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Evidence that angiotensin II, endothelins and nitric oxide regulate mitogen-activated protein kinase activity in rat aorta

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Abstract

We measured the activity of mitogen-activated protein (MAP) kinases, enzymes believed to be involved in the pathway for cell proliferation, in rat aortic strips with or without endothelium, and examined effects of angiotensin receptor antagonists, endothelin receptor antagonists and nitric oxide (NO)-related agents. Endothelium removal produced an activation of MAP kinase activity in the strips, whereas the enzyme activity was not affected in the adventitia. The MAP kinase activation was inhibited by either the angiotensin AT_1 receptor antagonist losartan or the endothelin ET_A receptor antagonist BQ 123. The combination of both antagonists caused an additive inhibition. The angiotensin AT_2 receptor antagonist PD 123,319 and the endothelin ET_B receptor antagonist BQ 788 did not affect the MAP kinase activation. The NO synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) caused an activation of MAP kinase in the endothelium-intact aorta and the MAP kinase activation was inhibited by losartan or BQ123. The NO releaser nitroprusside inhibited the MAP kinase activation induced by endothelium removal or angiotensin II. These results suggest that even in isolated arteries, NO of endothelial origin tonically exert MAP kinase-inhibiting effects and endogenous angiotensin II and endothelins in the media are tonically released to cause MAP kinase-stimulating effects in medial smooth muscle. © 1998 Elsevier Science B.V.

Keywords: Angiotensin; Endothelin; Nitric oxide (NO); Aorta, rat; MAP (mitogen-activated protein) kinase

1. Introduction

There is considerable interest in understanding mechanisms of vascular hypertrophy, since it appears to be a fundamental pathogenic factor for both hypertension and atherosclerosis (Folkow et al., 1982; Kubo, 1978; Owens and Schwartz, 1982). Endothelial and vascular smooth muscle cell integrity appears to be a crucial factor for maintenance of the structural property of the vascular wall. Indeed, endothelial injury by balloon catheterization causes blood vessel hypertrophy by promoting progressive vascular smooth muscle cell proliferation in the intima of muscular arteries (Clowes et al., 1983; Powell et al., 1989). Although vasoactive substances like angiotensin II, endothelins and nitric oxide (NO) are suggested to be related with such pathophysiological responses (Powell et al., 1989; Douglas et al., 1994), little is known of exact involvement of these substances in those vascular responses.

It is well known that angiotensin II and endothelin-1, vasoconstrictors, are released from the endothelium (Bobik and Campbell, 1993; Dzau and Gibbons, 1988; Marin and Sanchez-Ferrer, 1990; Peiro et al., 1995). These substances released are considered to act at the media to promote vascular smooth muscle cell growth (Bobik et al., 1990; Dzau and Gibbons, 1988; Janakidevi et al., 1992; Yanagisawa et al., 1988). NO is an important vasorelaxing substance of endothelial origin and has been shown to have a growth-inhibiting effect (Dubey, 1994; Ignarro et al., 1987; Moncada et al., 1991; Palmer et al., 1987). In addition, vascular smooth muscle cells themselves have several autocrine systems. For example, the vasoconstrictor angiotensin II appears to be released from vascular smooth muscle cells, since vascular smooth muscle cells express angiotensinogen mRNA (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).

Mitogen-activated protein (MAP) kinases are members of a family of serine/threonine-specific protein kinases

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(Kosako et al., 1992). MAP kinase activation is commonly induced by a variety of growth-stimulating factors, including angiotensin II and endothelin-1 (Koide et al., 1992; Tsuda et al., 1992). These growth factors activate MAP kinases via phosphorylation on both tyrosine and serine/threonine residues (Kosako et al., 1992). MAP kinases stimulate ribosomal protein S6 kinase II and protein synthesis in vascular smooth muscle cells (Sturgill et al., 1988). In addition, MAP kinases also phosphorylate the products of the c-*jun*, c-*fos* and c-*myc* proto-oncogenes (Alvarez et al., 1991; Pulverer et al., 1991). Therefore, MAP kinases are considered to play an important role in mediating signals from the growth factor receptors to the ribosomes and nucleus. However, little is known of the regulation of MAP kinase activity by vasoactive substances in blood vessels.

The objectives of the present study were to examine how endogenous angiotensin II, endothelins and NO in the blood vessel wall are involved in the vascular vessel growth regulation after endothelial injury. Using rat aortic strips, we measured the activity of MAP kinases, enzymes believed to be involved in the pathway for cell proliferation, in the vessel strips with or without endothelium, and examined effects of angiotensin II receptor antagonists, endothelin receptor antagonists and NO related agents.

2. Materials and methods

Male Wistar rats (200–260 g) were killed by over doses of ether. The thoracic aorta was removed and incubated at 4°C in Tyrode solution, comprised of 137 mM NaCl, 2.7 mM KCl, 1.8 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.4 mM NaH_2PO_4, 11.9 mM $NaHCO_3$, 5.5 mM glucose. Connective tissues were gently cleaned under a dissecting microscope using sterile conditions according to the method of Ross (1971). The endothelium was removed by rubbing gently the intimal surface with a fine forceps. The aorta was washed twice and cut into six to eight strips (approximately 3×4 mm each). In some experiments, the adventitia was carefully removed under a dissecting microscope to obtain mainly media portion and mainly adventitia portion. The complete removal of the endothelium was confirmed immunohistochemically. Endothelium-denuded aortic strips consistently had no cells stained positively to the endothelium antibody, endothelium mouse IgG_1 antibody (BMA Biomedicals, Augst, Switzerland), whereas intact aortic strips had endothelial cells stained to the antibody.

2.1. Tissue incubation and preparation of tissue extracts

The aortic strips were placed into plates (three to four strips in each plate) containing 1 ml of Dulbecco's modified Eagle's medium (DMEM) supplemented with 19 mM $NaHCO_3$, 0.58 mg/ml L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. The strips were preincubated in 37°C DMEM for 5 min for tissue equilibration, and then, a 10-min incubation or a 30-min incubation was started at 37°C in a moist tissue culture incubator containing an atmosphere of 95% air and 5% CO_2. Drugs were added into DMEM at the beginning of the preincubation or at the beginning of the incubations. Drugs were dissolved in physiological saline (0.9% NaCl) and added into DMEM in a volume of 10 μl. In preliminary experiments, MAP kinase activity in endothelium-denuded aortic strips was increased to a similar extent at 5 min and at 10 min after addition of angiotensin II (10^{-8} M) or endothelin-1 (10^{-8} M) to DMEM, but the extent of the increase in MAP kinase activity was decreased 30 min after the addition of these agents. Thus, we measured MAP kinase activity 10 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. Tissues were frozen in liquid nitrogen and stored at −80°C until required.

After defreezing, the tissues were lysed and homogenated in 0.3 ml of an ice-cold buffer, comprised 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). All further steps were performed at 4°C. Tissue homogenates were centrifuged at 15,000 rpm for 30 min and the supernatant retained to obtain cytoplasmic MAP kinase.

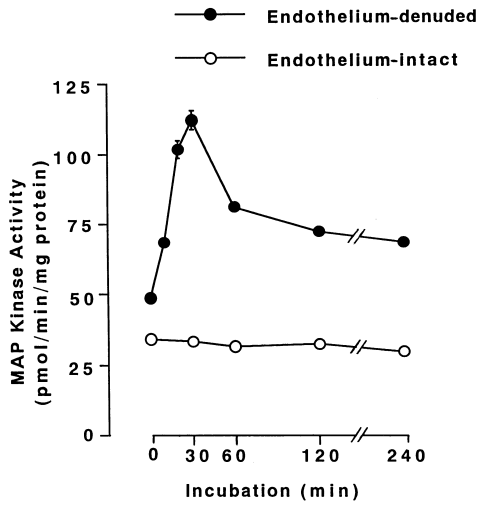

Fig. 1. Time-course of MAP kinase activity level after incubation in endothelium-intact (open circles) and endothelium-denuded (closed circles) aortic strips of rats. Aortic strips with or without endothelium were preincubated at 37°C for 5 min and then, incubations were started at 37°C in a moist tissue culture incubator containing an atmosphere of 95% air and 5% CO_2. MAP kinase activities were greater in endothelium-denuded aortic strips than those of endothelium-intact aortic strips in all the incubation periods ($P < 0.05$). Values are mean ± S.E.M. from 4 experiments.

2.2. Assay for MAP kinase activity

MAP kinase activity was assayed by using the p42/p44 MAP kinase enzyme assay system (Amersham) which is designed to detect MAP kinases in lysed tissues. Briefly, MAP kinase activity was measured by detecting the extent of protein phosphorylation, since the enzyme in the samples can catalyze the transfer of the γ-phosphate of adenosine-5′-triphosphate to the threonine group on a peptide. The peptide used as substrate contains the phosphorylation sequence Pro–Leu–Ser/Thr–Pro which p42/p44 MAP kinases recognize as a site for phosphorylation (Alvarez et al., 1991) but contains no other phosphorylation sites. Fifteen μl of samples, 10 μl of substrate in a buffer containing HEPES and sodium orthovanadate, and 5 μl of magnesium [^{32}P]ATP (200 μCi/ml) were mixed in tubes and incubated for 30 min with water bath at 30°C. The reaction was terminated by adding 10 μl of solution of orthophosphoric acid containing carmosine red. Then, 30 μl of terminated reaction mixture was pipetted on peptide binding papers. The papers were washed twice with 75 mM orthophosphoric acid and once with water. Each binding paper was placed in a scintillation vial, 10 ml liquid scintillation cocktail was added to each vial, and its scintillation was counted. Protein was measured by the method of Lowry et al. (1951).

Drugs used were angiotensin II acetate salt (Sigma, St. Louis, MO), endothelin-1 human, BQ 123, BQ 788, N^G-nitro-L-arginine methyl ester hydrochloride, PD123,319 ditrifluoroacetate (Research Biochemicals International, Natick, MA), L-arginine monohydrochloride, HEPES buffer (Wako, Osaka, Japan), leupeptin hemisulfate, aprotinin

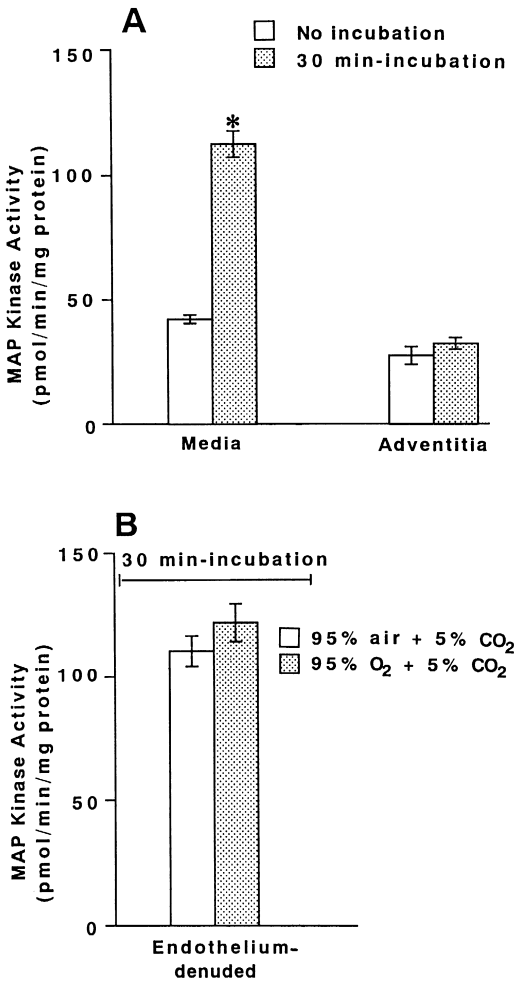

Fig. 2. (A) Effects of 30-min incubation on MAP kinase activity in mainly media portion (media) or mainly adventitia portion (adventitia) of aortic strips of rats. (B) Effects of oxygen (O_2) on the increase in MAP kinase activity 30 min after incubation in endothelium-denuded aortic strips of rats. Aortic strips without endothelium were incubated for 30 min in DMEM saturated with 95% air and 5% CO_2 or with 95% O_2 and 5% CO_2. Values are mean ± S.E.M. from 5 experiments. $* P < 0.05$, compared with no incubation.

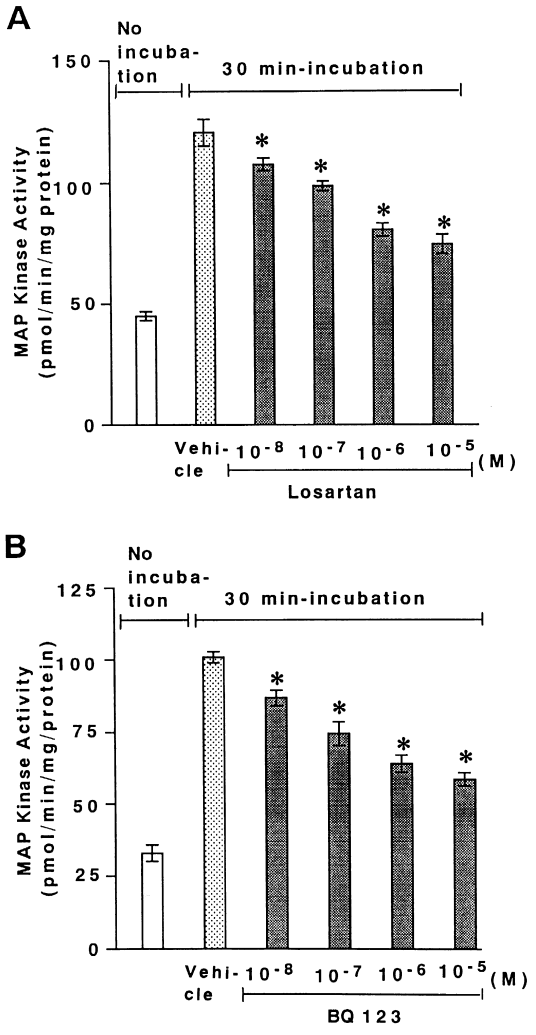

Fig. 3. Concentration-dependent effects of losartan (A) and BQ 123 (B) on the increase in MAP kinase activity 30 min after incubation in endothelium-denuded aortic strips of rats. Aortic strips were preincubated for 5 min and then, a 30-min incubation was started. Saline (vehicle), losartan and BQ 123 were added into DMEM at the beginning of the 30-min incubation. MAP kinase activity levels without incubation were also measured (open columns). Values are mean ± S.E.M. from 4 experiments. $* P < 0.05$, compared with 30 min-incubation vehicle.

(Sigma), and DMEM (Dainihon Pharmaceuticals, Osaka, Japan). Losartan was generously supplied by Dupont-Merck Pharmaceuticals (Wilmington, DE).

The results are expressed as mean ± S.E.M. Statistical studies, using analysis of variance and Student's *t*-test for individual differences, were performed. Differences were considered significant at $P < 0.05$.

3. Results

3.1. Effects of endothelium removal on MAP kinase activity

Following a 5-min preincubation at 37°C in DMEM, incubation of aortic strips with or without endothelium was started at 37°C in a moist tissue culture incubator containing an atmosphere of 95% air and 5% CO_2. MAP kinase activity in endothelium-denuded aortic strips was increased time-dependently after incubation (Fig. 1). The enzymes were gradually stimulated after incubation and their activity reached a maximum level at 20–30 min after incubation. The enzyme activity began to decrease at 60 min after incubation but still was higher even 240 min after incubation than the 0-min value. On the other hand, no activation of the enzymes was found in endothelium-intact aortic strips during all the incubation periods. MAP kinase activities were greater in endothelium-denuded aortic atrips than those of endothelium-intact aortic strips during all the incubation periods.

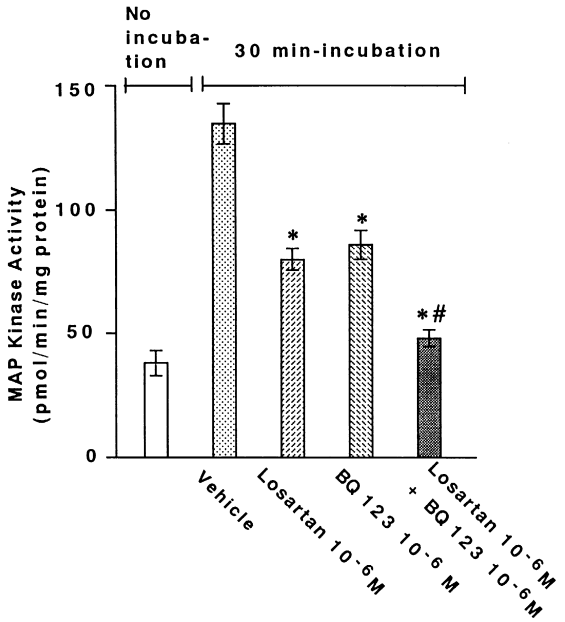

Fig. 4. Effects of the combination of losartan and BQ 123 on the increase in MAP kinase activity 30 min after incubation in endothelium-denuded aortic strips of rats. Aortic strips were preincubated for 5 min and then, a 30-min incubation was started. Saline (vehicle), losartan and BQ 123 were added into DMEM at the beginning of the 30-min incubation. MAP kinase activity levels without incubation were also measured (open column). Values are mean ± S.E.M. from 5 experiments. $* P < 0.05$, compared with 30 min-incubation vehicle. $^{\#}P < 0.05$, compared with losartan alone and BQ 123 alone.

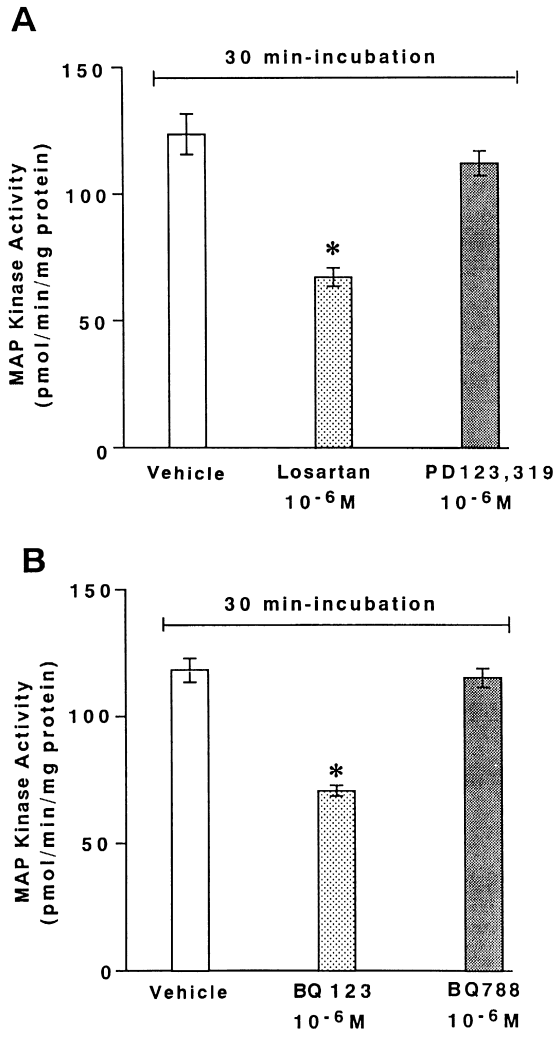

Fig. 5. Effects of the angiotensin receptor antagonists losartan and PD 123,319 (A) or the endothelin receptor antagonists BQ 123 and BQ 788 (B) on the increase in MAP kinase activity 30 min after incubation in endothelium-denuded aortic strips of rats. Aortic strips were preincubated for 5 min and then, a 30-min incubation was started. Saline (vehicle) and drugs were added into DMEM at the beginning of the 30-min incubation. Values are mean ± S.E.M. from 5 experiments. $* P < 0.05$, compared with vehicle.

Endothelium-denuded aortic strips consist of the media and the adventitia. To examine whether the increase in MAP kinase activity occurs in the media or in the adventitia, mainly media portion and mainly adventitia portion were incubated at 37°C for 30 min. MAP kinases were again stimulated in the media portion, whereas there was no increase of the enzyme activity in the adventitia portion (Fig. 2A).

It has been recognized that hypoxia can exert some direct effects on vascular smooth muscle (Arnqvist et al., 1983). To examine whether the MAP kinase activation observed in endothelium-denuded strips results from

anoxia, the endothelium-denuded strips were incubated at 37°C for 30 min in DMEM saturated with 95% O_2 and 5% CO_2. The increase in MAP kinase activity in the strips was almost the same as that incubated with DMEM saturated with 95% air and 5% CO_2 (Fig. 2B).

3.2. Effects of angiotensin receptor antagonists and endothelin receptor antagonists on the endothelium removal-induced MAP kinase activation

The angiotensin AT_1 receptor antagonist losartan (10^{-8}–10^{-5} M) inhibited the increase in MAP kinase activity 30 min after incubation concentration-dependently in endothelium-denuded aortic strips (Fig. 3A). The endothelin ET_A receptor antagonist BQ 123 (10^{-8}–10^{-5} M) also inhibited it in a concentration-dependent manner (Fig. 3B). The combination of losartan (10^{-6} M) and BQ 123 (10^{-6} M) caused a greater inhibition of the MAP kinase activation than losartan (10^{-6} M) alone or BQ 123 (10^{-6} M) alone (Fig. 4). On the other hand, the angiotensin AT_2 receptor antagonist PD 123,319 (10^{-6} M) (Fig. 5A) and the endothelin ET_B receptor antagonist BQ 788 (10^{-6} M) (Fig. 5B) did not affect the endothelium removal-induced increase of MAP kinase activity.

Both angiotensin II (10^{-8} M) and endothelin-1 (10^{-8} M) caused an increase in MAP kinase activity 10 min after

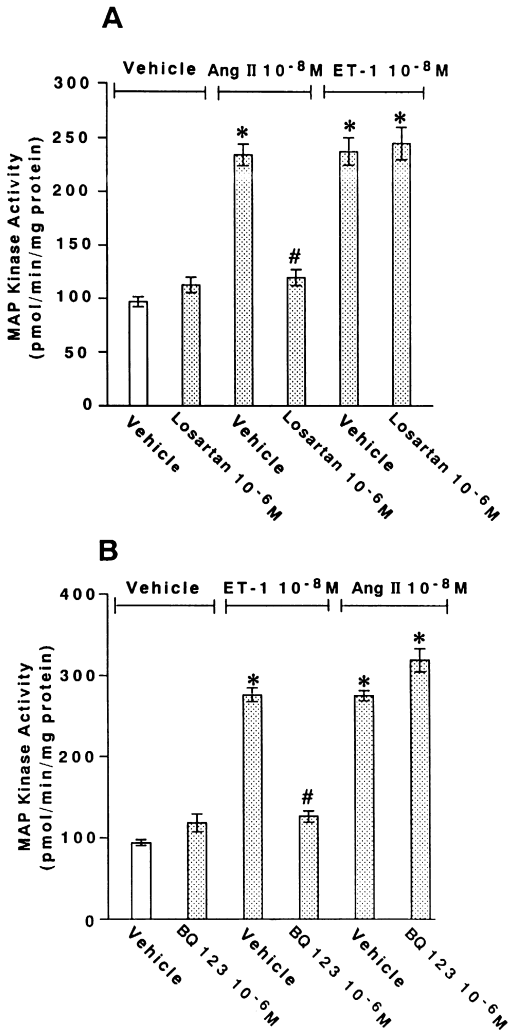

Fig. 6. Effects of losartan (A) and BQ 123 (B) on angiotensin II (Ang II)- and endothelin-1(ET-1)-induced increases in MAP kinase activity in endothelium-denuded aortic strips of rats. Aortic strips were preincubated for 5 min and then, a 10-min incubation was started. Saline (vehicle), losartan and BQ 123 were added into DMEM at the beginning of the 5-min preincubation. Saline (vehicle), Ang II and ET-1 were added into DMEM at the beginning of the 10-min incubation. Values are mean ± S.E.M. from 5 experiments. $* P < 0.05$, compared with vehicle–vehicle. $^{\#}P < 0.05$, compared with vehicle–Ang II (A) or vehicle–ET-1 (B).

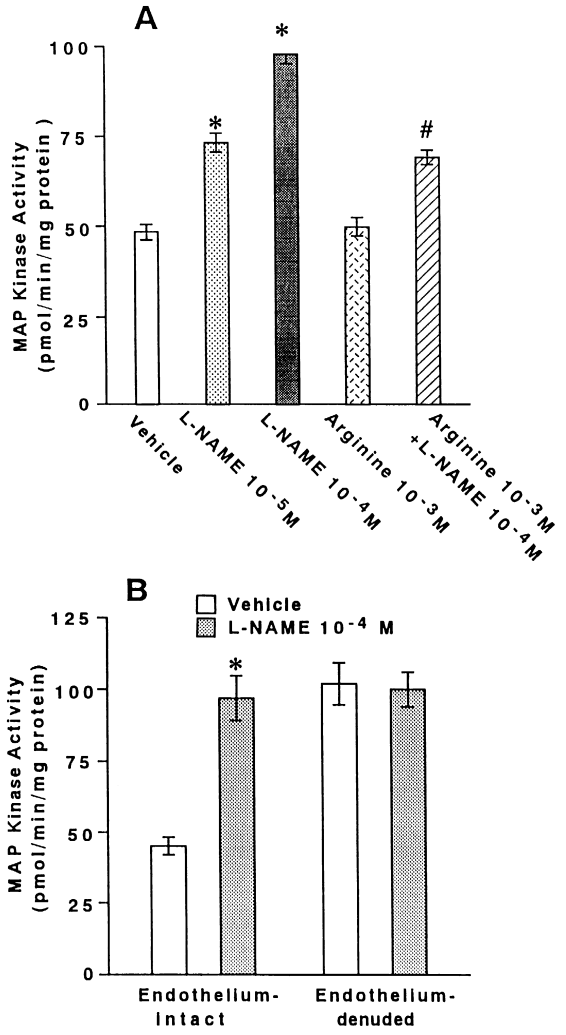

Fig. 7. (A) Effects of L-NAME on MAP kinase activity and antagonistic effect by L-arginine on the L-NAME (10^{-4} M)-induced increase of MAP kinase activity in endothelium-intact aortic strips of rats. Aortic strips were preincubated for 5 min and then, a 30-min incubation was started. Arginine was added into DMEM at the beginning of the 5-min preincubation. Saline (vehicle) and L-NAME were added into DMEM at the beginning of the 30-min incubation. (B) Effects of L-NAME on MAP kinase activity in endothelium-intact and endothelium-denuded aortic strips of rats. Saline (vehicle) and L-NAME were added into DMEM at the beginning of the 30-min incubation. Values are mean ± S.E.M. from 5 experiments. $* P < 0.05$, compared with vehicle. $^{\#}P < 0.05$, compared with L-NAME 10^{-4} M alone.

incubation in endothelium-denuded strips (Fig. 6A,B). The angiotensin II-induced increase in MAP kinase activity was inhibited by losartan (10^{-6} M) but not by BQ 123 (10^{-6} M) (Fig. 6B), suggesting that losartan is specific to angiotensin II. The endothelin-1-induced increase in MAP kinase activity was inhibited by BQ 123 (10^{-6} M) (Fig. 6B) but not by losartan (10^{-6} M) (Fig. 6A), suggesting that BQ 123 is specific to endothelins. On the other hand, losartan (10^{-6} M) and BQ 123 (10^{-6} M) did not affect basal MAP kinase activity after 10 min (Fig. 6A,B).

3.3. Effects of NAME in endothelium-intact aortic strips

Next, we determined effects of the NO synthase inhibitor N^{G}-nitro-L-arginine methyl ester hydrochloride (L-NAME) in endothelium-intact aortic strips, in order to examine whether the endothelium removal-induced increase in MAP kinase activity results from the lack of the growth-inhibiting factor, nitric oxide (NO) of endothelial origin. L-NAME (10^{-5} and 10^{-4} M) caused a concentration-dependent increase in MAP kinase activity in endothelium-intact strips (Fig. 7A), whereas this agent (10^{-4} M) did not affect MAP kinase activity in endothelium-denuded aortic strips (Fig. 7B). The increase in MAP kinase activity induced by L-NAME (10^{-4} M) was inhibited by

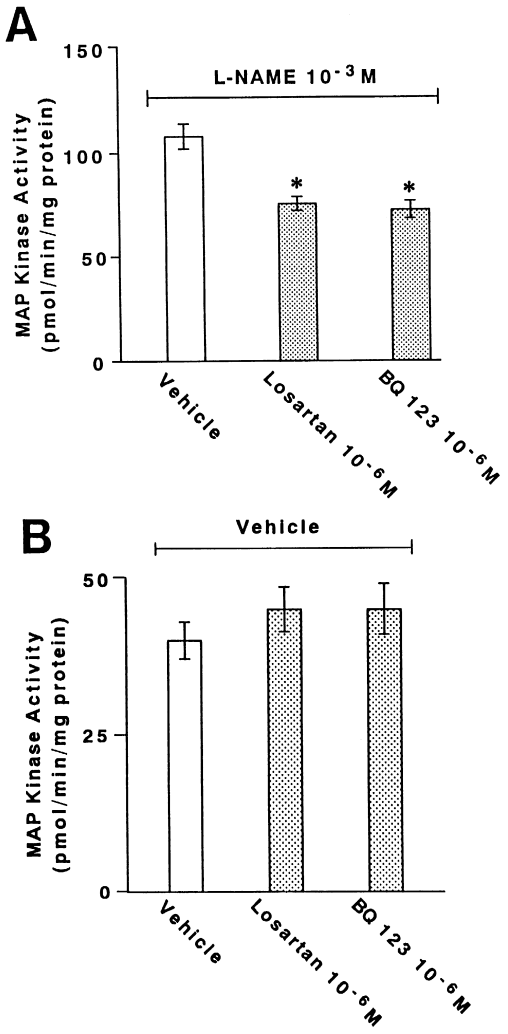

Fig. 8. (A,B) Effects of losartan and BQ 123 on the L-NAME (10^{-3} M)-induced increase of MAP kinase activity (A) and on basal MAP kinase activity (B) in endothelium-intact aortic strips of rats. Aortic strips were preincubated for 5 min and then, a 30-min incubation was started. L-NAME (A) and saline (vehicle) (B) were added at the beginning of the 5-min preincubation. Saline (vehicle), losartan and BQ 123 were added into DMEM at the beginning of the 30-min incubation. Values are mean ± S.E.M. from 5 experiments. $* P < 0.05$, compared with vehicle.

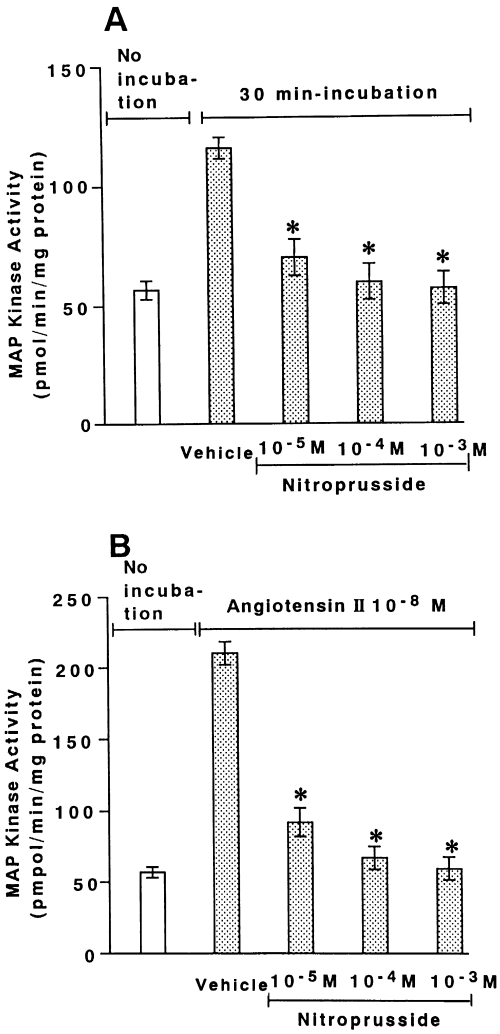

Fig. 9. (A) Effects of nitroprusside on the endothelium removal-induced increase in MAP kinase activity in aortic strips of rats. Aortic strips were preincubated for 5 min and then, a 30-min incubation was started. Saline (vehicle) and nitroprusside were added into DMEM at the beginning of the 30-min incubation. (B) Effects of nitroprusside on the angiotensin II-induced increase of MAP kinase activity in endothelium-denuded aortic strips of rats. Aortic strips were preincubated for 5 min and then, a 10-min incubation was started. Saline (vehicle) and nitroprusside were added into DMEM at the beginning of the 5-min preincubation. Angiotensin II was added into DMEM at the beginning of the 10-min incubation. MAP kinase activity levels without incubation were also measured (open column). Values are mean ± S.E.M. from 5 experiments. $* P < 0.05$, compared with vehicle.

preincubation with the precursor of NO, L-arginine (10^{-3} M) (Fig. 7A), suggesting that the L-NAME-induced increase in MAP kinase activity is due to an inhibition of NO synthesis. The L-NAME (10^{-4} M)-induced increase in MAP kinase activity in endothelium-intact strips was almost the same as that induced by endothelium removal (Fig. 7B). The L-NAME-induced increase in MAP kinase activity was again inhibited by either losartan (10^{-6} M) or BQ 123 (10^{-6} M)(Fig. 8A). Losartan (10^{-6} M) and BQ 123 (10^{-6} M) did not affect MAP kinase activity in endothelium-intact strips in the absence of L-NAME (Fig. 8B).

3.4. Effects of nitroprusside on the endothelium removal-induced MAP kinase activation

Next, we determined effects of the NO releaser nitroprusside in endothelium-denuded aortic strips to investigate whether NO can indeed inhibit MAP kinase activity. Nitroprusside (10^{-5}–10^{-3} M) caused a concentration-dependent inhibition of the MAP kinase activation after endothelium removal (Fig. 9A). Nitroprusside (10^{-5}–10^{-3} M) also caused a concentration-dependent inhibition of the angiotensin II-induced activation of MAP kinases (Fig. 9B).

4. Discussion

In the present study, endothelium removal in aortic strips from rats caused an increase of MAP kinase activity after incubation in DMEM saturated with 95% air and 5% CO_2. The MAP kinase activation occurred in the media portion but not in the adventitia portion. On the other hand, no activation of the enzymes was found after incubation in endothelium-intact strips. These results suggest that in isolated arteries, MAP kinases are tonically activated in the medial layer of the vessel wall and this MAP kinase activation is tonically inhibited by the endothelium. In the present study, a similar endothelium removal-induced increase in MAP kinase activity was found after incubation in DMEM saturated with 95% O_2 and 5% CO_2, suggesting that the increase of MAP kinase activity is not due to anoxia. MAP kinase activity was greater in endothelium-denuded than that in endothelium-intact even at 0 min incubation. This difference may be due to an increase in MAP kinase activity induced during the preincubation period. In addition, the physical process of dissection could cause artifactual activation of signal transduction pathways.

The increase in MAP kinase activity after endothelium removal was inhibited by either the angiotensin receptor antagonist losartan or the endothelin receptor antagonist BQ 123. Losartan was indeed specific to angiotensin II and BQ 123 was specific to endothelins. These findings suggest that the increase in MAP kinase activity after endothelium removal is mediated through angiotensin II and endothelins.

Angiotensin II is known to act on, at least, two distinct receptors termed AT_1 and AT_2 (Timmermans et al., 1991). Although the angiotensin AT_1 receptor antagonist losartan inhibited the MAP kinase activation after endothelium removal, the angiotensin AT_2 ligand PD 123,319 had no effect. Thus, the AT_1 receptor subtype appears to be mainly involved in mediation of the enzyme activation after endothelium removal. On the other hand, endothelins are known to act on, at least, two distinct receptors termed ET_A and ET_B (Masaki et al., 1991). The endothelin ET_A receptor antagonist BQ 123 inhibited the increase in MAP kinases, whereas the endothelin ET_B receptor antagonist BQ 788 had no effect. Thus, the ET_A receptor subtype appears to mainly mediate the enzyme activation effect. The results of the present study are compatible with those of previous findings (Bunkenburg et al., 1992; Chiu et al., 1991; Sung et al., 1994) showing that angiotensin II produces MAP kinase activation and hypertrophic responses, and these responses are mediated through activation of the angiotensin AT_1 receptor subtype in rat vascular smooth muscle cells. Similarly, endothelin-1-induced activation of MAP kinases has been reported to be inhibited by the endothelin ET_A receptor antagonist BQ 123 in rat vascular smooth muscle cells (Sung et al., 1994).

Although the endothelium is believed to be the major source for generation of angiotensin II and endothelins in the vasculature (Dzau and Gibbons, 1988; Goto et al., 1996; Masaki et al., 1991; Ryan et al., 1976), from the results of the present study, it seems likely that the media also supplies both peptides. Probably, vascular smooth muscle cells, which compose the media, may be a source for both peptides, because vascular smooth muscle cells can express angiotensinogen mRNA (Naftilan et al., 1991), angiotensin converting enzyme protein and its mRNA (Fishel et al., 1995), and endothelin-1 mRNA (Sung et al., 1994). In addition, cultured vascular smooth muscle cells have been shown to release endothelins (Sung et al., 1994). Alternatively, it is probable that both peptides and/or their precursors released from the endothelium are deposited within the media and slowly released there. Nevertheless, we cannot rule out the possibility that angiotensin II and endothelins released from a few residual endothelial cells may contribute, in part, to the MAP kinase activation.

In the present study, MAP kinase activity was first increased and subsequently decreased in endothelium-denuded strips. Similar increase and subsequent decrease of MAP kinase activity is observed in cultured vascular smooth muscle cells after addition of angiotensin II or endothelin-1 (Koide et al., 1992; Tsuda et al., 1992). It has been reported that in addition to activation of MAP kinase, angiotensin II inactivates MAP kinases probably via activation of MAP kinase phosphatase, an enzyme for dephosphorylation of MAP kinases (Duff et al., 1995). Further,

the decrease in MAP kinase activity observed in endothelium-denuded strips might results partly from decreased release of angiotensin II and/or endothelins. In the present study, losartan and BQ 123 did not affect basal MAP kinase activity after 10 min incubation. Thus, it seems likely that there is a time-dependency of the effects of these antagonists on MAP kinase activity. It is possible that involvement of angiotensin II and endothelin-1 in the increase of MAP kinase activity is small at the early phase. Exact mechanisms of the time-dependency remain to be settled.

The fact that endothelium removal caused an increase in MAP kinase activity in the aortic strips also suggests that growth-inhibiting substances may be tonically released from the endothelium into the media. A possible growth-inhibiting factor involved is nitric oxide (NO), since the endothelium of healthy arteries is known to release NO (Ignarro et al., 1987; Moncada et al., 1991; Palmer et al., 1987). In the present study, indeed, application of the NO synthase inhibitor L-NAME resulted in an activation of MAP kinases in endothelium-intact aortic strips, whereas the NO synthase inhibitor did not affect MAP kinase activity in endothelium-denuded aortic strips. In addition, the L-NAME-induced MAP kinase activation was inhibited by the NO precursor L-arginine. Further, the NO releaser nitroprusside caused an inhibition of the MAP kinase activation after endothelium removal. These results suggest that even in such isolated arteries, the endothelium tonically releases NO and this gas acts to inhibit MAP kinases in the media. The L-NAME-induced MAP kinase activation was again inhibited by both losartan and BQ 123, suggesting that the MAP kinase activation is also through angiotensin II and endothelins.

In the present study, the L-NAME-induced increase in MAP kinase activity was comparable to that induced by endothelium removal. Thus, it appears that NO is mainly involved in mediation of growth-inhibiting effects of the endothelium in this experimental condition. In contrast, Peiro et al. (1995) have reported that coculture of vascular smooth muscle cells with endothelial cells results in a reduction of DNA synthesis but the reduction of DNA synthesis is not affected by L-NAME, suggesting that the endothelium can also exert its growth-inhibiting effects via other factors than NO.

Thus, the results of the present study suggest the existence of cross-talk among endogenous NO, angiotensin II and endothelins within the blood vessel wall. However, it is uncertain how NO of endothelial origin cross-talks with angiotensin and endothelin systems in the media of the vessel wall. It is possible that NO antagonizes angiotensin II and endothelins at the intracellular signaling pathway level of vascular smooth muscle cells in the media. In the present study, we found that the NO releaser nitroprusside indeed can inhibit the angiotensin II-induced MAP kinase activation. Similar counteracting effects of NO and angiotensin II have been reported in vascular smooth muscle cell proliferation and migration (Dubey, 1994; Dubey et al., 1995; Garg and Hassid, 1989). Alternatively, NO may cause an inhibition of release of these peptides. Clearly, more studies will be needed to elucidating mechanisms responsible for the cross-talk.

It has been reported that in rat aortic smooth muscle cells, angiotensin II mediates cell proliferation and this proliferation is partly 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 BQ 123 and the endothelin-1-induced MAP kinase activation was not inhibited by the angiotensin receptor antagonist losartan. Thus, it seems likely that angiotensin II and endothelins act independently in this experimental condition.

It has been demonstrated that endothelial injury by balloon catheterization causes myointimal thickening in the rat carotid and the rat aorta (Daemen et al., 1991; Janiak et al., 1992; Powell et al., 1989, 1990). Chronic treatment with angiotensin converting enzyme inhibitors and the angiotensin AT_1 receptor antagonist losartan have been shown to reduce the neointima formation (Capron et al., 1991; Daemen et al., 1991; Janiak et al., 1992; Osterrieder et al., 1991; Powell et al., 1989, Powell et al., 1990). Chronic administration of the non-peptide endothelin receptor antagonist SB 209670 has been also reported to protect the neointima formation following rat carotid artery balloon angioplasty (Douglas et al., 1994). From the results of the present study, it can be considered that the endothelial injury unmasks and thus results in, an activation of angiotensin and endothelin systems in the media, and this activation of both systems is partly involved in the myointima formation.

In the present study, the endothelium removal-induced MAP kinase activation was additively inhibited by losartan and BQ 123. Thus, it could be expected that the combination of a drug inhibiting angiotensin systems and a drug inhibiting endothelin systems will be more effective in the treatment of vascular diseases based on endothelial injury. In other words, inhibition of angiotensin systems alone or endothelin systems alone may be only partly effective for the treatment. In addition, the results of the present study suggest that NO releasing agents like nitroprusside may be also useful for the treatment of such diseases.

In summary, the results of the present study demonstrate that in rat aortic strips, endothelium removal produces an activation of MAP kinase activity in the media and the MAP kinase activation is mediated through angiotensin II and endothelins. The NO synthase inhibitor L-NAME also caused an activation of MAP kinase activity through angiotensin II and endothelins in endothelium-intact strips. These findings would provide evidence for cross-talk responsible for vascular vessel growth regulation among endogenous NO, angiotensin II and endothelins, and thus provide an important information for mechanisms responsible for vascular hypertrophy.

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