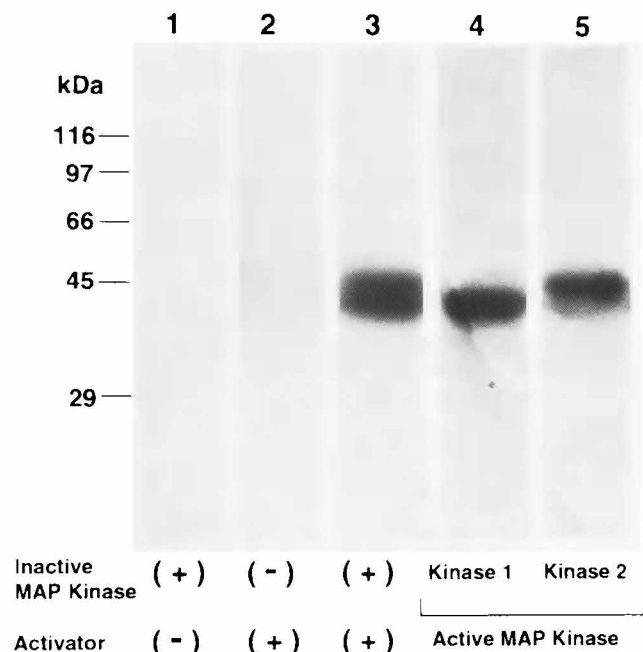


Fig. 3. The kinase assay in a polyacrylamide gel (12%) containing MBP. The inactive MAP kinase preparation was incubated with the MAP kinase activator for 15 min. As controls, the inactive MAP kinase preparation or the MAP kinase activator was separately incubated. Samples were subjected to the kinase assay in polyacrylamide gel containing MBP (12% gel). Authentic MAP kinases were partially purified from ang II-stimulated VSMC and subjected to the same assay. (Lane 1) Inactive MAP kinase preparation; (lane 2) MAP kinase activator; (lane 3) inactive MAP kinase preparation and MAP kinase activator; (lane 4) partially purified Kinase 1; (lane 5) partially purified Kinase 2. Arrows indicate the positions of MAP kinases.

ments, we used, as the MAP kinase activator, a preparation thus purified from the second peak of MAP kinase activator activity on Mono Q column chromatography. Similar results were obtained when preparations purified from the first or third peaks were used as the MAP kinase activator.

The inactive MAP kinase preparation was incubated with the MAP kinase activator and subjected to the kinase assay in polyacrylamide gels containing MBP (Fig. 3). MAP kinase activity was detected in two polypeptides with apparent molecular masses of 40 and 45 kDa, which migrated to the identical positions with the authentic Kinase 1 and Kinase 2, respectively, indicating that both Kinase 1 and Kinase 2 were activated by the MAP kinase activator. The activation of MAP kinases by the MAP kinase activator was dependent on the presence of ATP during the incubation period (data not shown). No MAP kinase activity was detected when the inactive MAP kinase preparation or the MAP kinase activator was separately incubated.

When the inactive MAP kinase preparation was incubated with the MAP kinase activator in the presence of [ $\gamma$ - $^{32}$ P]ATP, two polypeptides with apparent molecular masses of 40 and 45 kDa became phosphorylated (Fig. 4). Immunoblot analysis revealed that these two radio-

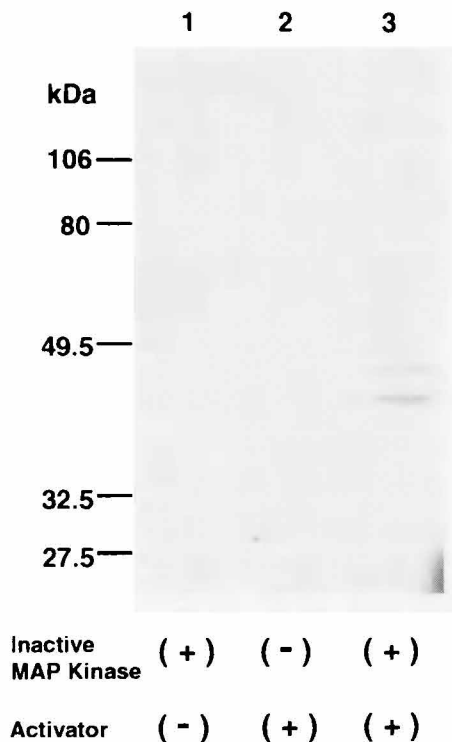


Fig. 4. Effect of the MAP kinase activator on MAP kinase phosphorylation. The inactive MAP kinase preparation was incubated with the MAP kinase activator in the presence of  $40 \mu\text{M}$   $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  for 15 min at  $30^\circ\text{C}$ . As controls, the inactive MAP kinase preparation or the MAP kinase activator was separately incubated. The reaction was terminated by the addition of SDS sample buffer followed by SDS-PAGE (12% gel). Separated proteins were electrophoretically transferred to a PVDF membrane followed by autoradiography. (Lane 1) Inactive MAP kinase preparation; (lane 2) MAP kinase activator; (lane 3) inactive MAP kinase preparation and MAP kinase activator. Arrows indicate the positions of MAP kinases.

active bands were recognized by the anti-MAP kinase antibody (data not shown), indicating that both Kinase 1 and Kinase 2 were phosphorylated during the *in vitro* activation by the MAP kinase activator. No phosphorylated bands were detected when the inactive MAP kinase preparation or the MAP kinase activator was separately incubated. The radioactive Kinase 1 and Kinase 2 bands were excised and subjected to phosphoamino acid analysis (Fig. 5). Kinase 1 and Kinase 2 phosphorylated *in vitro* contained phosphotyrosine, phosphothreonine and, to a lesser extent, phosphoserine.

#### 4. DISCUSSION

The results of the present study provide the first demonstration that the MAP kinase activators are involved in the activation mechanism of MAP kinases by extracellular signals whose receptors are coupled to phospholipase C via the GTP-binding protein, Gp. In a preceding report, we have demonstrated that ang II activates two MAP kinases mainly through the protein ki-

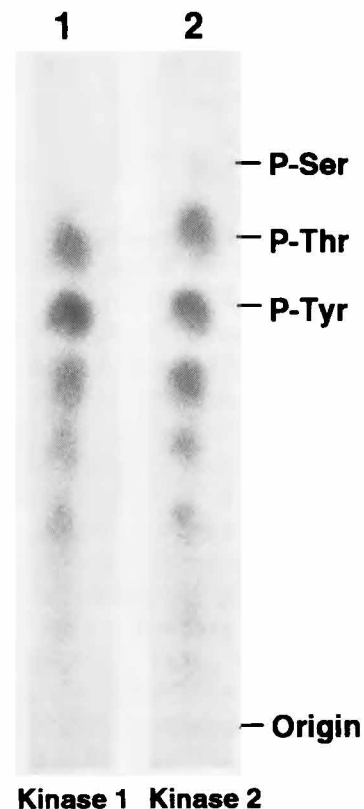


Fig. 5. Phosphoamino acid analysis of MAP kinases phosphorylated after incubation with the MAP kinase activator. The inactive MAP kinase preparation was incubated with the MAP kinase activator in the presence of  $40 \mu\text{M}$   $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  for 15 min at  $30^\circ\text{C}$ . The reaction was terminated by the addition of SDS sample buffer followed by SDS-PAGE. Separated proteins were electrophoretically transferred to a PVDF membrane followed by immunoblotting with the anti-MAP kinase antibody. Immunoreactive bands were excised and subjected to phosphoamino acid analysis. (Lane 1) Kinase 1; (lane 2) Kinase 2. The positions of origin, phosphotyrosine (P-Tyr), phosphothreonine (P-Thr) and phosphoserine (P-Ser) were indicated to the right.

nase C-signalling pathway in VSMC [17]. We also described here that the effect of ang II on the MAP kinase activators was mimicked by the protein kinase C-activating phorbol ester, PMA. It has been shown that the MAP kinase activator activity is regulated via the protein-serine/threonine phosphorylation reaction [18]. Although it remains to be clarified whether protein kinase C directly phosphorylates the MAP kinase activators, it seems likely that protein kinase C plays a role in the activation mechanism of the MAP kinase activators by ang II.

The ang II-stimulated MAP kinase activator activity was separated into three peaks on Mono Q anion-exchange column chromatography. They had similar molecular masses, as assessed by gel filtration, and similar activator activities to each other. Similarly, two peaks of MAP kinase activator activities on the same anion-exchange column chromatography were described in EGF-stimulated fibroblasts and NGF-stimulated PC12 cells [11,18]. Although the relationship between these

peaks of the MAP kinase activators remains to be clarified, they may represent multiple isoforms of the MAP kinase activators.

Although the molecular mechanism by which the MAP kinase activators induce tyrosine and serine/threonine phosphorylation of MAP kinases has not yet been clarified, some possibilities have been proposed. Since MAP kinases undergo autophosphorylation on both tyrosine and serine/threonine residues, the possibility is proposed that the MAP kinase activators function by enhancing the ability of MAP kinases to undergo autophosphorylation [23–25]. Another possibility is that the MAP kinase activators are protein kinases with specificity for either tyrosine or serine/threonine; either type of phosphorylation by the MAP kinase activators enhances another type of autophosphorylation of MAP kinases. However, recent observations obtained using the kinase-defective or autophosphorylation site-defective mutants of MAP kinases raise the possibility that the MAP kinase activators are protein kinases capable of phosphorylating both tyrosine and serine/threonine residues of MAP kinases [26,27]. Purification and molecular cloning of the MAP kinase activators will clarify which of these is the case.

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