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Mitogen-activated protein kinase activity regulation role of angiotensin and endothelin systems in vascular smooth muscle cells

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Abstract

To examine whether angiotensin II and endothelins produced in vascular smooth muscle cells can play roles in the regulation of mitogen-activated protein (MAP) kinase activity in vascular smooth muscle cells, we measured the activity of MAP kinases in cultured vascular smooth muscle cells, and determined effects of renin–angiotensin and endothelin systems activators and inhibitors. Angiotensin II and endothelin-1 produced an activation of MAP kinase activity in vascular smooth muscle cells, whereas the angiotensin receptor antagonist, losartan and the endothelin receptor antagonist, cyclo (D-α-aspartyl-L-prolyl-D-valyl-L-leucyl-D-tryptophyl, BQ123) inhibited the enzyme activity. MAP kinase activity in vascular smooth muscle cells was also inhibited either by the renin inhibitor pepstatin A or by the angiotensin-converting enzyme inhibitor captopril. The degree of the inhibition of MAP kinase activity by pepstatin A, captopril and losartan was almost the same. Renin produced a considerable increase in MAP kinase activity and the renin-induced MAP kinase activation was inhibited by pepstatin A. The endothelin precursor big endothelin-1 produced an increase of MAP kinase activity in vascular smooth muscle cells, whereas the endothelin-converting enzyme inhibitor phosphoramidon inhibited the enzyme activity. These findings suggest that functional renin–angiotensin system and endothelin system are present in vascular smooth muscle cells and these systems tonically serve to increase MAP kinase activity. It appears that renin or renin-like substances play the determining role in the regulation of renin–angiotensin system in vascular smooth muscle cells.

Keywords: Renin; Angiotensin; Endothelin-1; MAP (mitogen-activated protein kinase); Smooth muscle cells

1. Introduction

Vascular hypertrophy is a fundamental pathogenic factor for both hypertension and atherosclerosis (Kubo, 1978; Folkow et al., 1982; Owens and Schwartz, 1982; Bader et al., 1994). Endothelial and vascular smooth muscle cell integrity appears to be a crucial factor for maintenance of the structural property of the vascular wall. Specifically vascular smooth muscle cell growth is an important event in the development of vascular hypertrophy. Vasoactive substances like angiotensin II and endothelins are suggested to be related with such pathophysiological responses (Dzau and Gibbons, 1988; Powell et al., 1989; Douglas et al., 1994).

Mitogen-activated protein (MAP) kinases, serin/ethreonin-specific protein kinases (Kosako et al., 1992), are a family of ubiquitously expressed enzymes that are highly conserved and play a central role in the signaling events leading to growth responses in a wide variety of cell types (Sturgill et al., 1988; Alvarez et al., 1991; Pulverer et al., 1991). Angiotensin II and endothelins stimulate MAP kinases, leading to stimulation of protein synthesis (Duff et al., 1992; Koide et al., 1992; Tsuda et al., 1992; Molloy et al., 1993). In previous studies (Kubo et al., 1998, 1999), we demonstrated that, in rat aorta strips, endothelium removal resulted in activation of MAP kinase activity. The MAP kinase activation was found in the media portion but not in the adventitia portion. MAP kinase activation was inhibited by either the angiotensin receptor antagonist, losartan, or by the endothelin receptor antagonist, cyclo (D-α-aspartyl-L-prolyl-D-valyl-L-leucyl-D-tryptophyl, BQ123). Although these findings suggest that endogenous angiotensin II and endothelins are tonically released to cause MAP kinase-stimulating effects in the medial portion of rat aorta strips, it remains to be settled how endogenous angiotensin II and endothelins are produced and released in the media portion of the vasculature.

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The media portion of blood vessels mainly consists of vascular smooth muscle cells. It has been reported that vascular smooth muscle cells express angiotensinogen mRNA (Campbell and Habener, 1986; Cassis et al., 1988; Naftilan et al., 1991), angiotensin-converting enzyme protein (Pipili-Synetos et al., 1990) and its mRNA (Fishel et al., 1995). Vascular smooth muscle cells also have been reported to express endothelin-1 mRNA and to release endothelins (Sung et al., 1994). Thus, it could be considered that angiotensin II and endothelins produced and released in vascular smooth muscle cells regulate MAP kinase activity in the media portion of the vasculature. In this study, using cultured vascular smooth muscle cells, we examined whether angiotensin II and endothelins were produced in vascular smooth muscle cells and whether both peptides released could play roles in the regulation of MAP kinase activity in vascular smooth muscle cells.

2. Materials and methods

2.1. Vascular smooth muscle cell preparation

Male Wistar rats weighing 200–250 g were used in this study. The rats were killed by overdoses of ether, and the thoracic aorta was removed. The thoracic aorta was incubated at 4°C in Tyrode solution. Connective tissues were gently cleaned under a dissecting microscope using sterile conditions according to the method of Ross (1971). The endothelium was removed by gently rubbing the intimal surface with a fine forceps. Next, the adventitia was carefully removed under a dissecting microscope to obtain media portion. The media portion was washed twice and cut into strips. Thereafter, the vessels were placed on polyethylene dishes (diameter 60 mm). Dulbecco's modified Eagle's medium (DMEM) containing 20% fetal calf

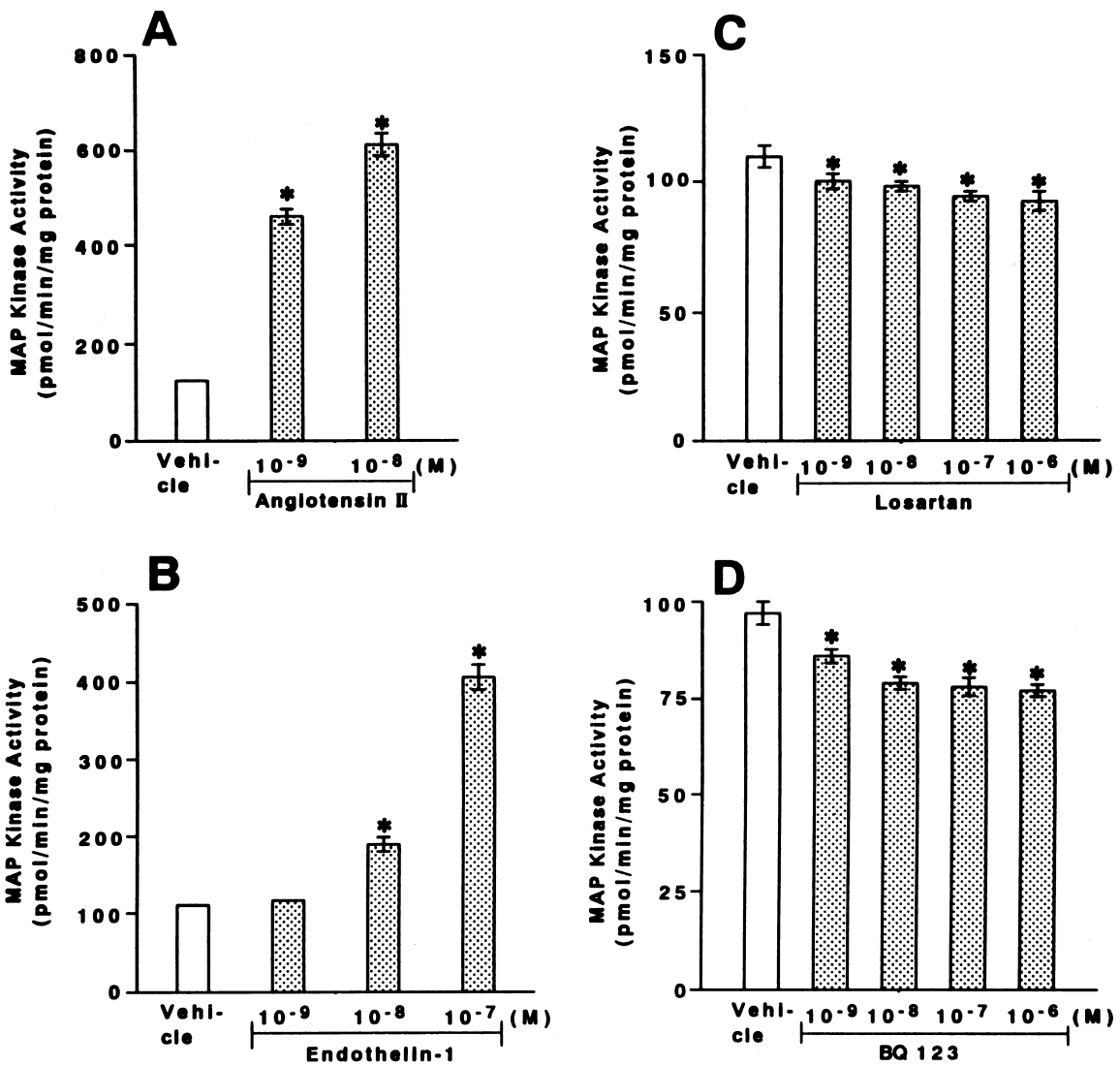

Fig. 1. Effects of angiotensin II (A), endothelin-1 (B), losartan (C) and BQ123 (D) on MAP kinase activity in vascular smooth muscle cells. Drugs were added into DMEM and a 5-min incubation (A and B) or a 30-min incubation (C and D) was started. Values are mean ± S.E.M. from four experiments. * $P < 0.05$, compared with vehicle.

serum, 100 U/ml penicillin and 100 μg/ml streptomycin was added to the dishes to cover the aortae without disturbing the explants. The dishes were kept at 37°C in a humidified atmosphere of 95% air and 5% CO_2. The cells, which grew from the explants, reached confluence after about 14 days and were harvested by brief exposure to

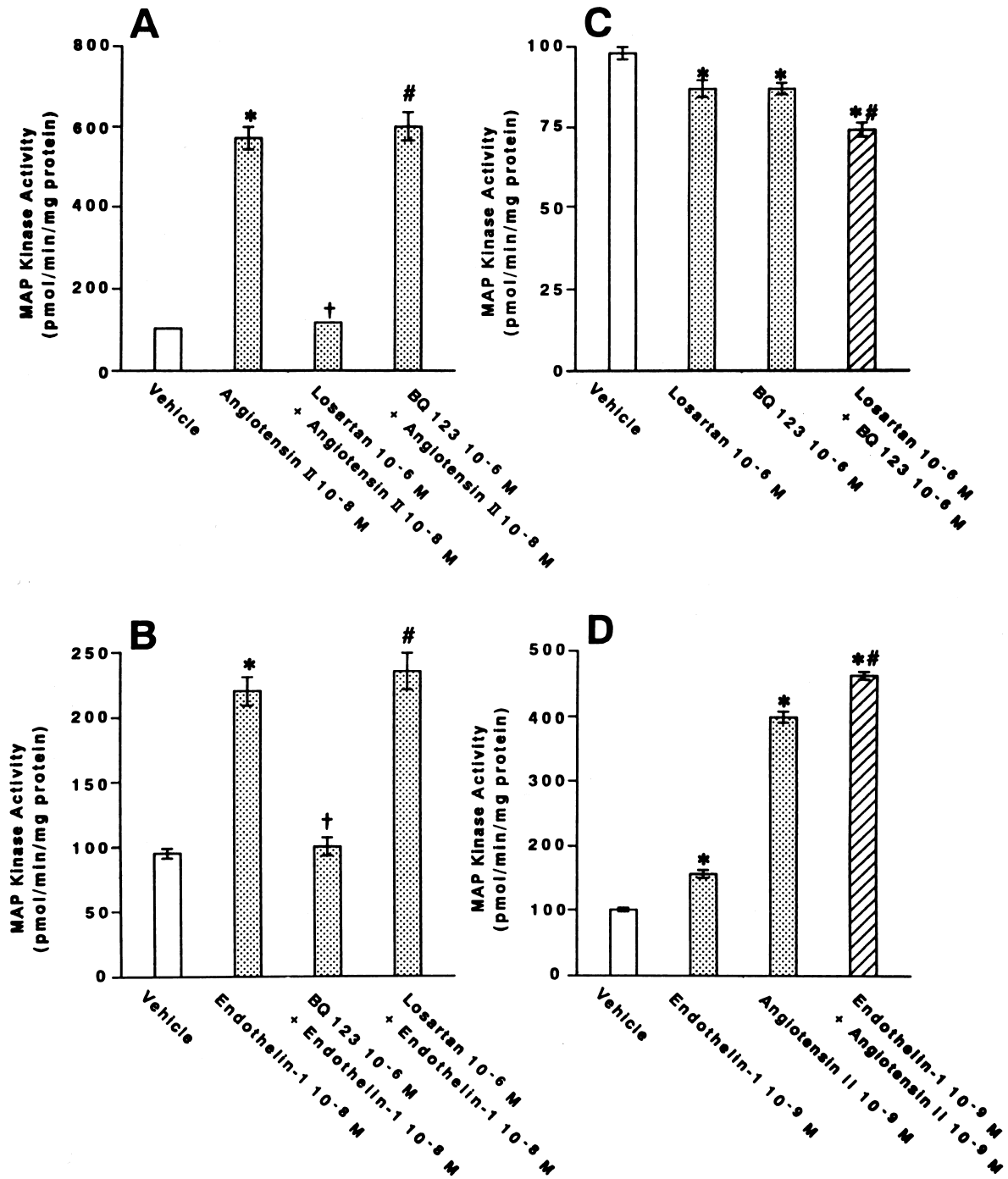

Fig. 2. (A, B) Effects of losartan and BQ123 on angiotensin II (A) induced and endothelin-1 (B) induced increase in MAP kinase activity in vascular smooth muscle cells. Angiotensin II and endothelin-1 were added into DMEM and a 5-min incubation was started. Losartan and BQ123 were added into DMEM 5 min before the 5-min incubation. Values are mean ± S.E.M. from four experiments. $^{*}P < 0.05$, compared with vehicle. $+P < 0.05$, compared with angiotensin II or endothelin-1 alone. $\#P > 0.05$, compared with angiotensin II or endothelin-1 alone. (C, D) Effects of the combination of losartan and BQ123 (C) or endothelin-1 and angiotensin II (D) on MAP kinase activity in vascular smooth muscle cells. Angiotensin II and endothelin-1 were added into DMEM and a 5-min incubation was started. Losartan and BQ123 were added into DMEM and a 30-min incubation was started. Values are mean ± S.E.M. from four experiments. $^{*}P < 0.05$, compared with vehicle. $\#P < 0.05$, compared with losartan or BQ123 alone (C) and with angiotensin II or endothelin-1 alone (D).

Hanks' medium supplemented with 0.25% trypsin and 0.02% ethylenediaminetetraacetic acid (EDTA)-sodium salt and transferred to fresh dishes. The properties of the cells cultured showed a typical hills-and-valleys growth pattern and α-actin molecule upon immunohistochemical analysis using mouse anti-α-actin (smooth muscle) (Zymed Laboratories). These characteristics indicated that the cells were grown from vascular smooth muscle cells.

2.2. Determination of MAP kinase activity in quiescent confluent vascular smooth muscle cells

Cells of passage 3 were used. They were plated in 60 mm culture dishes (1×10^5 cells per dish) containing DMEM with 10% fetal calf serum. After 7 days, the cells were rendered quiescent by a 48-h serum deprivation period. The serum-free medium was replaced by 1 ml fresh

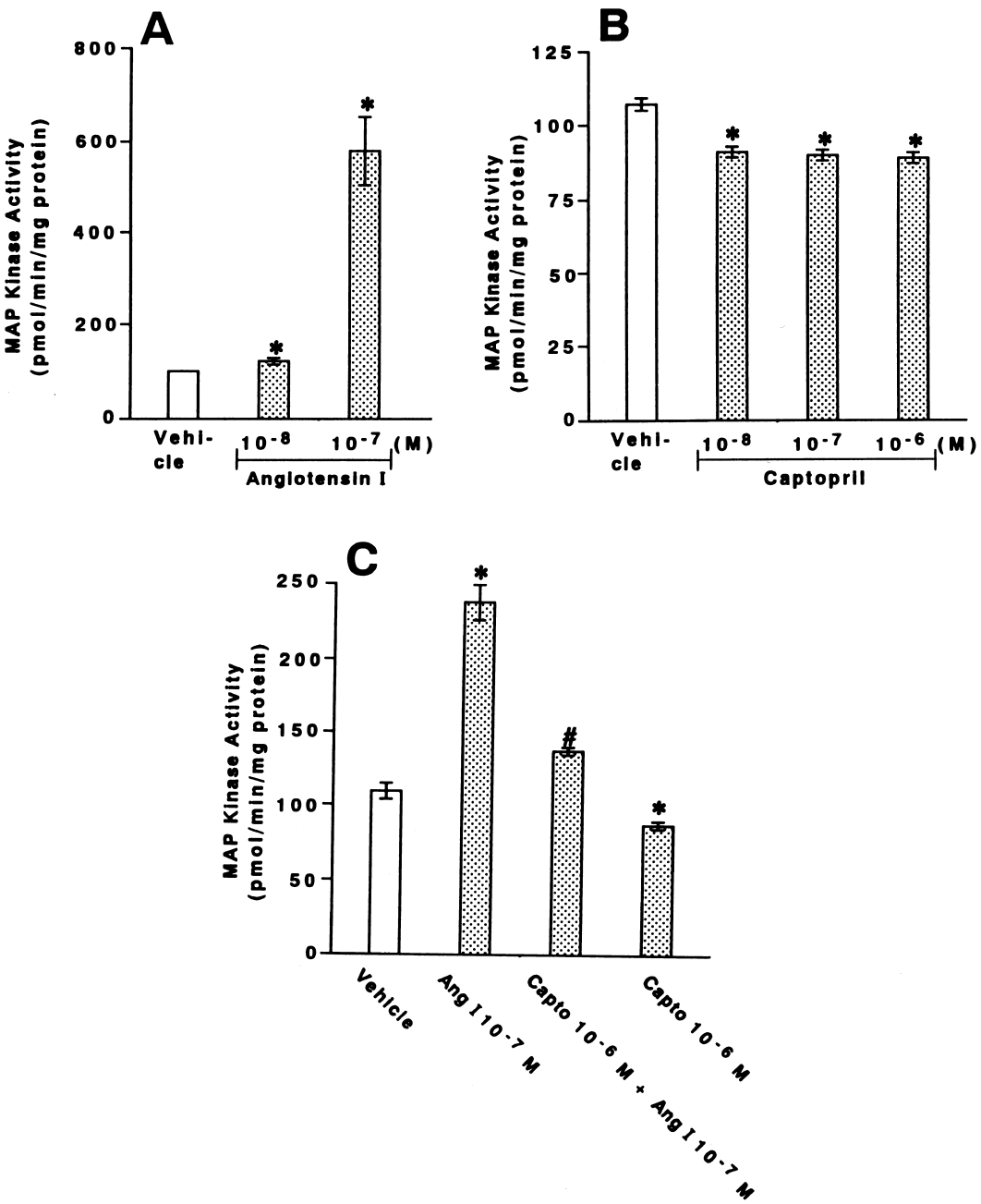

Fig. 3. (A, B) Effects of angiotensin I (A) and captopril (B) on MAP kinase activity in vascular smooth muscle cells. Drugs were added into DMEM and a 5-min incubation (A) or a 30-min incubation (B) was started. (C) Effects of captopril on angiotensin I-induced increase in MAP kinase activity in vascular smooth muscle cells. Angiotensin I (Ang I) was added into DMEM and a 5-min incubation was started. Captopril (Capto) was added into DMEM 5 min before the 5-min incubation. Values are mean ± S.E.M. from four experiments. $^{*}P < 0.05$, compared with vehicle. $\#P < 0.05$, compared with Ang I alone.

serum-free DMEM, and after 3 h, drugs were added and a 5-min incubation or a 30-min incubation was started. Drugs were dissolved in physiological saline (0.9% NaCl) and added into DMEM in a volume of 10 μl. In case of pepstatin A, it was dissolved in dimethylsulfoxide (DMSO) and added into DMEM in a volume of 1 μl. In preliminary experiments, MAP kinase activity in vascular smooth muscle cells was increased to a maximal level at 5 min after addition of angiotensin II (10^{-8} M) or endothelin-1 (10^{-8} M) into DMEM, but the extent of the increase in MAP kinase activity was decreased 10 min after the addition. Thus, we measured MAP kinase activity 5 min after addition of angiotensin II or endothelin-1. The reaction was terminated by chilling the plates on ice and washing twice with ice-cold phosphate-buffered saline.

The cells were lysed and homogenized in 0.3 ml ice-cold buffer, consisting of 10 mM Tris, 150 mM NaCl, 2 mM EGTA, 2 mM dithiothreitol, 1 mM orthovanadate, 1 mM (*p*-amidinophenyl)methanesulphonyl fluoride, 10 mg/ml leupeptin and 10 mg/ml aprotinin (pH 7.4). Cellular debris was precipitated by centrifuging at 15 000 rpm for 30 min and the supernatant retained to obtain cytoplasmic MAP kinases.

MAP kinase activity was assayed with the p42/p44 MAP kinase enzyme assay system (Amersham) which is designed to detect MAP kinases in lysed cells, as described elsewhere (Kubo et al., 1998). Protein was measured by the method of Lowry et al. (1951).

Drugs used were angiotensin II acetate salt, leupeptin hemisulfate, aprotinin, big endothelin 39 rat, porcine renin (Sigma, St. Louis, MO), HEPES buffer (Wako, Osaka, Japan), endothelin-1 human, BQ123, captopril (Research Biochemicals International, Natick, MA), pepstatin A (Chemicon International, CA) and Dulbecco's modified Eagle's medium (DMEM, Dainihon Pharmaeuticals, Osaka, Japan). Losartan was generously supplied by Dupont-Merck Pharmaceuticals (Wilmington, DE).

The results are expressed as means ± S.E.M. All results were analyzed by either Student's *t*-test or one-way analysis of variance combined with Dunnett's test for post hoc analysis for intergroup comparisons. Differences were considered significant at $P < 0.05$.

3. Results

3.1. Effects of angiotensin II, endothelin-1, losartan and BQ123 on MAP kinase activity in quiescent confluent vascular smooth muscle cells

Angiotensin II (10^{-9} and 10^{-8} M) and endothelin-1 (10^{-9} to 10^{-7} M) caused an increase in MAP kinase activity 5 min after application in vascular smooth muscle cells in a concentration-dependent manner (Fig. 1A and B). The angiotensin receptor antagonist losartan (10^{-9} to 10^{-6} M) and the endothelin receptor antagonist BQ123 (10^{-9} to 10^{-6} M) produced a decrease in MAP kinase activity 30 min after application in vascular smooth muscle cells in a concentration-dependent manner (Fig. 1C and D). The angiotensin II (10^{-8} M)-induced MAP kinase activation was inhibited by losartan (10^{-6} M) but not by BQ123 (10^{-6} M) (Fig. 2A). The endothelin-1 (10^{-8} M)-induced MAP kinase activation was inhibited by BQ123 (10^{-6} M) but not by losartan (10^{-6} M) (Fig. 2B). The

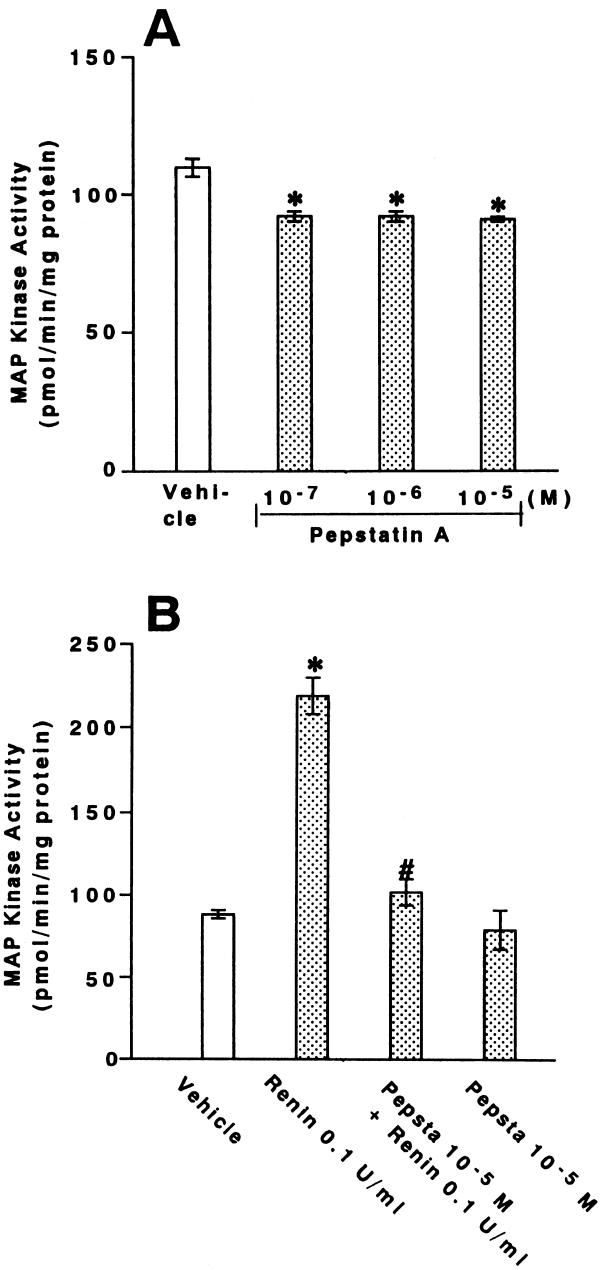

Fig. 4. (A) Effects of pepstatin A on MAP kinase activity in vascular smooth muscle cells. Drugs were added into DMEM and a 30-min incubation was started. (B) Effects of pepstatin A on renin-induced increase in MAP kinase activity in vascular smooth muscle cells. Renin was added into DMEM and a 5-min incubation was started. Pepstatin A (Pepsta) was added into DMEM 5 min before the 5-min incubation. Values are mean ± S.E.M. from four experiments. $^{*}P < 0.05$, compared with vehicle. $\#P < 0.05$, compared with renin alone.

combination of losartan (10^{-6} M) and BQ123 (10^{-6} M) caused a greater inhibition of the MAP kinase activity than losartan (10^{-6} M) alone or BQ123 (10^{-6} M) alone (Fig. 2C). The combination of endothelin-1 (10^{-9} M) and angiotensin II (10^{-9} M) caused an additive effect on MAP kinase activity (Fig. 2D).

3.2. Effects of angiotensin I, captopril, pepstatin A and renin on MAP kinase activity in quiescent confluent vascular smooth muscle cells

Angiotensin I (10^{-8} and 10^{-7} M) caused a concentration-dependent increase in MAP kinase activity in vascular smooth muscle cells, whereas the angiotensin-converting enzyme inhibitor captopril (10^{-8} to 10^{-6} M) caused decreases in MAP kinase activity in VSMCs (Fig. 3A and B). The angiotensin I (10^{-7} M)-induced increase in MAP kinase activity was inhibited by captopril (10^{-6} M) (Fig. 3C).

The renin inhibitor pepstatin A also caused a concentration-dependent decrease in MAP kinase activity in vascular smooth muscle cells (Fig. 4A). Renin (0.1 U/ml) caused an increase in MAP kinase activity in vascular smooth muscle cells and the renin-induced increase in MAP kinase activity was inhibited by pepstatin A (10^{-5} M) (Fig. 4B). The inhibitions of MAP kinase activity induced by pepstatin A (10^{-5} M), captopril (10^{-6} M) and losartan (10^{-6} M) were almost the same (Fig. 5).

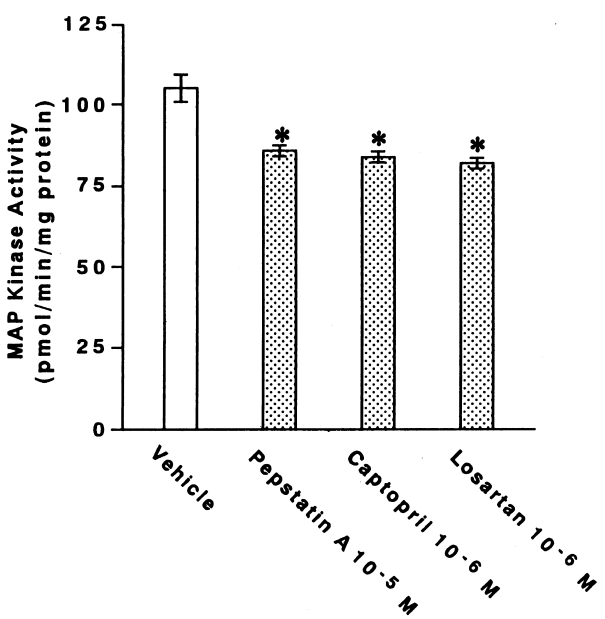

Fig. 5. Effects of pepstatin A, captopril and losartan on MAP kinase activity in vascular smooth muscle cells. Drugs were added into DMEM and a 30-min incubation was started. Values are mean ± S.E.M. from four experiments. $^{*}P < 0.05$, compared with vehicle.

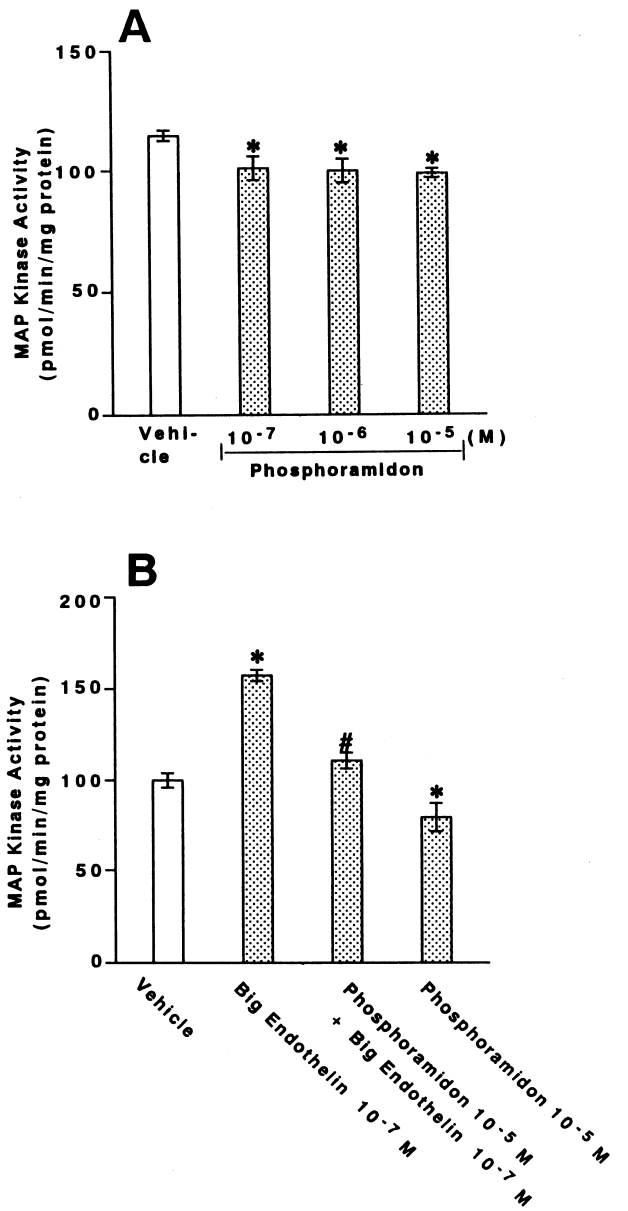

Fig. 6. (A) Effects of phosphoramidon on MAP kinase activity in vascular smooth muscle cells. Phosphoramidon was added into DMEM and a 30-min incubation was started. (B) Effects of phosphoramidon on big endothelin-induced increase in MAP kinase activity in vascular smooth muscle cells. Big endothelin was added into DMEM and a 5-min incubation was started. Phosphoramidon was added into DMEM 5 min before the 5-min incubation. Values are mean ± S.E.M. from four experiments. $^{*}P < 0.05$, compared with vehicle. $\#P < 0.05$, compared with big endothelin alone.

3.3. Effects of phosphoramidon and big endothelin on MAP kinase activity in quiescent confluent vascular smooth muscle cells

The endothelin-converting enzyme inhibitor phosphoramidon (10^{-7} to 10^{-5} M) caused decreases in MAP kinase activity in vascular smooth muscle cells (Fig. 6A). The endothelin precursor big endothelin (10^{-7} M) caused an increase in MAP kinase activity in vascular smooth

muscle cells and the big endothelin-induced increase in MAP kinase activity was inhibited by phoshoramidon (10^{-5} M) (Fig. 6B).

4. Discussion

In the present study, angiotensin II and endothelin-1 caused an increase of MAP kinase activity. The MAP kinase activation induced by angiotensin II and endothelin-1 were inhibited by the angiotensin receptor antagonist losartan and by the endothelin receptor antagonist BQ123, respectively. Losartan alone or BQ123 alone caused decreases in MAP kinase activity. These results suggest that MAP kinases are tonically activated via endogenous angiotensin II and endothelins in vascular smooth muscle cells.

Vascular smooth muscle cells express angiotensinogen mRNA (Naftilan et al., 1991), renin (Re et al., 1982), angiotensin-converting enzyme protein (Pipili-Synetos et al., 1990) and its mRNA (Fishel et al., 1995), suggesting that vascular smooth muscle cells can synthesize and release angiotensins in vascular smooth muscle cells. In the present study, indeed, renin and angiotensin I increased MAP kinase activity in vascular smooth muscle cells, whereas the renin inhibitor pepstatin A and the angiotensin-converting enzyme inhibitor captopril decreased MAP kinase activity in vascular smooth muscle cells. These findings suggest that vascular smooth muscle cells tonically produce and release angiotensins to activate MAP kinase activity.

Controversy exists whether vascular smooth muscle cells synthesize renin. One report indicated the expression of the renin gene in aortic tissues (Samani et al., 1988), while this was not confirmed in other studies (Ekker et al., 1989). In addition, Holycross et al. (1992) have demonstrated that in aortic smooth muscle, cathepsins D and E but not renin are the predominant aspartic proteinases, suggesting that these cathepsins may play angiotensinogen cleaving role in place of renin in vascular smooth muscle cells.

In the present study, the renin inhibitor pepstatin A, the angiotensin-converting enzyme inhibitor captopril and the angiotensin receptor antagonist losartan at respective maximally effective doses caused a similar extent of inhibition of basal MAP kinase activity in vascular smooth muscle cells. In addition, renin application onto vascular smooth muscle cells caused an increase of MAP kinase activity and this effect was inhibited by pepstatin A. Pepstatin A can inhibit not only renin but also cathepsins A and E. Thus, the results of the present study suggest that renin or renin-like substances are important factors in determining angiotensin generation in vascular smooth muscle cells.

Vascular smooth muscle cells are also reported to express endothelin-1 mRNA and to release endothelins (Sung et al., 1994). In the present study, the endothelin precursor big endothelin produced an increase in MAP kinase activity, whereas the endothelin-converting enzyme inhibitor phosphoramidon caused a decrease in MAP kinase activity in vascular smooth muscle cells. The big endothelin-induced MAP kinase activation was inhibited by phosphoramidon. These findings suggest that vascular smooth muscle cells can tonically produce and release endothelins to activate MAP kinase activity.

It has been reported that in rat vascular smooth muscle cells, angiotensin II mediates cell proliferation and this proliferation is partially mediated via endothelins (Sung et al., 1994). In the present study, however, the angiotensin II-induced MAP kinase activation was not inhibited by the endothelin receptor antagonist BQ123. In addition, basal MAP kinase activity in vascular smooth muscle cells was additively inhibited by losartan and BQ123. The combination of angiotensin II and endothelin-1 caused an additive increase in MAP kinase activity in vascular smooth muscle cells. Thus, it seems likely that angiotensin II and endothelins essentially act independently for MAP kinase activation in vascular smooth muscle cells.

Previously, we have demonstrated that in rat aortic strips, endothelium removal produces an activation of MAP kinase activity through angiotensin II and endothelins, probably released in the media layer of the vascular wall (Kubo et al., 1998). The results of the present study demonstrate that vascular smooth muscle cells, which compose the media, tonically produce and release angiotensin II and endothelins, suggesting that vascular smooth muscle cells may be a source for both peptides in intact vessel media. Thus, the current findings would provide potential explanations for the previous data of us suggesting the presence of intrinsic, complete angiotensin II and endothelin generating systems in intact vessel media. To our knowledge, this is the first work to simultaneously demonstrate the co-existence of these systems in such a model.

Nevertheless, it should be considered that the presence and activity of angiotensin and endothelin systems in isolated cells in culture are not necessarily a reflection of the situation in the intact vascular wall. Endothelium removal, the stress of cell layer stretching, serum deprivation, glucose levels, the presence or absence of other stimulating factors, all have been reported to affect responses to the angiotensin and endothelin systems (Jackson and Schwartz, 1992). Vascular smooth muscle cells in culture rapidly undergo phenotypic modulation, resulting in the up-regulation of multiple growth-mediating pathways (Sarzani et al., 1991). Further studies to relate the basal MAP kinase activity found in this study to a physiologically relevant activity in the intact vascular wall will be needed.

In summary, this study provides evidence suggesting that apparently functional renin–angiotensin system and endothelin system are present in vascular smooth muscle cells and these systems serve to increase MAP kinase

activity. It appears that renin or renin-like substances play the determining role in the regulation of renin–angiotensin system in vascular smooth muscle cells.

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