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Angiotensin II mediates pressure loading-induced mitogen-activated protein kinase activation in isolated rat aorta

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Abstract

Vascular hypertrophy occurs during chronic hypertension and contributes to the elevation of peripheral vascular resistance in hypertension. In this study, we examined whether acute pressure overloading of the vascular wall produces activation of mitogen-activated protein (MAP) kinases, enzymes believed to be involved in the pathway for cell proliferation, in isolated perfused rat aortae, and examined whether the mechanical overloading-induced MAP kinase activation is mediated via the vascular angiotensin system. Aortae were perfused with Tyrode solution. Increases in perfusion pressure caused a pressure-dependent increase in MAP kinase activity in endothelium-intact aortae and in endothelium-denuded aortae. The increase in MAP kinase activity induced by pressure loading was inhibited by the angiotensin receptor antagonist, losartan, the renin inhibitor, pepstatin A, and the angiotensin-converting enzyme inhibitor, captopril. Ca^{2+} depletion and the Ca^{2+} channel antagonist, nifedipine, did not affect the pressure loading-induced MAP kinase activation. The results of the present study suggest that pressure loading of the vascular wall per se can activate MAP kinases in the vasculature and that the MAP kinase activation is mediated at least partly via the vascular angiotensin system. It seems unlikely that the pressure loading-induced increase in MAP kinase activity is mainly mediated via increases in Ca^{2+} influx in vascular cells.

Keywords: Angiotensin II; MAP (mitogen activated protein) kinase; Mechanical stress; Aorta; Losartan

1. Introduction

It is accepted that vascular hypertrophy occurs during chronic hypertension and contributes to the elevation of peripheral vascular resistance in the established phase of hypertension (Folkow et al., 1982; Mulvany et al., 1985; Owens et al., 1988). An increase in cell size, i.e., hypertrophy, or an increase in cell number, i.e., hyperplasia, is responsible for the vascular hypertrophy (Mulvany et al., 1985; Owens et al., 1988). For example, in studies with hypertensive animals, smooth muscle hypertrophy with polyploidy is seen in the aorta, whereas hyperplasia is observed in small arteries and arterioles (Mulvany et al., 1978; Owens, 1987). Mechanical strain to the vascular wall following hypertension initiates a series of biochemical events that could form the basis for hypertrophy and hyperplasia of smooth muscle cells. However, the exact mechanisms of this vascular response are not known.

Angiotensin II, a vasoconstrictor, is reported to be released from the endothelium in the vasculature (Dzau and Gibbons, 1988; Bobik and Campbell, 1993). This peptide is suggested to act at the media to promote vascular smooth muscle cell growth (Dzau and Gibbons, 1988). In addition, vascular smooth muscle cells in the media appear to release this peptide, 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). Therefore, angiotensin II may act as an initial mediator of hypertension-mediated hypertrophy and hyperplasia of vascular smooth muscle cells. Angiotensin-converting enzyme inhibitors and angiotensin AT_1 receptor antagonists are indeed reported to inhibit vascular hypertrophy in spontaneously hypertensive rats (Powell et al., 1989; Soltis, 1993).

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 kinases play an important role in mediating signals from growth factor receptors to ribosomes and the nucleus (Sturgill et al., 1988; Alvarez et al., 1991; Pulverer et al., 1991). These enzymes are believed to be involved in the pathway for cell proliferation and thus in vascular hypertrophy (Tsuda et al., 1992; Molloy et al., 1993; Duff et al., 1995). In previous studies (Kubo et al., 1998a, 1999a, 1999b), we demonstrated that, in isolated rat aortae, endothelium removal produced an increase of MAP kinase activity and that the MAP kinase activation was found in the media portion but not in the adventitia portion. The endothelium removal-induced MAP kinase activation was inhibited by the angiotensin receptor antagonist, losartan, suggesting that, in rat aortae, endogenous angiotensin II is tonically released to cause MAP kinase-stimulating effects in medial smooth muscle.

Thus, it could be considered that mechanical stress to the vascular wall following hypertension may activate MAP kinases in blood vessels and that this MAP kinase activation may in turn lead to vascular hypertrophy. The objectives of the present study were to examine, using isolated rat aortae, whether acute pressure overloading to the vascular wall, per se, caused increases in MAP kinase activity and whether endogenous angiotensin II in the vasculature was involved in mediation of the pressure loading-induced MAP kinase activation. Stretching of aortic vascular smooth muscle cells enhances Ca^{2+} influx through a voltage-dependent dihydropyridine-sensitive Ca^{2+} entry pathway (Ruiz-Velasco et al., 1996) and the Ca^{2+} taken up into the cells may activate MAP kinases (Eguchi et al., 1996). Thus, we also examied the effects of Ca^{2+} deprivation and the voltage-dependent Ca^{2+} channel antagonist, nifedipine, on the pressure loading-induced MAP kinase activation.

2. Materials and methods

Male Wistar rats (200–260 g) were used in this study. They were kept under alternating 12-h periods of dark and light, and given standard rat chow and tap water ad libitum. They were killed with overdoses of ether.

2.1. Isolation of thoracic aortae

After sternotomy, 20 ml of saline containing 200 units of heparin was injected into the heart. A cannula (PE-60) was inserted into the thoracic aorta just next to the arcus aorta and a second cannula (PE-60) was inserted into the thoracic aorta just next to the abdominal aorta. The thoracic aorta was perfused with Tyrode solution (millimolar concentrations: NaCl 137, KCl 2.7, $CaCl_2$, 1.8, $MgCl_2$ 0.5, NaH_2PO_4 0.4, $NaHCO_3$ 11.9, glucose 5.5). All the branches of the thoracic aorta were ligated, and the aorta was isolated and placed in a dish filled with Tyrode solution maintained at 30°C.

The thoracic aorta was perfused with Tyrode solution maintained at 30°C and gassed with a mixture of 95% oxygen and 5% carbon dioxide. The aorta was perfused at a constant flow rate of 2 ml/min with a roller pump (Atto, Tokyo), and perfusion pressure was monitored by a pressure transducer connected to a polygraph. The end of the outlet cannula was connected to a silicone tube and the silicone tube was constricted by a clamp to elevate perfusion pressure.

2.2. Tissue perfusion and preparation of tissue extracts

First, the thoracic aorta was perfused without constriction of the silicone tube. After 60 min of the equilibration perfusion at basal pressure (10 mm Hg), in the perfusion pressure elevation groups, the perfusion pressure was elevated by constricting the silicone tube, but, in the basal pressure groups, perfusion pressure was not changed. In a control group, all the branches of the thoracic aorta were ligated but no perfusion was performed. Drugs were dissolved in the perfusion buffer solution and applied for 5 min before and during the elevation of perfusion pressure. We used losartan 10^{-6} M, pepstatin A 10^{-5} M and captopril 10^{-6} M, because these doses of the drugs produced a maximal inhibition of MAP kinase activity in isolated endothelium-denuded aortae of rats (Kubo et al., 1998a, 1999b).

The tissues were homogenized and sonicated in 0.3 ml of an ice-cold buffer (10 mM Tris, 150 mM NaCl, 2 mM EGTA, 2 mM dithiothreitol, 1 mM orthovanadate, 1 mM (*p*-amidinophenyl)methansulphonyl fluoride, 10 μg/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 was retained to obtain cytoplasmic MAP kinases.

In some experiments, before cannulation, the endothelium was removed by rubbing the intimal surface with a cotton swab. After experiments, a piece of each endothelium-denuded aorta was fixed in formalin, frozen, cut into thin sections, and stained with hematoxylin and eosin.

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

Drugs used were angiotensin II acetate salt, leupeptin hemisulfate, aprotinin (Sigma, St. Louis), HEPES buffer (Wako, Osaka, Japan), captopril (Research Biochemicals International, Natick, MA) and pepstatin A (Chemicon International, CA). 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. Pressure loading-induced increase of MAP kinase activity in rat aortae

After 60 min of equilibration perfusion at basal pressure (10 mm Hg), the perfusion pressure was elevated for 30 min by constricting a silicone tube connected to the end of the outlet cannula in the isolated rat aortae. Increases in perfusion pressure (100–200 mm Hg) caused a pressure-dependent increase in MAP kinase activity in aortae, when compared with MAP kinase activity level in aortae perfused at the basal pressure instead of the elevated perfusion pressure (Fig. 1A). The MAP kinase activity in aortae perfused at the basal pressure was greater than that of aortae, which were not perfused. The pressure loading (200 mm Hg)-induced increase in MAP kinase activity was time dependent (Fig. 1B). The enzymes were moderately stimulated 10 min after pressure loading and their activity reached a maximum of 30 min after pressure loading. Then, the increase in enzyme activity decreased 60 min after pressure loading. In the aortae perfused at basal pressure, MAP kinase activities were not different among the three time groups.

To examine the role of the endothelium in the pressure loading-induced MAP kinase activation, we next removed the endothelium by rubbing the intimal surface with a cotton swab. The MAP kinase activity 30 min after perfusion at basal pressure in endothelium-denuded aortae (Fig. 2) was greater than that seen in endothelium-intact aortae (Fig. 1) ($P < 0.05$). Elevation of perfusion pressure (200 mm Hg) further produced a small but significant increase in MAP kinase activity in aortae (Fig. 2). After the experiments, we cut slices of tissue and stained them with hematoxylin and eosin. Endothelium-denuded aortae consistently had no endothelial cells (data not shown).

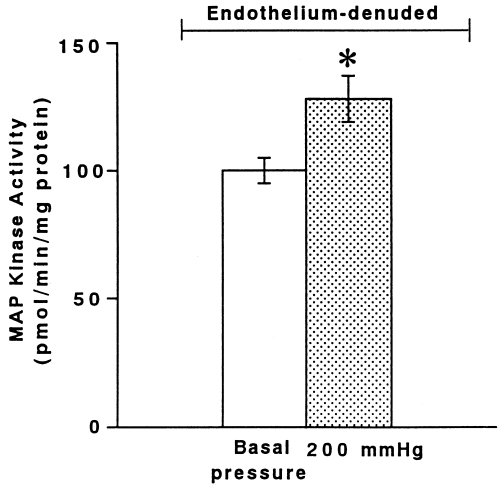

Fig. 2. MAP kinase activity 30 min after perfusion at basal pressure and at 200 mm Hg in isolated endothelium-denuded aortae of rats. Values are means ± S.E.M. from six experiments. * $P < 0.05$, compared with basal.

3.2. Effects of losartan, pepstatin A, and captopril on pressure loading-induced increases in MAP kinase activity in rat aortae

Increases in perfusion pressure (200 mm Hg) for 20 min also caused an increase in MAP kinase activity in isolated aortae. All further experiments were performed using this experimental condition, i.e., 200 mm Hg for 20 min. The angiotensin receptor antagonist losartan (10^{-7} and 10^{-6}

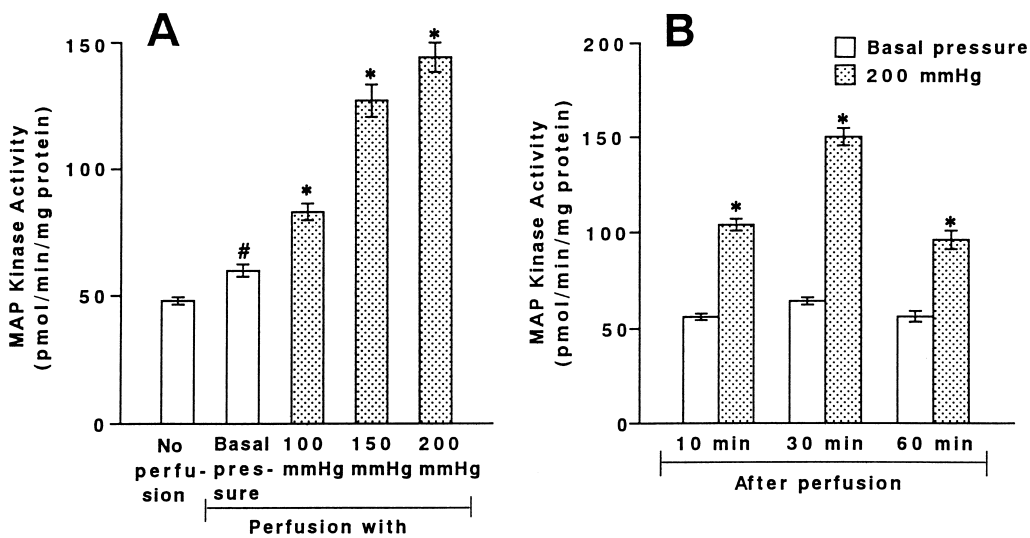

Fig. 1. (A) Effects of perfusion pressure on MAP kinase activity in isolated aortae of rats. First, the aorta was perfused for 60 min without constriction of a silicone tube connected to the end of the outlet cannula. Then, the silicone tube was constricted for 30 min to elevate perfusion pressure to 100, 150 or 200 mm Hg. In the basal perfusion pressure group (basal perfusion), the silicone tube was not constricted and the perfusion pressure was 10 mmHg. In the no perfusion group, aortae were isolated but not perfused. Values are means ± S.E.M. from four experiments. * $P < 0.05$, compared with basal. # $P < 0.05$, compared with no perfusion. (B) Time course of MAP kinase activity after perfusion at basal pressure and at 200 mm Hg in isolated aortae of rats. Values are means ± S.E.M. from four experiments. * $P < 0.05$, compared with respective basal.

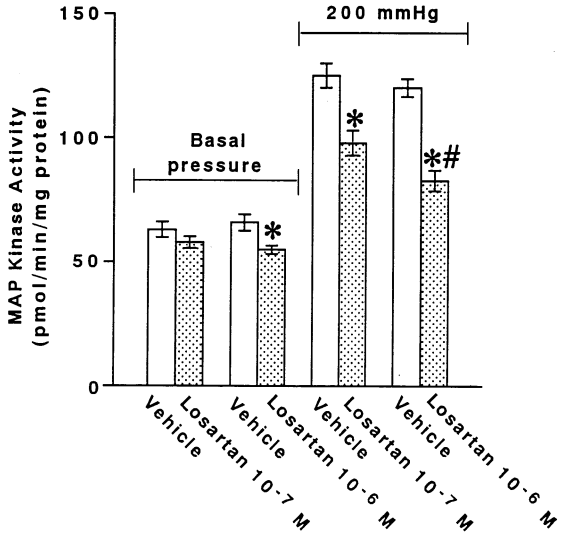

Fig. 3. Effects of losartan on MAP kinase activity 20 min after perfusion at basal pressure and at 200 mm Hg in isolated aortae of rats. Saline (vehicle) and losartan were dissolved in the perfusion buffer and perfused 5 min before and during perfusion at basal pressure or at 200 mm Hg. Values are means ± S.E.M. from five experiments with losartan 10^{-7} M and from 10 experiments with losartan 10^{-6} M. $^{*}P < 0.05$, compared with respective vehicle. $\#P < 0.05$, compared with basal pressure-losartan 10^{-6} M.

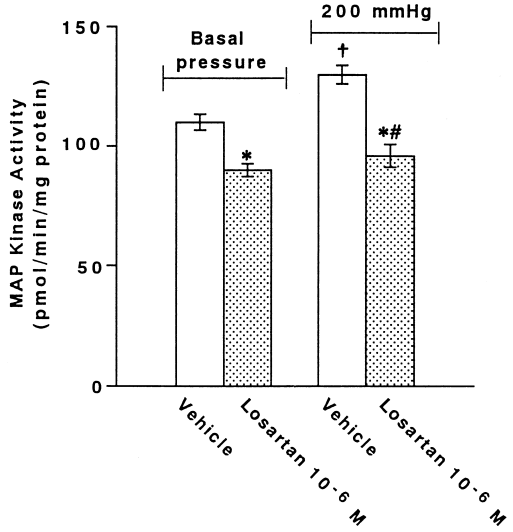

Fig. 5. Effects of losartan on MAP kinase activity 20 min after perfusion at basal pressure and at 200 mm Hg in isolated endothelium-denuded aortae of rats. Saline (vehicle) and losartan were dissolved in the perfusion buffer and perfused 5 min before and during perfusion at basal pressure or at 200 mm Hg. Values are means ± S.E.M. from six experiments. $^{*}P < 0.05$, compared with respective vehicle. $\#P > 0.05$, compared with basal pressure-losartan. †$P < 0.05$, compared with basal pressure-vehicle.

M) inhibited the pressure loading-induced increase in MAP kinase activity in isolated aortae, and this antagonist minimally inhibited the MAP kinase activity in aortae perfused at basal pressure (Fig. 3). The MAP kinase activity in losartan (10^{-6} M)-treated aortae perfused at 200 mm Hg was still greater than that of losartan (10^{-6} M)-treated aortae perfused at basal pressure. The renin inhibitor pepstatin A 10^{-5} M and the angiotensin converting enzyme inhibitor captopril 10^{-6} M also inhibited the pressure loading-induced increase in MAP kinase activity in isolated aortae, whereas these agents minimally inhibited MAP kinase activity in aortae perfused at basal pressure (Fig. 4A and B).

In isolated perfused endothelium-denuded aortae, losartan 10^{-6} M also inhibited MAP kinase activity in aortae perfused at basal pressure and in aortae perfused at 200 mm Hg (Fig. 5). The MAP kinase activity in losartan-

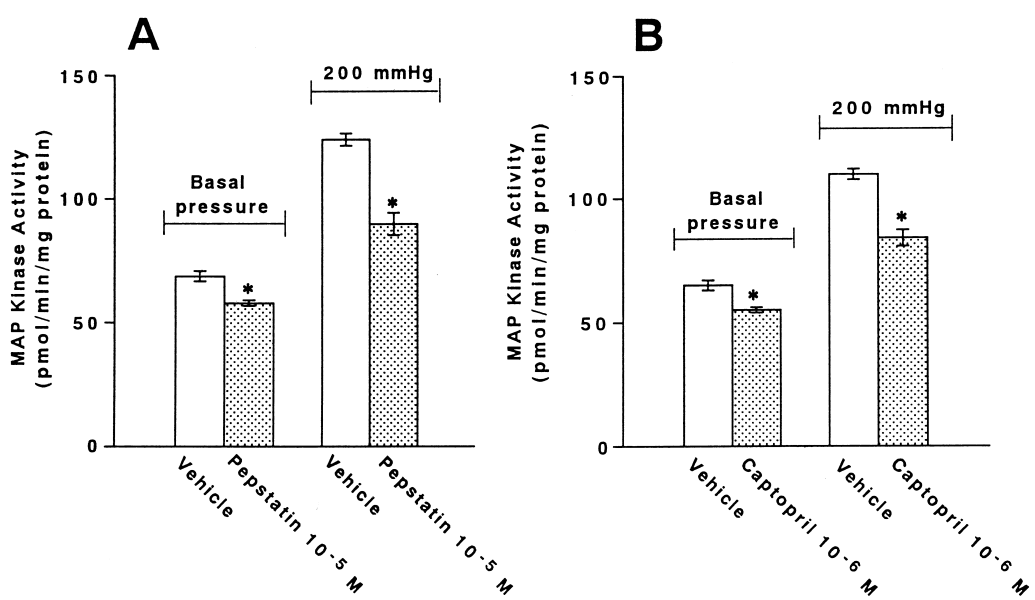

Fig. 4. Effects of pepstatin A (A) and captopril (B) on MAP kinase activity 20 min after perfusion at basal pressure and at 200 mm Hg in isolated aortae of rats. Saline (vehicle), pepstatin A, and captopril were dissolved in the perfusion buffer and perfused 5 min before and during perfusion at basal pressure or at 200 mm Hg. Values are means ± S.E.M. from six experiments. $^{*}P < 0.05$, compared with respective vehicle.

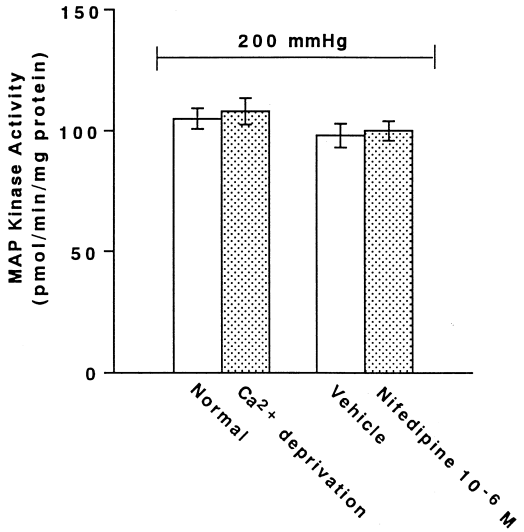

Fig. 6. Effects of Ca^{2+} deprivation and nifedipine on MAP kinase activity 20 min after perfusion at 200 mmHg in isolated aortae of rats. In Ca^{2+} deprivation experiments, Ca^{2+}-free and EGTA 2 mM-containing buffer or normal buffer was perfused 5 min before and during perfusion at 200 mm Hg. Saline (vehicle) and nifedipine were dissolved in the perfusion buffer and perfused 5 min before and during the perfusion at 200 mm Hg. Values are means ± S.E.M. from six experiments.

treated aortae perfused at 200 mm Hg was almost the same as that of losartan-treated aortae perfused at basal pressure.

3.3. Effects of Ca^{2+} deprivation and nifedipine on pressure loading-induced increases in MAP kinase activity in rat aortae

Ca^{2+} depletion of the perfusion medium did not affect the pressure loading-induced increase in MAP kinase activity in isolated aortae (Fig. 6). The voltage-dependent Ca^{2+} channel antagonist, nifedipine, also did not affect the pressure loading-induced MAP kinase activation in isolated aortae (Fig. 6).

4. Discussion

In the present study, elevation of the perfusion pressure in isolated rat aortae caused a pressure-dependent increase of MAP kinase activity. Thus, the results of the present study suggest that pressure loading of the vascular wall can directly activate MAP kinases in the vasculature. Perfusion flow would cause both pressure stress and shear stress to the vascular wall. Since we did not change the perfusion flow rate in this study, shear stress would not be different in aortae perfused at basal pressure and at elevated pressures. Thus, the pressure loading-induced elevation of MAP kinase activity in aortae seems to be mainly due to pressure stress.

In the present study, increases in MAP kinase activity induced by pressure loading in isolated aortae were inhibited by the angiotensin receptor antagonist, losartan, the renin inhibitor, pepstatin A, and the angiotensin converting enzyme inhibitor, captopril. Losartan 10^{-6} M, pepstatin A 10^{-5} M, and captopril 10^{-6} M used in this study nearly abolished MAP kinase activation induced by angiotensin II 10^{-8} M, renin 0.1 unit/ml, and angiotensin I 10^{-7} M, respectively, and maximally inhibited MAP kinase activation induced by endothelium removal in isolated rat aortae (Kubo et al., 1998a, 1999b). In addition, losartan did not inhibit endothelin-1-induced MAP kinase activation, pepstatin A did not inhibit angiotensin I- and angiotensin II-induced MAP kinase activation, and captopril did not inhibit angiotensin II-induced MAP kinase activation in isolated rat aortae (Kubo et al., 1998a, 1999b). These findings suggest that losartan antagonizes angiotensin receptors, pepstatin A inhibits renin enzyme activity, and captopril inhibits angiotensin converting enzyme activity, specifically (Kubo et al., 1998a, 1999b). Thus, the results of the present study suggest that the pressure loading-induced MAP kinase activation is mediated, at least in part, via the production and release of angiotensin II in isolated aortae.

MAP kinase activity in aortae perfused with basal pressure (10 mm Hg) was greater than that of aortae which were not perfused, suggesting that perfusion at basal pressure also activates these enzymes. In the present study, losartan, pepstatin A, and captopril produced a decrease in MAP kinase activity level in aortae perfused at basal pressure. In a previous study (Kubo et al., 1998a), we found that losartan did not affect MAP kinase activity in aortae which were not perfused. Thus, the basal pressure-induced MAP kinase activation is also partly mediated via the vascular angiotensin system.

The vascular wall is composed of endothelial cells, smooth muscle cells, and fibroblasts. Endothelial and vascular smooth muscle cell integrity is thought to be a crucial factor in the maintenance of the structural properties of the vascular wall (Clowes et al., 1983; Powell et al., 1989). In the present study, endothelial denudation of the aorta activated MAP kinases and attenuated the pressure-induced MAP kinase activation. Thus, it seems likely that the pressure effect on MAP kinase activity is largely mediated by the endothelium. It is probable that pressure blunts the inhibitory influence of endothelial cells on local angiotensin synthesis in vascular smooth muscle cells which in turn activates MAP kinases. The inhibitory factor involved may be nitric oxide (see Kubo et al., 1998a).

Nevertheless, the MAP kinase activation induced by increases in perfusion pressure was still partly found in endothelium-denuded aortae. Thus, it seems likely that smooth muscle cells and/or fibroblasts also, at least in part, mediate the pressure effect on MAP kinase activity. It has been reported that mechanical strain to cultured vascular smooth muscle cells induces mitogenesis and proliferation (Wilson et al., 1993; Li et al., 1997).

The MAP kinase activity in losartan (10^{-6} M)-treated aortae perfused at 200 mm Hg was still greater than that in

losartan-treated aortae perfused at basal pressure. The dose 10^{-6} M of losartan was enough to abolish the angiotensin II-induced MAP kinase activation completely (Kubo et al., 1998a, 1999b). Thus, the results of the present study suggest that angiotensin II is involved in the pressure loading-induced MAP kinase activation but mechanisms other than those affecting the angiotensin system are also involved in the MAP kinase activation. In endothelium-denuded aortae, the MAP kinase activity in losartan (10^{-6} M)-treated aortae perfused at 200 mm Hg was almost the same as that in losartan-treated aortae perfused at basal pressure. These findings suggest that in endothelium-denuded aortae, angiotensin II is mainly involved in the pressure loading-induced MAP kinase activation.

Vascular smooth muscle cell membranes have stretch-activated channels which allow Ca^{2+} ions to enter (Ohya et al., 1998). It has been demonstrated that stretching of cultured aortic vascular smooth muscle cells enhances Ca^{2+} uptake through a voltage-dependent dihydropyridine-sensitive Ca^{2+} entry pathway (Ruiz-Velasco et al., 1996). The influx of Ca^{2+} could activate MAP kinases to alter the rate of cell proliferation (Eguchi et al., 1996). In the present study, however, Ca^{2+} deprivation and the dihydropyridine-sensitive Ca^{2+} channel antagonist, nifedipine, did not affect the pressure loading-induced increase of MAP kinase activity. Nifedipine at 10^{-6} M blocks voltage-dependent Ca^{2+} channels in vascular smooth muscle cells (Kubo et al., 1998b). These findings suggest that Ca^{2+} entry is not the main event involved in the pressure loading-induced MAP kinase activation in isolated aortae. The main source of Ca^{2+} as mediator of the action of angiotensin in vascular smooth muscle is the intracellular stores, which are not inhibited by EGTA and nifedipine. The possible involvement of the release of Ca^{2+} from intracellular stores in the pressure-induced MAP kinase activation remains to be settled.

From the results of the present study, it can be speculated that pressure overloading following hypertension activates MAP kinases in the vascular wall and that this MAP kinase activation may be responsible for vascular hypertrophy. MAP kinase activation is indeed followed by c-*fos* and c-*jun* gene expression (Alvarez et al., 1991; Pulverer et al., 1991). The activities of c-*Fos* and c-*Jun* proteins are modulated through their phosphorylation by the MAP kinases. Fos and Jun proteins combine to form stable AP-1 heterodimers, which bind to AP-1 consensus sequences present in genes associated with cell proliferative and hypertrophic responses (Bishopric et al., 1992).

Xu et al. (1996) have demonstrated that an acute elevation of blood pressure activates MAP kinases in rat arteries (aorta, carotid, and femoral arteries). Since they used angiotensin II and phenylephrine, vasoconstrictors, to elevate blood pressure, it could not be ruled out that angiotensin II and phenylephrine directly contribute to the activation of MAP kinases in the arterial wall through mechanisms other than mechanical stress. Indeed, angiotensin II activates MAP kinases in vascular smooth muscle cells or in vascular wall (Tsuda et al., 1992; Duff et al., 1995; Kubo et al., 1998a), and phenylephrine activates MAP kinases in cardiac muscle cells (Thorburn, 1994). Our findings provide direct evidence that mechanical stress itself serves as a stimulus for MAP kinase activation in the arterial wall.

From the results of the present study, the origins of angiotensin II responsible for the pressure loading-induced MAP kinase activation in rat aortae are unclear. It is reported that this peptide is released from the endothelium (Dzau and Gibbons, 1988; Bobik and Campbell, 1993; Peiro et al., 1995). In addition, angiotensin II also exists in the media of the vascular wall and is released to cause MAP kinase-stimulating effects in medial smooth muscle cells (Kubo et al., 1998a). Li et al. (1997) have shown that the proliferation of cultured vascular smooth muscle cells induced by cyclic stretching is suppressed by saralasin, an angiotensin receptor antagonist, and by captopril, suggesting that angiotensin II produced in vascular smooth muscle cells is involved in the stretch-induced proliferation of the cells.

In summary, in the present study, pressure loading of the vascular wall caused a pressure-dependent increase of MAP kinase activity in isolated rat aortae. The increase in MAP kinase activity induced by pressure loading was inhibited by losartan, pepstatin A, and captopril. These findings suggest that pressure overloading of the vascular wall, per se, can activate MAP kinases in the vasculature. The MAP kinase activation is mediated at least partly via the vascular renin–angiotensin system.

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