ELSEVIER

European Journal of Pharmacology 445 (2002) 247–256

www.elsevier.com/locate/ejphar

Involvement of p44/42 mitogen-activated protein kinases in regulating angiotensin II- and endothelin-1-induced contraction of rat thoracic aorta

Akira Ishihata [a,*], Katsunari Tasaki [b], Yumi Katano [b]

[a] *Department of Physiology I, Yamagata University School of Medicine, 2-2-2, Iida-Nishi, Yamagata, 990-9585, Japan*
[b] *Division of Theoretical Nursing and Pathophysiology, Yamagata University School of Medicine, 2-2-2, Iida-Nishi, Yamagata, 990-9585, Japan*

Received 21 January 2002; received in revised form 1 May 2002; accepted 6 May 2002

Abstract

In order to elucidate the signal transduction pathway of vascular smooth muscle contraction induced by the activation of receptors for angiotensin II and endothelin-1, we examined whether tyrosine kinases and mitogen-activated protein (MAP) kinases are involved in the development of force of contraction in the rat aorta. Isolated aortic smooth muscles without endothelium were incubated in a modified Krebs–Henseleit solution and stimulated with angiotensin II (100 nM) or endothelin-1 (10 nM). A tyrosine kinase inhibitor genistein (10 μM) reduced the angiotensin II- and endothelin-1-induced aortic contraction, while 10 μM of daidzein (an inactive analogue of genistein) did not. The K^+ depolarization-induced contraction was not attenuated by 10 μM of genistein. Selective inhibitors of MAP kinase/extracellular signal-regulated kinase (Erk) kinase (MEK) such as PD98059 [2-(2-amino-3-methoxyphenyl)-4*H*-1-benzopyran-4-one] and U0126 [1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene] inhibited the angiotensin II- and endothelin-1-induced vasocontraction. The p44/42 MAP kinases were phosphorylated in cultured aortic smooth muscle cells and in physiologically contracted aortic vessels stimulated with angiotensin II and endothelin-1 for 5 min. The angiotensin II- and endothelin-1-induced phosphorylations of p44/42 MAP kinases were inhibited by PD98059 as well as U0126 in the intact aorta. These results suggest that the activation of genistein-sensitive tyrosine kinases and p44/42 MAP kinases is involved in the angiotensin II- and endothelin-1-induced rat aortic contraction.

Keywords: MAP kinase; U0126; PD98059; Angiotensin II; Endothelin-1; Contraction

1. Introduction

Angiotensin II is a potent vasoconstricting peptide that mediates a variety of effects such as stimulation of vascular smooth muscle proliferation and cardiac muscle hypertrophy (Sadoshima et al., 1995), release of catecholamine from autonomic nerve endings and secretion of adrenal aldosterone and cardiac positive inotropic effect, although there is a species-dependent difference in the action of angiotensin II (Ishihata and Endoh, 1993, 1995). Endothelin-1 is another very potent vasoconstrictor (Yanagisawa et al., 1988), and stimulates proliferation of vascular smooth muscle cells (Bobik et al., 1990). A variety of effects induced by angiotensin II and endothelin-1 are mediated by several signal transduction pathways such as stimulation of phospholipase C and protein kinase C (Force et al., 1991; Koide et al., 1992). Angiotensin II and endothelin-1 activate phospholipase C, which hydolyses phosphatidylinositol-4,5-bisphosphate (PIP_2) to generate inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol. IP_3 induces Ca^{2+} mobilization from intracelullar stores, while diacylglycerol activates protein kinase C (Nishizuka, 1984; Berridge, 1993). These pathways are considered to be important for both vascular contraction and cell growth. In addition, the vasocontraction evoked by angiotensin II is markedly inhibited by a voltage-dependent Ca^{2+} channel blocker nifedipine (Watts et al., 1998), indicating the involvement of Ca^{2+} channel activation in vasocontraction. However, recent studies suggest that the activation of G-protein coupled receptors may possess more complicated pathways for vascular contraction than these well-known signal transduction pathways. For example, it has been suggested that tyrosine kinases may be linked to agonists-induced smooth muscle contraction (DiSalvo et al., 1993; Hollenberg, 1994; Abebe and Agrawal,

* Corresponding author. Tel.: +81-23-628-5214; fax: +81-23-628-5433.
E-mail address: ishihata@med.id.yamagata-u.ac.jp (A. Ishihata).

0014-2999/02/$ - see front matter
PII: S0014-2999(02)01790-9

1995; Gould et al., 1995). Furthermore, intracellular Ca^{2+} transients evoked by G-protein coupled receptors are modulated by tyrosine kinase (Liu and Sturek, 1996; Touyz and Schiffrin, 1996). Several target molecules such as mitogen-activated protein (MAP) kinases seem to play a physiological role in tyrosine kinase-mediated signal transduction (Giasson and Meloche, 1995; Sadoshima et al., 1995; Turner et al., 1995). MAP kinases are serine/threonine kinases which will be activated by MAP kinase/extracellular signal regulated kinase (Erk) kinase (MEK). MAP kinases exist downstream of the pathway of tyrosine kinase, Src kinase and Janus kinase (Zubkov et al., 2000), and play an important role in cell growth in many cell types (Thomas et al., 1996; Force and Bonventre, 1998). In addition to the growth-stimulating effects, it has been suggested that activation of MAP kinase may be involved in 5-hydroxytryptamine (5-HT)- and endothelin-1-induced vasocontraction (Florian and Watts, 1998; Watts, 1998; Zubkov et al., 2000). However, dissociation of the angiotensin II-induced MAP kinase phosphorylation from vasocontraction was observed in a previous study (Watts et al., 1998); therefore, the role of MAP kinase in vascular contraction is controversial.

In this study, we hypothesized that the activation of tyrosine kinase and MEK is involved in the signal transduction pathway of angiotensin II- and endothelin-1-induced contraction in rat thoracic aorta. We indicated that the angiotensin II- and endothelin-1-induced contraction as well as phosphorylations of p44/42 MAP kinases were inhibited by PD98059 and U0126 in physiologically contracted aortic preparations, in addition to cultured vascular smooth muscle cells.

2. Materials and methods

2.1. Measurement of aortic contraction

Male Fischer 344 rats (210–300 g) obtained from Charles River Japan (Atsugi, Japan) were used. Experiments were performed in accordance with the Guide for Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication No. 85-23, revised 1996) and under the regulations of the Animal Care Committee of Yamagata University School of Medicine. Rats were killed by cervical dislocation under anesthesia with ether. Aortas were isolated and excess fat and connective tissues were removed. Vessels were cut into rings 3 mm long, which were cut open, and endothelium was removed by gentle rubbing the intimal surface with a moistened cotton swab. Isolated aortic strips were suspended in organ baths containing 10 ml physiological salt solution. The solution contained 118 mM NaCl, 4.7 mM KCl, 24.9 mM $NaHCO_3$, 1.18 mM $MgSO_4$, 1.18 mM KH_2PO_4, 11.1 mM glucose, 1.8 mM $CaCl_2$ and 0.057 mM ascorbic acid. High concentration of K^+ solution was made by substituting NaCl with equimolar KCl. These solutions were saturated with 95% O_2 and 5% CO_2 mixture at 37 °C to pH 7.4. The developed tension was recorded with an isometric force transducer (7T-15-240, Orientec, Tokyo, Japan) for measurement of changes in the contractile force. The preparation was stretched to a resting tension of 0.8 g, and the solution was changed every 15 min. After an equilibration period of 1 h, each strip was contracted with 66.7 mM KCl (high K^+) repeatedly until reproducible contraction was obtained. Removal of the endothelium was verified by the absence of relaxation with acetylcholine (1 μM) in strips precontracted with 100 nM phenylephrine. Then, each strip was washed and one of the following inhibitors (genistein, daidzein, PD98059 and U0126) was added 15 min before and during stimulation with an agonist, because it has been shown that these inhibitors attenuated the intact smooth muscle contraction with the incubation time of 10–15 min (Filipeanu et al., 1995; Jin et al., 1996; Yang et al., 1999, 2000; Yousufzai et al., 2000). These drugs were dissolved in dimethylsulfoxide (DMSO) to make stock solutions. Each aortic strip was challenged with only one concentration of angiotensin II (100 nM) or endothelin-1 (10 nM), because they have the effect of tachyphylaxis in rat aortic contraction.

2.2. Culture of rat aortic vascular smooth muscle cells

Rat aortic vascular smooth muscle cells were isolated from male Fischer 344 rats by the method previously reported (Ishihata et al., 1998). Briefly, the thoracic aortas of 12-week-old rats were dissected from the surrounding tissues. After adipose tissues and endothelial cells were removed, aortas were digested in Dulbecco's modified Eagle's medium (DMEM) containing 0.7 mg/ml collagenase (type IA, Sigma), 0.25 mg/ml elastase (type III, Sigma), 0.4 mg/ml soybean trypsin inhibitor and 1 mg/ml bovine serum albumin for 30 min at 37 °C. Dissociated vascular smooth muscle cells were seeded into plastic tissue culture dishes and grown in DMEM containing 10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C under 5% CO_2–95% air. The medium was changed every 3–4 days. Cultured vascular smooth muscle cells were used at 5–10 passages.

2.3. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and Western blot analysis in cultured vascular smooth muscle cells

Aortic vascular smooth muscle cells were cultured in DMEM with 10% fetal calf serum. When cells were grown to confluent, the medium was changed to serum-free DMEM. After 24 h, cells were washed three times and incubated in Hanks-balanced salt solution (HBSS) for 1 h. At the same time, inhibitors or vehicle (DMSO) were added and equilibrated. After cells were stimulated with angiotensin II or endothelin-1, culture dishes were placed on ice, and

HBSS was aspirated. Cells were washed three times with ice-cold HBSS containing sodium orthovanadate (1 mM), then lysed in a detergent solution and harvested with a rubber policeman. The lysate was centrifuged at 14,000 × *g* for 10 min at 4 °C, and the supernatant was used for sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. Proteins in each sample were separated on a 10% SDS-polyacrylamide gel, and electrotransferred to polyvinilidene difluoride (PVDF) membranes (Immobilon-P; Millipore, Bedford, MA, USA) in 25 mM Tris–HCl, 192 mM glycine, 0.1% SDS and 10% methanol for 1 h at constant current (100 mA). The phosphorylated MAP kinases were immunodetected as follows. Nonspecific binding of the membranes was blocked in TBS-T buffer (50 mM Tris, 150 mM NaCl and 0.1% Tween 20, pH 7.4) containing 5% nonfat dry milk. After incubation for 1 h at room temperature, membranes were washed three times in TBS-T buffer for 5 min, then incubated with rabbit anti-phosphorylated MAP kinases (New England BioLab, Beverly, MA, USA) diluted 1:1000 in blocking buffer at 4 °C overnight. After washing three times, the membranes were treated with anti-rabbit IgG conjugated to horseradish peroxidase with gentle agitation for 1 h at room temperature, then the corresponding antibody-labeled bands were detected by enhanced chemiluminescent reaction kit (Amersham).

2.4. SDS-polyacrylamide gel electrophoresis and Western blot analysis in vascular strips in vivo

Strips of endothelium-denuded aorta were mounted in the organ bath filled with Krebs–Henseleit buffer equilibrated with 95% O_2–5% CO_2 at 37 °C. After 1 h incubation for stabilization, tissues were exposed to phenylephrine and then washed several times. In the experiment to determine the agonist-induced protein phosphorylation, the aortic strips were stimulated with angiotensin II or endothelin-1 for 5 min and quickly frozen in the liquid nitrogen. The kinase inhibitors were added 15 min before stimulation. Then, strips were pulverized in ice-cold homogenization buffer [25 mM Tris–HCl, 50 mM NaCl, 1 mM ethylenediamine-*N*,*N*,*N*′,*N*′-tetraacetic acid (EDTA), 100 mM sodium fluoride, 1% Nonidet P-40 (NP-40), 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, pH 7.4] and were put on ice for 30 min. The homogenate was sonicated for 30 s and centrifuged at 14,000 × *g* for 10 min to remove debris, and aliquots of supernatant were taken for measurement of protein concentration. The lysates containing equal amounts of total proteins (15 μg) were solubilized in Laemmli's sample buffer (60 mM Tris–HCl, 2% SDS, 10% glycerol, 0.008% bromophenol-blue, 5% β-mercaptoethanol) and boiled for 5 min. Proteins in each sample were separated on a 10% SDS-polyacrylamide gel, and then electrically transferred to PVDF membranes (Immobilon-P; Millipore) in 25 mM Tris–HCl, 192 mM glycine, 0.1% SDS and 10% methanol for 1 h at constant current (100 mA). The phosphorylated MAP kinases were immunodetected as described above.

2.5. Statistical analysis

Data were expressed as means ± S.E.M. When comparing two paired groups, paired Student's *t*-test was used. To compare three or more groups, analysis of variance (ANOVA) followed by Scheffe's test was used. The difference was defined to be statistically significant when a *P* value was less than 0.05.

2.6. Drugs used

Acetylcholine chloride was obtained from Daiichi Pharmaceutical (Tokyo, Japan). Genistein was from Wako (Osaka, Japan). Daidzein and PD98059 [2-(2-amino-3-methoxyphenyl)-4*H*-1-benzopyran-4-one] were from Sigma (St. Louis, MO, USA). U0126 [1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene] was from Promega (Madison, WI, USA). Bovine serum albumin was obtained from Sigma. Dulbecco's modified Eagle's medium (DMEM), phosphate-buffered saline (PBS) and fetal calf serum were from Gibco (Grand Island, NY). Angiotensin II and endothelin-1 were from Peptide Institute (Osaka, Japan). Endothelin-1 was dissolved in distilled water containing 0.01% bovine serum albumin. Genistein, daidzein, PD98059 and U0126 were dissolved in DMSO.

3. Results

3.1. Effect of a tyrosine kinase inhibitor on the aortic contraction

3.1.1. Effect of genistein on the angiotensin II- and endothelin-1-induced contraction

Angiotensin II (100 nM) contracted the rat aorta to 74.2 ± 6.6% of the maximal contraction induced by 66.7 mM KCl (KCl_{max}). The maximal contraction was obtained 5.0 ± 0.5 min after application of angiotensin II, then the contractile force was declined to the basal level. In order to elucidate whether tyrosine kinases are involved in the angiotensin II-induced vasocontraction, a tyrosine kinase inhibitor genistein was used as treatment before angiotensin II was applied. As shown in Fig. 1A, pretreatment with genistein (10 and 30 μM) inhibited the angiotensin II-induced contraction, although the contractile responses to angiotensin II with low concentrations of genistein (1–3 μM) were not significantly different from control. The maximal response to angiotensin II was significantly inhibited by 10 and 30 μM of genistein to 50.7 ± 4.4% of KCl_{max} ($n = 9$, $P < 0.01$) and 22.7 ± 7.0% of KCl_{max} ($n = 6$, $P < 0.001$), respectively. To make sure that the inhibition

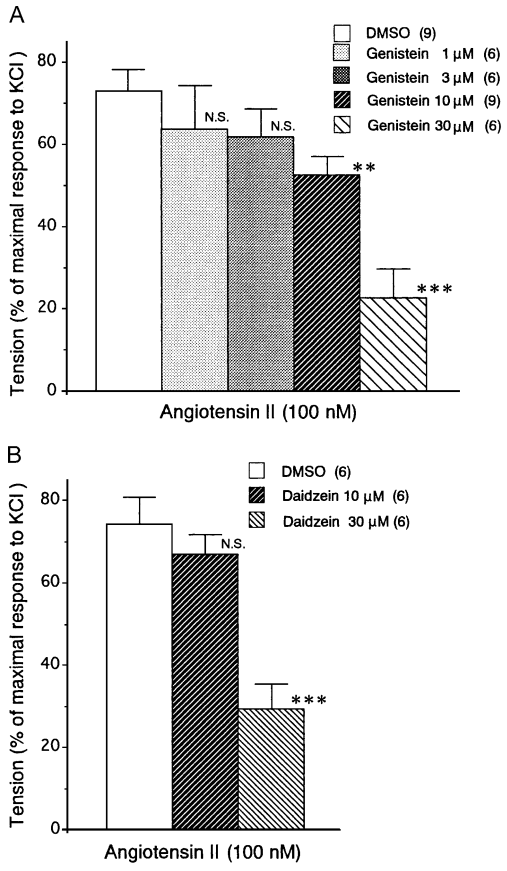

Fig. 1. Effects of (A) genistein (1–30 μM) and (B) daidzein (10–30 μM) on the contraction of endothelium-denuded rat thoracic aorta stimulated with angiotensin II (100 nM). Genistein and daidzein were added 15 min before stimulation with angiotensin II. Values are the mean ± S.E.M. The number of experiments is indicated in parentheses. $^{**}P<0.01$, $^{***}P<0.001$ vs. control. N.S.: not significant.

was due to specific effects of genistein on tyrosine kinases, effects of daidzein (an inactive analogue of genistein) on the angiotensin II-induced contraction was studied. The contractile response to angiotensin II in daidzein (10 μM)-treated aorta was not significantly different from control ($67.1 \pm 4.8\%$ of KCl_{max}, $n=6$, $P>0.05$). However, pretreatment with a higher concentration of daidzein (30 μM) significantly inhibited the angiotensin II-induced contraction ($29.2 \pm 6.3\%$ of KCl_{max}, $n=6$, $P<0.001$), suggesting the existence of nonspecific effects of genistein in higher concentrations (Fig. 1B).

Endothelin-1 caused a slow onset and sustained vasocontraction in the rat aorta. The maximal response was obtained 30 min after application of endothelin-1, which reached to $120.0 \pm 6.3\%$ of KCl_{max} ($n=6$). The developed tension was significantly reduced by pretreatment with low concentration (10 μM) of genistein ($92.5 \pm 7.3\%$, $n=6$, $P<0.01$, Fig. 2).

In order to elucidate whether tyrosine kinases modulate angiotensin II-induced contraction through voltage-dependent calcium channels, effects of genistein (10 μM) and

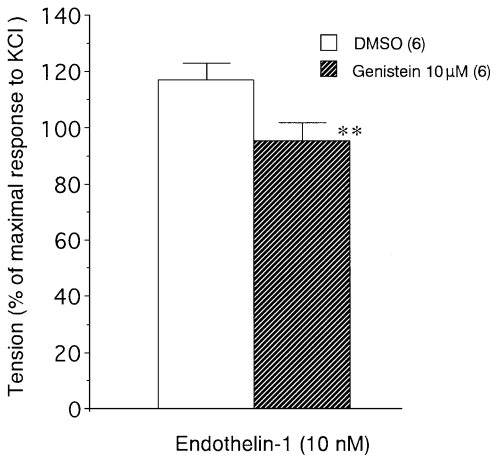

Fig. 2. Effects of genistein (10 μM) on endothelin-1 (10 nM)-induced contraction in rat aorta. Genistein was added 15 min before stimulation with endothelin-1. Values are the mean ± S.E.M. The number of experiments is indicated in parentheses. $^{**}P<0.01$ vs. control.

verapamil (1 μM) were examined. Genistein (10 μM) alone attenuated the angiotensin II-induced contraction to $50.7 \pm 4.4\%$ of KCl_{max} (Fig. 1A). Verapamil inhibited the angiotensin II-induced contraction ($35.3 \pm 2.2\%$ of KCl_{max}, $n=5$, $P<0.001$). However, when genistein and verapamil were pretreated together, the angiotensin II-induced contraction was attenuated more extensively compared with the inhibition by verapamil alone ($19.8 \pm 2.0\%$, $n=6$, $P<0.001$).

3.1.2. Effect of genistein on the high K^+-induced contraction

To investigate whether tyrosine kinase activation influences the depolarization-induced contraction in rat aorta, the effect of genistein on high K^+-induced contraction was studied. As shown in Fig. 3, genistein (10 μM) did not

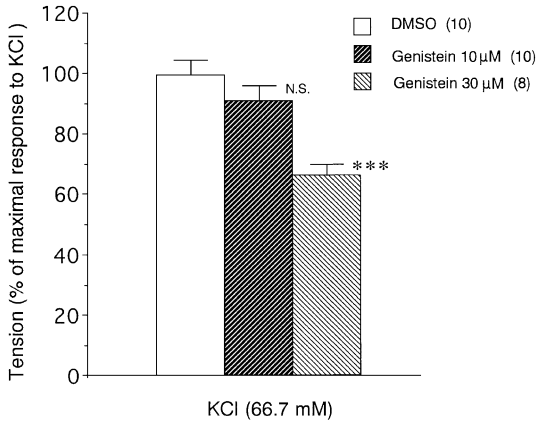

Fig. 3. Effects of genistein (10–30 μM) on KCl (66.7 mM)-induced contraction in rat aorta. Genistein was added 15 min before stimulation with KCl (66.7 mM). $^{***}P<0.001$ vs. control. N.S.: not significant.

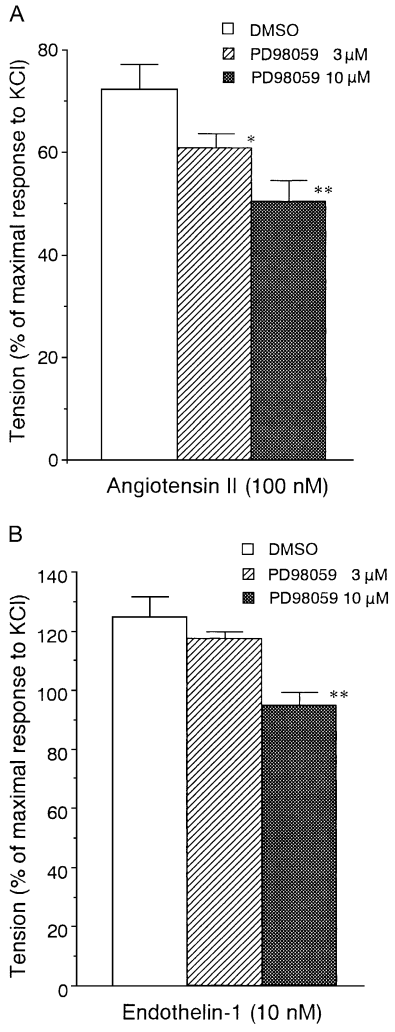

Fig. 4. (A) Effects of PD98059 (3–10 μM) on angiotensin II (100 nM)-induced contraction in the endothelium-denuded rat aorta. Values are the mean ± S.E.M. of seven independent experiments. (B) Effects of PD98059 (3–10 μM) on endothelin-1 (10 nM)-induced contraction in the endothelium-denuded rat aorta. Values are the mean ± S.E.M. of six independent experiments. *$P<0.05$, **$P<0.01$ vs. control.

significantly inhibit high K^+-induced contraction (90.0 ± 4.0%, $n=10$, $P>0.05$). However, 30 μM of genistein significantly reduced high K^+-induced contraction to 66.5 ± 3.6% of control ($n=8$, $P<0.001$). This inhibition was considered to be due to the nonspecific effect of genistein, because 30 μM of daidzein also inhibited the high K^+-induced contraction.

3.2. Effect of MEK inhibitors PD98059 and U0126 on the angiotensin II- and endothelin-1-induced contractions

In order to elucidate whether the activated p44/42 MAP kinases are involved in angiotensin II- and endothelin-1-induced contraction, effect of a MEK inhibitor PD98059 on the contraction was examined. As shown in Fig. 4A, PD98059 (3–10 μM) significantly inhibited the angiotensin II-induced contraction. In the control, the maximal contractile response was 71.9 ± 5.9% of KCl_{max}. When vessels were treated with 3 and 10 μM of PD98059, contractile responses were reduced to 62.1 ± 2.5% and 49.8 ± 4.9% of KCl_{max}, respectively ($n=7$). Fig. 4B shows the effect of PD98059 on the endothelin-1-induced contraction. The control response was 124.6 ± 7.1% of KCl_{max}, while the contractions were reduced by pretreatment with 3 and 10 μM of PD98059 to 117.5 ± 2.5% and 94.8 ± 4.6% of KCl_{max}, respectively ($n=6$).

Another MEK inhibitor, U0126 (3–10 μM), concentration-dependently inhibited the angiotensin II-induced contraction (Fig. 5A). In control, the angiotensin II-induced contraction was 68.2 ± 3.2% of KCl_{max}. When treated with 3 and 10 μM U0126, the maximal angiotensin II-induced

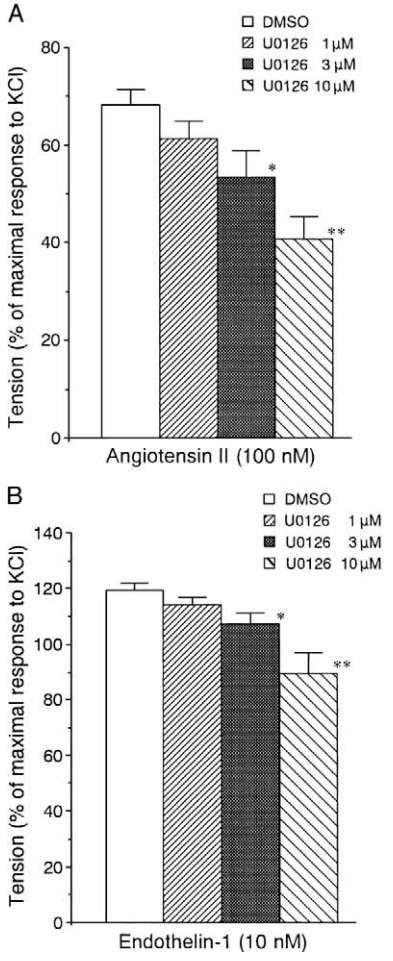

Fig. 5. (A) Effects of U0126 (1–10 μM) on angiotensin II (100 nM)- and endothelin-1 (10 nM)-induced contraction in the endothelium-denuded rat aorta. Values are the mean ± S.E.M. of 11 independent experiments. (B) Effects of U0126 (1–10 μM) on endothelin-1 (10 nM)-induced contraction in the endothelium-denuded rat aorta. Values are the mean ± S.E.M. of five independent experiments. *$P<0.05$, **$P<0.01$ vs. control.

vasocontractions were 53.6 ± 5.5% and 40.8 ± 4.7% of KCl_{max}, respectively ($n = 11$). In addition, endothelin-1-induced contraction was 119.3 ± 3.0% of KCl_{max} in the control, while contraction was inhibited by 3 μM U0126 (107.1 ± 4.3% of KCl_{max}) and 10 μM U0126 (89.5 ± 7.8% of KCl_{max}, $n = 5$) (Fig. 5B).

3.3. Effect of angiotensin II- and endothelin-1 on the phosphorylation of p44/42 MAP kinases in cultured vascular smooth muscle cells and in aortic strips (Figs. 6 and 7)

The activation of p44/42 MAP kinases by stimulation with angiotensin II and endothelin-1 was examined in cultured vascular smooth muscle cells. The activation status of p44/42 MAP kinases was determined by Western blot analysis with phospho-specific antibody to p44/42 MAP kinases. Stimulation with angiotensin II (100 nM) or endothelin-1 (10 nM) for 5 min increased the phosphorylation of p44/42 MAP kinases in cultured vascular smooth muscle cells (Fig. 6). Pretreatment with MEK inhibitors PD98059 (10 μM) as well as U0126 (10 μM) inhibited the phosphorylation of p44/42 MAP kinases induced by angiotensin II and endothelin-1.

To elucidate whether phosphorylation of p44/42 MAP kinases was contributing to the agonists-induced aortic contraction in vivo, angiotensin II- and endothelin-1-induced phosphorylation of p44/42 MAP kinases was determined in aortic strips incubated under a physiological condition in an organ bath. Stimulation with angiotensin II as well as endothelin-1 for 5 min increased the activation of p44/42 MAP kinases in rat aorta (Fig. 7). When the vessels were preincubated with PD98059 and U0126, angiotensin II- and endothelin-1-induced phosphorylation of p44/42 MAP kinases was inhibited.

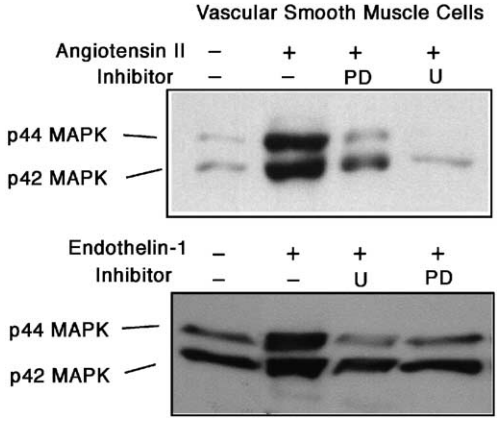

Fig. 6. Effects of angiotensin II (100 nM) and endothelin-1 (10 nM) on the phosphorylation of p44/42 MAP kinases in cultured aortic vascular smooth muscle cells. MEK inhibitors PD98059 (10 μM) as well as U0126 (10 μM) were added 15 min before stimulation with agonists. The experiment was repeated at least three times with similar results. The degree of phosphorylation of MAP kinases was determined by Western blot analysis with phospho-specific antibody against MAP kinases as described in Materials and methods. PD: PD98059, U: U0126.

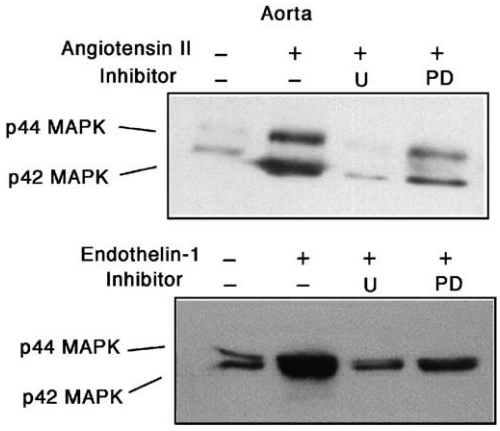

Fig. 7. Effects of angiotensin II (100 nM) and endothelin-1 (10 nM) on the phosphorylation of p44/42 MAP kinases in rat thoracic aorta incubated under a physiological condition. MEK inhibitors PD98059 (10 μM) as well as U0126 (10 μM) were added 15 min before stimulation with agonists. The experiment was repeated at least three times with similar results. The degree of phosphorylation of MAP kinases was determined by Western blot analysis with phospho-specific antibody against MAP kinases as described in Materials and methods. PD: PD98059, U: U0126.

4. Discussion

In this study, involvement of tyrosine kinase and MAP kinases in the angiotensin II- and endothelin-1-induced contraction in rat aorta was investigated. First, we examined the effect of genistein, a nonselective tyrosine kinase inhibitor, on the angiotensin II- and endothelin-1-induced contraction. As shown in Figs. 1 and 3, genistein (10 μM) inhibited the angiotensin II- and endothelin-1-induced contraction, while the high K^+-induced contraction was not significantly reduced by 10 μM genistein (Fig. 2). However, a higher concentration (30 μM) of genistein inhibited the high K^+-induced contraction. These findings are consistent with a previous study in which 10 μM of genistein did not inhibit the high K^+-induced contraction (Abebe and Agrawal, 1995). These results suggest that a tyrosine kinase-dependent pathway which does not influence the voltage-dependent Ca^{2+} channel is involved in angiotensin II- and endothelin-1-induced contraction. In contrast to our present results, Watts et al. (1998) reported previously that genistein did not inhibit the angiotensin II-induced vasocontraction in rat aorta. However, in that experiment, the concentration of genistein that they utilized (5 μM) was lower than what we used. Actually, when a lower concentration of genistein (1–3 μM) was pretreated, the contractile response to angiotensin II was not significantly different from the control response in our present study (Fig. 1A). In addition, angiotensin II was applied cumulatively to determine the concentration–response curve in the previous

study. On the other hand, angiotensin II was added in a single dose (100 nM) in this study because the contractile response of angiotensin II exhibited an apparent tachyphylaxis in rat aorta. These differences in experimental conditions may account for the discrepancy between results of other groups and ours.

It is not fully understood how tyrosine kinase activation induces vascular contraction. One possibility is an effect of tyrosine kinase on calcium sensitivity of smooth muscles. In intact artery, tyrphostin A23 inhibited endothelin-1-induced contraction, and in rat mesenteric arteries permeabilized with *Staphylococcus* α-toxin, endothelin-1 increased calcium sensitivity of contractile apparatus through tyrosine kinase activation (Ohanian et al., 1997). Alternatively, tyrosine kinase has been considered to modulate L-type Ca^{2+} channels. Angiotensin II- and endothelin-1-induced contractions are dependent on the elevated intracellular Ca^{2+} concentration via activation of L-type Ca^{2+} channels (Jackson and Garrison, 1995). Actually, in our previous study, angiotensin II- and endothelin-1-induced contractions were strongly inhibited in Ca^{2+}-free solution, and a voltage-dependent Ca^{2+} channel inhibitor verapamil inhibited the angiotensin II- and endothelin-1-induced contractions (Katano et al., 1998). These results suggest that influx of external Ca^{2+} through Ca^{2+} channels plays an important role in angiotensin II- and endothelin-1-induced contractions. It may be possible that tyrosine kinases directly activate L-type Ca^{2+} channels (Suzuki et al., 1996). In this study, however, 30 μM of genistein significantly inhibited the high K^+-induced contraction (Fig. 2), while 10 μM of genistein by which angiotensin II-induced contraction was significantly inhibited did not attenuate the high K^+-induced contraction (Figs. 1 and 2). Therefore, it is suggested that higher concentration (30 μM) of genistein inhibited not only tyrosine kinases but also L-type Ca^{2+} channels, while 10 μM of genistein selectively inhibited tyrosine kinases without modulating L-type Ca^{2+} channels. This implication was supported by the experiment with daidzein, an inactive analogue of genistein (Fig. 1B). Angiotensin II- and K^+-induced contractions were not inhibited by 10 μM of daidzein, while a higher concentration (30 μM) of daidzein strongly inhibited the contraction (Fig. 1B). These results suggest that 30 μM of genistein had nonspecific effects other than tyrosine kinase inhibition. In addition, when genistein and verapamil were pretreated together, the angiotensin II-induced contraction was attenuated more extensively than that pretreated with verapamil alone. These results suggest that activations of tyrosine kinase and L-type Ca^{2+} channel are not linked directly.

In this study, we examined the effects of MEK inhibitors on angiotensin II- and endothelin-1-induced contractions. MEK1/2 phosphorylate and activate p44/42 MAP kinases which play an important role in cell growth and proliferation (Watts, 1996). There are evidences that stimulation of several G-protein coupled receptors resulted in an activation of p44/42 MAP kinases in cultured cells. For example, histamine H_1 receptor activation time- and concentration-dependently stimulated MAP kinases through a pertussis toxin-sensitive way in DDT_1MF-2 cells. The activation was blocked by kinase inhibitors including PD98059 and genistein (Robinson and Dickenson, 2001). In rat aortic vascular smooth muscle cells, angiotensin II-activated MAP kinases are reported to be involved in cell proliferation (Duff et al., 1992; Molloy et al., 1993; Schmitz et al., 1998). Furthermore, consistent with our present results that endothelin-1 could induce p44/42 MAP kinase activation in rat aortic vascular smooth muscle (Fig. 6), stimulation of endothelin receptors activated MAP kinases in intact canine pulmonary artery smooth muscle (Yamboliev et al., 1998) and in cultured porcine aortic vascular smooth muscle cells (Chevalier et al., 2000). In addition to the phosphorylation in cultured vascular smooth muscle cells, we revealed that these agonists could stimulate p44/42 MAP kinase activation in intact vessels under a physiological condition (Fig. 7). Recently, it has been reported that 5-HT-induced contraction was dependent on tyrosyl phosphorylation of MAP kinase in rat aorta and tail artery (Florian and Watts, 1998). Indeed, we also observed previously that the aortic contraction induced by 5-HT was attenuated by pretreatment with a MEK inhibitor (unpublished data). Furthermore, tyrphostin A23 and PD98059 inhibited the angiotensin II-induced formation of second messengers and vasocontraction in human small arteries (Touyz et al., 1999). However, another study in which PD98059 was used as a MEK inhibitor showed the dissociation of the angiotensin II-induced phosphorylation of MAP kinase from aortic contraction (Watts et al., 1998). In addition, PD98059 did not reduce histamine and phorbol 12, 13-dibutyrate-elicited contraction in intact swine carotid artery (Gorenne et al., 1998). In our present study, a possible role of MAP kinase in vascular contraction was investigated by using another newer MEK inhibitor, U0126 (Duncia et al., 1998; Favata et al., 1998) in addition to PD98059 (Pang et al., 1995). U0126 is demonstrated to inhibit both MEK1 and MEK2 in a specific manner with IC_{50} of 60−70 nM (Duncia et al., 1998). Because it has been reported that PD98059 and genistein could inhibit L-type Ca^{2+} channels (Wijetunge et al., 1992; Ekinci et al., 1999), it is important to make sure that the inhibitory effect of PD98059 was mediated through its direct action on MEK, especially when it was used in a high concentration. In this study, the high K^+-induced contraction was not attenuated by low concentration of PD98059 (10 μM), while both the angiotensin II- and endothelin-1-induced contractions were significantly inhibited by 10 μM of PD98059 (Fig. 4). The angiotensin II- and endothelin-1-induced contractions were also inhibited by pretreatment with U0126 (Fig. 5). As shown in Fig. 7, stimulation with angiotensin II as well as endothelin-1 increased the phosphorylation of p44/42 MAP kinases in intact aorta. The aortic phosphorylation of p44/42 MAP kinases was inhibited by pretreatment with PD98059 or U0126. These results suggest that activated MAP kinase

may be involved in the vascular contraction evoked by angiotensin II and endothelin-1.

The downstream signaling of MAP kinase activation to vasocontraction is not clear. One possible substrate in smooth muscle is a thin-filament binding protein caldesmon, which phosphorylation is suggested to be regulated by MAP kinase (Katoch and Moreland, 1995; Pohl et al., 1997; Dessy et al., 1998). Caldesmon blocked the binding of myosin to actin and inhibited actin-dependent myosin ATPase activity, while the phosphorylated caldesmon reversed the inhibition to increase muscle tension (Zhang et al., 1994). However, the role of MAP kinase–caldesmon pathway in vascular smooth muscle contraction is controversial. For example, there are evidences that activated MAP kinase does not lead to disinhibition of actin–myosin-activated ATPase activity in vitro (Krymsky et al., 1999). In addition, phosphorylation of caldesmon neither induced contraction nor affected the Ca^{2+}-sensitivity in permeabilized smooth muscle (Nixon et al., 1995). Alternatively, Na^+/H^+ exchanger may be another target of MAP kinase for vascular contraction. Stimulation of Na^+/H^+-exchanger-1 by angiotensin II is reported to be an early event that is required for vascular contraction as well as smooth muscle cell growth (Berk et al., 1988; Berk and Corson, 1997). Because MAP kinase can regulate the p90 ribosomal S6 kinase activity, and p90 ribosomal S6 kinase phosphorylates Na^+/H^+-exchanger-1 (Takahashi et al., 1997), it appears likely that MAP kinases are important in regulating angiotensin II-mediated Na^+/H^+-exchanger-1 activation (Wang et al., 1997; Kusuhara et al., 1998). The exact role of caldesmon as well as Na^+/H^+-exchanger-1 in vascular smooth muscle contraction still needs to be determined.

The agonists-induced contraction may be affected by genistein and MEK inhibitors through influencing the phosphorylation level of smooth muscle surface receptors. Indeed, it is reported that surface receptors are phosphorylated by activation of tyrosine kinase and MAP kinases to modulate the receptor signaling. For example, angiotensin II caused phosphorylation of AT_1 receptors via MAP kinases in neuronal cultures of rat brains, and PD98059 attenuated the angiotensin II-induced AT_1 receptor phosphorylation (Yang et al., 1997). In HEK293 cells, PD98059 not only prevented MAP kinase activation but also inhibited agonist-promoted μ-opioid receptor phosphorylation (Schmidt et al., 2000). Although receptor phosphorylation may be involved in the process of the uncoupling of receptors to effectors or desensitization in these cases, it is possible that modulation of receptor phosphorylation by genistein and MEK inhibitors may lead to the attenuation of angiotensin II- and endothelin-1-induced aortic contraction.

In conclusion, in this study, we examined whether tyrosine kinases and MAP kinases are involved in the development of force of contraction by angiotensin II and endothelin-1 in rat intact aorta. A tyrosine kinase inhibitor genistein as well as MEK inhibitors PD98059 and U0126 significantly attenuated the angiotensin II- and endothelin-1-induced contraction. Angiotensin II and endothelin-1-induced phosphorylation of MAP kinases in the intact aorta was inhibited by PD98059 and U0126. It is suggested that tyrosine kinases and MAP kinases play, at least in part, a physiological role in the angiotensin II- and endothelin-1-induced contraction in rat aorta.

Acknowledgements

This study was supported by the Grant-in-Aid for Scientific Research (C) (No. 11672254 to A. Ishihata) and the Grant-in-Aid for Scientific Research (C) (No. 11670658 to Y. Katano) from the Ministry of Education, Science, Sports and Culture, Japan.

References

Abebe, W., Agrawal, D.K., 1995. Role of tyrosine kinases in norepinephrine-induced contraction of vascular smooth muscle. J. Cardiovasc. Pharmacol. 26, 153–159.

Berk, B.C., Corson, M.A., 1997. Angiotensin II signal transduction in vascular smooth muscle: role of tyrosine kinases. Circ. Res. 80, 607–616.

Berk, B.C., Canessa, M., Vallega, G., Alexander, R.W., 1988. Agonist-mediated changes in intracellular pH: role in vascular smooth muscle cell function. J. Cardiovasc. Pharmacol. 12 (Suppl. 5), S104–S114.

Berridge, M.J., 1993. Inositol trisphosphate and calcium signalling. Nature 361, 315–325.

Bobik, A., Grooms, A., Millar, J.A., Mitchell, A., Grinpukel, S., 1990. Growth factor activity of endothelin on vascular smooth muscle. Am. J. Physiol. 258, C408–C415.

Chevalier, D., Thorin, E., Allen, B.G., 2000. Simultaneous measurement of ERK, p38, and JNK MAP kinase cascades in vascular smooth muscle cells. J. Pharmacol. Toxicol. Methods 44, 429–439.

Dessy, C., Kim, I., Sougnez, C.L., Laporte, R., Morgan, K.G., 1998. A role for MAP kinase in differentiated smooth muscle contraction evoked by alpha-adrenoceptor stimulation. Am. J. Physiol. 275, C1081–C1086.

DiSalvo, J., Steusloff, A., Semenchuk, L., Satoh, S., Kolquist, K., Pfitzer, G., 1993. Tyrosine kinase inhibitors suppress agonist-induced contraction in smooth muscle. Biochem. Biophys. Res. Commun. 190, 968–974.

Duff, J.L., Berk, B.C., Corson, M.A., 1992. Angiotensin II stimulates the pp44 and pp42 mitogen-activated protein kinases in cultured rat aortic smooth muscle cells. Biochem. Biophys. Res. Commun. 188, 257–264.

Duncia, J.V., Santella, J.B., Higley, C.A., Pitts, W.J., Wityak, J., Frietze, W.E., Rankin, F.W., Sun, J.H., Earl, R.A., Tabaka, A.C., Teleha, C.A., Blom, K.F., Favata, M.F., Manos, E.J., Daulerio, A.J., Stradley, D.A., Horiuchi, K., Copeland, R.A., Scherle, P.A., Trzaskos, J.M., Magolda, R.L., Trainor, G.L., Wexler, R.R., Hobbs, F.W., Olson, R.E., 1998. MEK inhibitors: the chemistry and biological activity of U0126, its analogs, and cyclization products. Bioorganic Med. Chem. Lett. 8, 2839–2844.

Ekinci, F.J., Malik, K.U., Shea, T.B., 1999. Activation of the L voltage-sensitive calcium channel by mitogen-activated protein (MAP) kinase following exposure of neuronal cells to beta-amyloid. MAP kinase mediates beta-amyloid-induced neurodegeneration. J. Biol. Chem. 274, 30322–30327.

Favata, M.F., Horiuchi, K.Y., Manos, E.J., Daulerio, A.J., Stradley, D.A., Feeser, W.S., Van, D.D., Pitts, W.J., Earl, R.A., Hobbs, F., Copeland, R.A., Magolda, R.L., Scherle, P.A., Trzaskos, J.M., 1998. Identifica-

tion of a novel inhibitor of mitogen-activated protein kinase kinase. J. Biol. Chem. 273, 18623–18632.
Filipeanu, C.M., Brailoiu, E., Huhurez, G., Slatineanu, S., Baltatu, O., Branisteanu, D.D., 1995. Multiple effects of tyrosine kinase inhibitors on vascular smooth muscle contraction. Eur. J. Pharmacol. 281, 29–35.
Florian, J.A., Watts, S.W., 1998. Integration of mitogen-activated protein kinase kinase activation in vascular 5-hydroxytryptamine 2A receptor signal transduction. J. Pharmacol. Exp. Ther. 284, 346–355.
Force, T., Bonventre, J.V., 1998. Growth factors and mitogen-activated protein kinases. Hypertension 31, 152–161.
Force, T., Kyriakis, J.M., Avruch, J., Bonventre, J.V., 1991. Endothelin, vasopressin, and angiotensin II enhance tyrosine phosphorylation by protein kinase C-dependent and -independent pathways in glomerular mesangial cells. J. Biol. Chem. 266, 6650–6656.
Giasson, E., Meloche, S., 1995. Role of p70 S6 protein kinase in angiotensin II-induced protein synthesis in vascular smooth muscle cells. J. Biol. Chem. 270, 5225–5231.
Gorenne, I., Su, X., Moreland, R.S., 1998. Inhibition of p42 and p44 MAP kinase does not alter smooth muscle contraction in swine carotid artery. Am. J. Physiol. 275, H131–H138.
Gould, E.M., Rembold, C.M., Murphy, R.A., 1995. Genistein, a tyrosine kinase inhibitor, reduces Ca^{2+} mobilization in swine carotid media. Am. J. Physiol. 268, C1425–C1429.
Hollenberg, M.D., 1994. Tyrosine kinase pathways and the regulation of smooth muscle contractility. Trends Pharmacol. Sci. 15, 108–114.
Ishihata, A., Endoh, M., 1993. Pharmacological characteristics of the positive inotropic effect of angiotensin II in the rabbit ventricular myocardium. Br. J. Pharmacol. 108, 999–1005.
Ishihata, A., Endoh, M., 1995. Species-related differences in inotropic effects of angiotensin II in mammalian ventricular muscle: receptors, subtypes and phosphoinositide hydrolysis. Br. J. Pharmacol. 114, 447–453.
Ishihata, A., Uno, S., Guo, D.F., Katano, Y., Inagami, T., 1998. Inhibition of the expression of the gene for the angiotensin AT_1 receptor by angiotensin II in the rat adrenal gland. Eur. J. Pharmacol. 350, 129–139.
Jackson, E.K., Garrison, J.C., 1995. Renin and Angiotensin, 9th ed. McGraw-Hill, New York, NY.
Jin, N., Siddiqui, R.A., English, D., Rhoades, R.A., 1996. Communication between tyrosine kinase pathway and myosin light chain kinase pathway in smooth muscle. Am. J. Physiol. 271, H1348–H1355.
Katano, Y., Tasaki, K., Ishihata, A., 1998. Role of calcium and cyclooxygenase products in age-related changes in response to angiotensin II. Naunyn-Schmiedeberg's Arch. Pharmacol. 358 (Suppl. 1), R247.
Katoch, S.S., Moreland, R.S., 1995. Agonist and membrane depolarization induced activation of MAP kinase in the swine carotid artery. Am. J. Physiol. 269, H222–H229.
Koide, M., Kawahara, Y., Tsuda, T., Ishida, Y., Shii, K., Yokoyama, M., 1992. Endothelin-1 stimulates tyrosine phosphorylation and the activities of two mitogen-activated protein kinases in cultured vascular smooth muscle cells. J. Hypertens. 10, 1173–1182.
Krymsky, M.A., Chibalina, M.V., Shirinsky, V.P., Marston, S.B., Vorotnikov, A.V., 1999. Evidence against the regulation of caldesmon inhibitory activity by p42/p44 Erk mitogen-activated protein kinase in vitro and demonstration of another caldesmon kinase in intact gizzard smooth muscle. FEBS Lett. 452, 254–258.
Kusuhara, M., Takahashi, E., Peterson, T.E., Abe, J., Ishida, M., Han, J., Ulevitch, R., Berk, B.C., 1998. p38 Kinase is a negative regulator of angiotensin II signal transduction in vascular smooth muscle cells: effects on Na^+/H^+ exchange and ERK1/2. Circ. Res. 83, 824–831.
Liu, C.Y., Sturek, M., 1996. Attenuation of endothelin-1-induced calcium response by tyrosine kinase inhibitors in vascular smooth muscle cells. Am. J. Physiol. 270, C1825–C1833.
Molloy, C.J., Taylor, D.S., Weber, H., 1993. Angiotensin II stimulation of rapid protein tyrosine phosphorylation and protein kinase activation in rat aortic smooth muscle cells. J. Biol. Chem. 268, 7338–7345.
Nishizuka, Y., 1984. Turnover of inositol phospholipids and signal transduction. Science 225, 1365–1370.
Nixon, G.F., Iizuka, K., Haystead, C.M., Haystead, T.A., Somlyo, A.P., Somlyo, A.V., 1995. Phosphorylation of caldesmon by mitogen-activated protein kinase with no effect on Ca^{2+} sensitivity in rabbit smooth muscle. J. Physiol. 487, 283–289.
Ohanian, J., Ohanian, V., Shaw, L., Bruce, C., Heagerty, A.M., 1997. Involvement of tyrosine phosphorylation in endothelin-1-induced calcium-sensitization in rat small mesenteric arteries. Br. J. Pharmacol. 120, 653–661.
Pang, L., Sawada, T., Decker, S.J., Saltiel, A.R., 1995. Inhibition of MAP kinase kinase blocks the differentiation of PC-12 cells induced by nerve growth factor. J. Biol. Chem. 270, 13585–13588.
Pohl, J., Winder, S.J., Allen, B.G., Walsh, M.P., Sellers, J.R., Gerthoffer, W.T., 1997. Phosphorylation of calponin in airway smooth muscle. Am. J. Physiol. 272, L115–L123.
Robinson, A.J., Dickenson, J.M., 2001. Regulation of p42/p44 MAPK and p38 MAPK by the adenosine A_1 receptor in DDT_1MF-2 cells. Eur. J. Pharmacol. 413, 151–161.
Sadoshima, J., Qiu, Z., Morgan, J.P., Izumo, S., 1995. Angiotensin II and other hypertrophic stimuli mediated by G protein-coupled receptors activate tyrosine kinase, mitogen-activated protein kinase, and 90-kD S6 kinase in cardiac myocytes. The critical role of Ca^{2+}-dependent signaling. Circ. Res. 76, 1–15.
Schmidt, H., Schulz, S., Klutzny, M., Koch, T., Handel, M., Hollt, V., 2000. Involvement of mitogen-activated protein kinase in agonist-induced phosphorylation of the μ-opioid receptor in HEK 293 cells. J. Neurochem. 74, 414–422.
Schmitz, U., Ishida, T., Ishida, M., Surapisitchat, J., Hasham, M.I., Pelech, S., Berk, B.C., 1998. Angiotensin II stimulates p21-activated kinase in vascular smooth muscle cells: role in activation of JNK. Circ. Res. 82, 1272–1278.
Suzuki, A., Shinoda, J., Oiso, Y., Kozawa, O., 1996. Tyrosine kinase is involved in angiotensin II-stimulated phospholipase D activation in aortic smooth muscle cells: function of Ca^{2+} influx. Atherosclerosis 121, 119–127.
Takahashi, E., Abe, J., Berk, B.C., 1997. Angiotensin II stimulates p90rsk in vascular smooth muscle cells. A potential Na^+-H^+ exchanger kinase. Circ. Res. 81, 268–273.
Thomas, W.G., Thekkumkara, T.J., Baker, K.M., 1996. Cardiac effects of AII. AT_{1A} receptor signaling, desensitization, and internalization. Adv. Exp. Med. Biol. 396, 59–69.
Touyz, R.M., Schiffrin, E.L., 1996. Tyrosine kinase signaling pathways modulate angiotensin II-induced calcium ($[Ca^{2+}]_i$) transients in vascular smooth muscle cells. Hypertension 27, 1097–1103.
Touyz, R.M., He, G., Deng, L.Y., Schiffrin, E.L., 1999. Role of extracellular signal-regulated kinases in angiotensin II-stimulated contraction of smooth muscle cells from human resistance arteries. Circulation 99, 392–399.
Turner, C.E., Pietras, K.M., Taylor, D.S., Molloy, C.J., 1995. Angiotensin II stimulation of rapid paxillin tyrosine phosphorylation correlates with the formation of focal adhesions in rat aortic smooth muscle cells. J. Cell Sci. 108, 333–342.
Wang, H., Silva, N.L., Lucchesi, P.A., Haworth, R., Wang, K., Michalak, M., Pelech, S., Fliegel, L., 1997. Phosphorylation and regulation of the Na^+/H^+ exchanger through mitogen-activated protein kinase. Biochemistry 36, 9151–9158.
Watts, S.W., 1996. Serotonin activates the mitogen-activated protein kinase pathway in vascular smooth muscle: use of the mitogen-activated protein kinase kinase inhibitor PD098059. J. Pharmacol. Exp. Ther. 279, 1541–1550.
Watts, S.W., 1998. Activation of the mitogen-activated protein kinase pathway via the $5\text{-}HT_{2A}$ receptor. Ann. N.Y. Acad. Sci. 861, 162–168.
Watts, S.W., Florian, J.A., Monroe, K.M., 1998. Dissociation of angiotensin II-stimulated activation of mitogen-activated protein kinase kinase from vascular contraction. J. Pharmacol. Exp. Ther. 286, 1431–1438.
Wijetunge, S., Aalkjaer, C., Schachter, M., Hughes, A.D., 1992. Tyrosine kinase inhibitors block calcium channel currents in vascular smooth muscle cells. Biochem. Biophys. Res. Commun. 189, 1620–1623.

Yamboliev, I.A., Hruby, A., Gerthoffer, W.T., 1998. Endothelin-1 activates MAP kinases and c-Jun in pulmonary artery smooth muscle. Pulm. Pharmacol. Ther. 11, 205–208.

Yanagisawa, M., Kurihara, H., Kimura, S., Tomobe, Y., Kobayashi, M., Mitsui, Y., Yazaki, Y., Goto, K., Masaki, T., 1988. A novel potent vasoconstrictor peptide produced by vascular endothelial cells. Nature 332, 411–415.

Yang, H., Lu, D., Raizada, M.K., 1997. Angiotensin II-induced phosphorylation of the AT_1 receptor from rat brain neurons. Hypertension 30, 351–357.

Yang, Z., Altura, B.T., Altura, B.M., 1999. Low extracellular Mg^{2+} contraction of arterial muscle: role of protein kinase C and protein tyrosine phosphorylation. Eur. J. Pharmacol. 378, 273–281.

Yang, Z., Wang, J., Altura, B.T., Altura, B.M., 2000. Extracellular magnesium deficiency induces contraction of arterial muscle: role of PI3-kinases and MAPK signaling pathways. Pflügers Arch. Eur. J. Physiol. 439, 240–247.

Yousufzai, S.Y., Gao, G., Abdel, L.A., 2000. Mitogen-activated protein kinase inhibitors suppress prostaglandin $F_{2\alpha}$-induced myosin light chain phosphorylation and contraction in iris sphincter smooth muscle. Eur. J. Pharmacol. 407, 17–26.

Zhang, Y., Moreland, S., Moreland, R.S., 1994. Regulation of vascular smooth muscle contraction: myosin light chain phosphorylation dependent and independent pathways. Can. J. Physiol. Pharm. 72, 1386–1391.

Zubkov, A.Y., Rollins, K.S., Parent, A.D., Zhang, J., Bryan, R.J., 2000. Mechanism of endothelin-1-induced contraction in rabbit basilar artery. Stroke 31, 526–533.