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Local renin–angiotensin system and mitogen-activated protein kinase activation in rat aorta

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Abstract

We previously reported that endogenous angiotensin II is released to cause mitogen-activated protein (MAP) kinase stimulation in the media portion of the vasculature. In this study, we examined whether a functional renin–angiotensin system is indeed present within the media of the vasculature. In rat aortic strips, endothelium removal produced an increase of MAP kinase activity. The MAP kinase activation was inhibited either by the renin inhibitor pepstatin A or by the angiotensin-converting enzyme inhibitor captopril. The degree of the inhibition of the MAP kinase activation by pepstatin A, captopril and the angiotensin receptor antagonist losartan was almost the same. Pepstatin A inhibited MAP kinase activation induced by renin but not by angiotensin I and angiotensin II. Captopril inhibited the MAP kinase activation induced by angiotensin I but not by angiotensin II. In nephrectomized rat aortic strips, endothelium removal also produced an increase in MAP kinase activity, but the MAP kinase activation was considerably small and minimally inhibited by losartan. Nephrectomy produced a marked decrease in plasma renin activity. These findings suggest that an apparently fully intact and functional renin–angiotensin system is present in the media of the rat vasculature and this system serves to increase MAP kinase activity. It appears that renin plays the determining role in the regulation of angiotensin generation also in the media and the major source of the renin is renin of kidney origin.

Keywords: Renin; Angiotensin; MAP (mitogen activated protein) kinase; Aorta, rat; Nephrectomy

1. Introduction

The renin–angiotensin system plays a critical role in blood-pressure regulation and fluid and electrolyte balance. Evidence demonstrates the presence of renin–angiotensin systems in local tissues, including the brain, heart, kidney and the vascular wall (Campbell, 1987; Dzau, 1988; Bader et al., 1994).

In the vascular wall, there exist angiotensinogen, renin, angiotensin-converting enzyme (Dzau and Gibbons, 1988) and renin binding protein (Takahashi et al., 1983). In addition, messenger RNAs (mRNAs) for angiotensinogen, renin and angiotensin-converting enzyme are present in the vascular wall (Campbell and Habener, 1986; Cassis et al., 1988; Samani et al., 1988; Naftilan et al., 1991; Fishel et al., 1995), suggesting that these components for angiotensin generation may be synthesized within the vascular wall. On the other hand, it has been reported that angiotensinogen and renin in circulation are taken up into blood vessels to generate angiotensins (Loudon et al., 1983; Kato et al., 1993). These lines of evidence suggest the presence of renin–angiotensin system in the vascular wall. However, little is known about the exact localization of local renin–angiotensin system and exact physiological roles of the system in the vascular wall.

Mitogen-activated protein (MAP) kinases, members of a family of serine/threonine-specific protein kinases (Kosako et al., 1992), are considered to play an important role in mediating signals from the growth factor receptors to ribosomes and nucleus (Sturgill et al., 1988; Pulverer et al., 1991; Tsuda et al., 1992). Previously, we demonstrated that in rat aortic strips, endothelium removal produced an increase of MAP kinase activity (Kubo et al., 1998). The MAP kinase activation was found in the media portion but not in the adventitia portion. These findings suggest that in arteries, MAP kinases are tonically activated in the medial layer of the vessel wall and this MAP kinase activation is

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tonically inhibited by the endothelium. The MAP kinase activation was inhibited by the angiotensin receptor antagonist losartan. In endothelium-intact aortic strips, the nitric oxide synthase inhibitor N^G-nitro-L-arginine methyl ester caused an activation of MAP kinases and this MAP kinase activation was again inhibited by losartan. From these results, we concluded that even in isolated arteries, endogenous angiotensin II is tonically released to cause MAP kinase-stimulation in the media and this angiotensin II-induced MAP kinase activation is tonically inhibited by nitric oxide released from the endothelium. These findings provide evidence for a physiological role of endogenous angiotensin II in the media portion of the vasculature. However, how the angiotensin II responsible for the MAP kinase activation is supplied in the media portion of the vasculature remains to be clarified, since a significant amount of angiotensin II is believed to be generated mainly within the endothelium of the vasculature (Dzau, 1988).

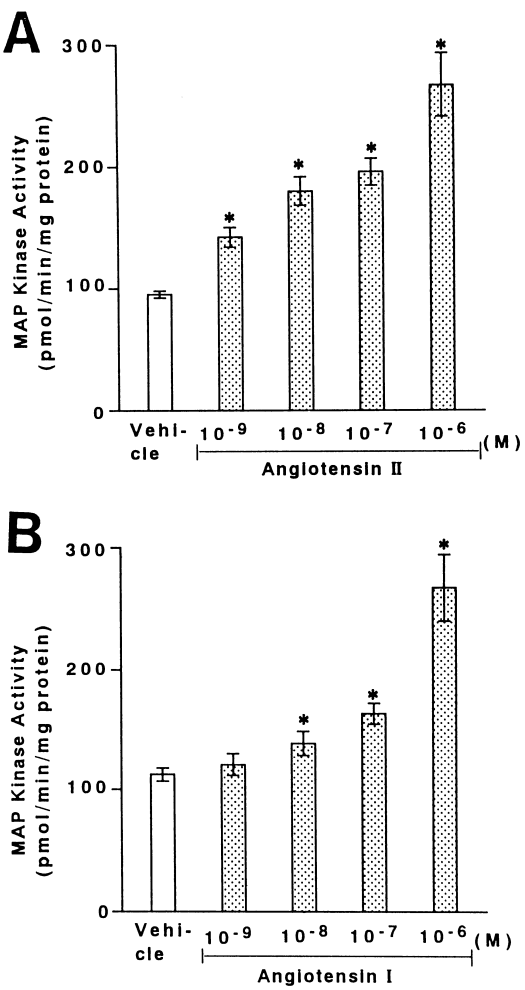

Fig. 1. Effects of angiotensin II (A) and angiotensin I (B) on MAP kinase activity in endothelium-denuded aortic strips of rats. Aortic strips were preincubated for 5 min in 37°C DMEM and then a 10-min incubation was started. Saline (vehicle), angiotensin II and angiotensin I were added into DMEM at the beginning of the 10-min incubation. Values are mean ± S.E.M. from four experiments. $* P < 0.05$, compared with vehicle.

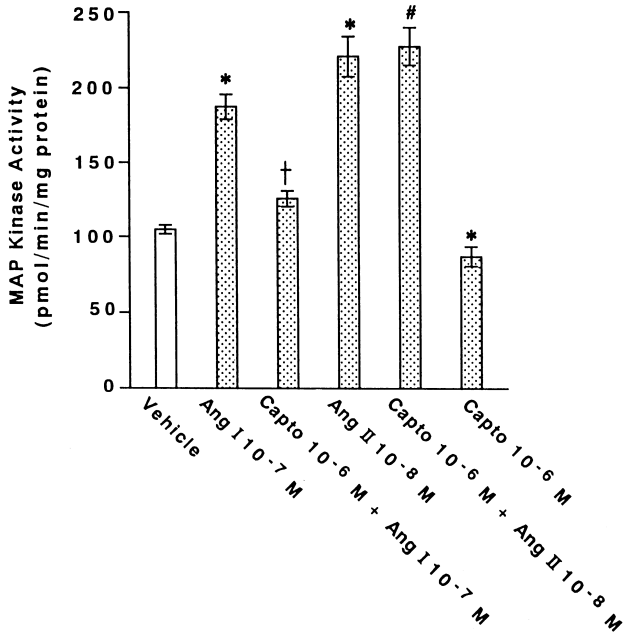

Fig. 2. Effects of captopril on angiotensin I- and angiotensin II-induced increases in MAP kinase activity in endothelium-denuded aortic strips of rats. Aortic strips were preincubated for 5 min in 37°C DMEM and then a 10-min incubation was started. Saline (vehicle) and captopril (capto) were added into DMEM at the beginning of the 5-min preincubation. Angiotensin I (Ang I) and angiotensin II (Ang II) were added into DMEM at the beginning of the 10-min incubation. Values are mean ± S.E.M. from five experiments. $* P < 0.05$, compared with vehicle. $^{+} P < 0.05$, compared with Ang I alone. $^{\#} P > 0.05$, compared with Ang II alone.

A possible mechanism responsible for the supply of angiotensin II in the media of the vasculature is that angiotensin II of endothelial origin is taken up and stored in the media of the vasculature, and that this angiotensin II is slowly released in the media. Alternatively, it is possible that the media portion itself of the vasculature has a local renin–angiotensin system and angiotensin II is supplied through the system within the media. Endothelium-denuded portion of arteries is indeed reported to still have angiotensin-converting enzyme activity (Pipili-Synetos et al., 1990).

In the present study, we measured the activity of MAP kinases in rat endothelium-denuded aortic strips, and examined effects of a renin inhibitor and an angiotensin-converting enzyme inhibitor, to obtain evidence for the existence of local renin–angiotensin system responsible for the regulation of MAP kinase activity in the media of the vasculature. In addition, we examined effects of nephrectomy to investigate possible participation of renin of kidney origin in the local renin–angiotensin system.

2. Materials and methods

Male Wistar rats (200–260 g) were killed by overdoses of ether. In some experiments, rats were bilaterally

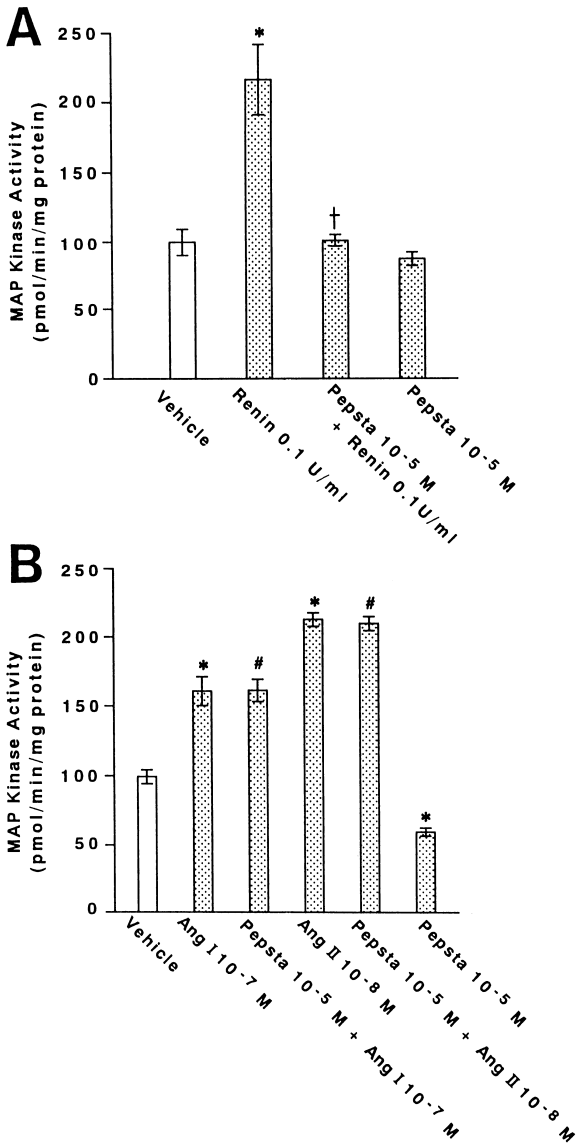

Fig. 3. Effects of renin (A) or pepstatin A (B) on MAP kinase activity in aortic strips of rats. In (A), effects of renin on MAP kinase activity and an antagonistic effect of pepstatin A on the renin-induced activation of MAP kinase activity in endothelium-denuded aortic strips of rats are shown. In (B), effects of pepstatin A on angiotensin I- and angiotensin II-induced increases in MAP kinase activity in endothelium-denuded aortic strips of rats are shown. Aortic strips were preincubated for 5 min in 37°C DMEM and then a 10-min incubation was started. DMSO (vehicle) and pepstatin A (pepsta) were added into DMEM at the beginning of the 5-min preincubation. Renin, angiotensin I (Ang I) and angiotensin II (Ang II) were added into DMEM at the beginning of the 10-min incubation. Values are mean ± S.E.M. from five experiments. $* P < 0.05$, compared with vehicle. $^{+} P < 0.05$, compared with renin alone. $^{\#} P > 0.05$, compared with Ang I alone and Ang II alone, respectively.

nephrectomized 24 or 36 h before sacrifice. 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 (Su et al., 1986). The aorta was washed twice and cut into six to eight strips (approximately 3 mm × 4 mm each). 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 immunoglobulin G1 antibody (BMA Biomedicals, Augst, Switzerland), whereas intact aortic strips had endothelial cells stained to the antibody (data not shown).

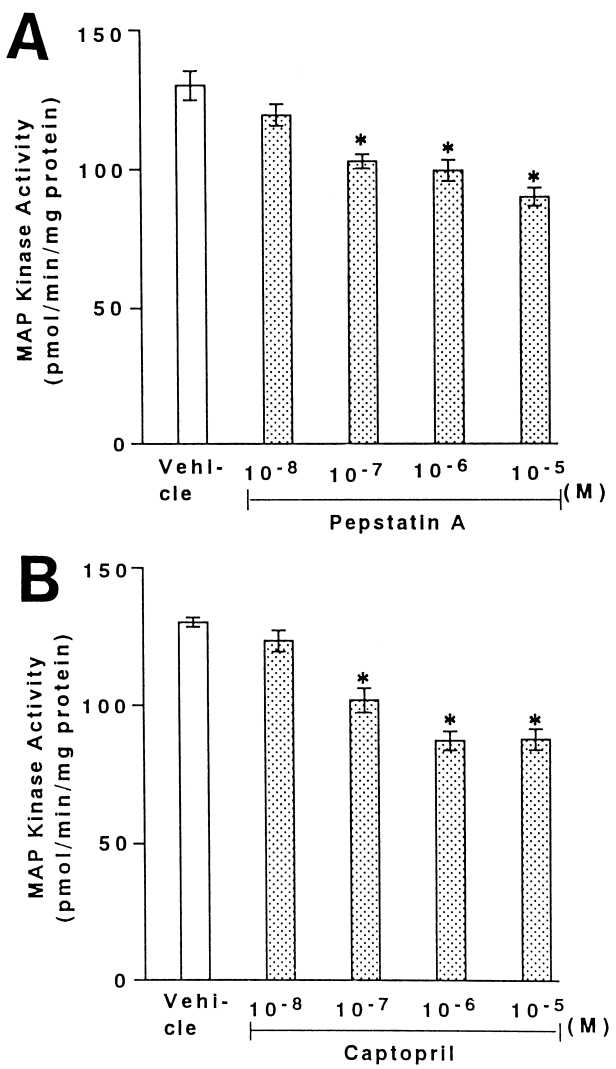

Fig. 4. Concentration-dependent effects of pepstatin A (A) and captopril (B) on the increase in MAP kinase activity after 30-min incubation in endothelium-denuded aortic strips of rats. Aortic strips were preincubated for 5 min in 37°C DMEM and then a 30-min incubation was started. Drugs were added into DMEM at the beginning of the 30-min incubation. Values are mean ± S.E.M. from five experiments. $* P < 0.05$, compared with vehicle.

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 incubator containing an atmosphere of 95% air and 5% CO_2. In preliminary experiments, endothelium-removal-induced MAP kinase activation was maximal 30 min after incubation, and angiotensin II-induced MAP kinase activation was maximal 5 and 10 min after angiotensin II (10^{-8} M). Thus, 10-min incubation and 30-min incubation were used in angiotensin II-induced MAP kinase activation experiments and endothelium removal-induced MAP kinase activation experiments, respectively. Drugs were added into DMEM either at the beginning of the incubations or at the beginning of the preincubation. 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. The reaction was terminated by chilling the plates on ice and washing twice with ice-cold phosphate-buffered saline.

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)methansulphonyl fluoride, 10 μg/ml leupeptin and 10 μg/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 kinases.

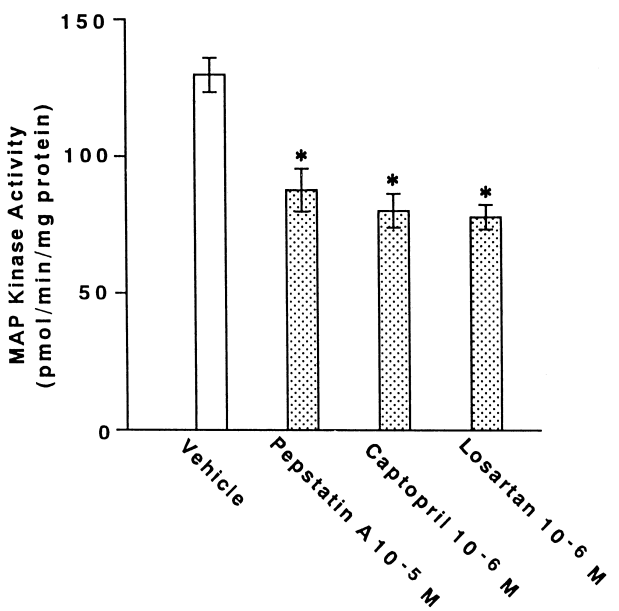

Fig. 5. Effects of pepstatin A, captopril and losartan on the increase in MAP kinase activity after 30-min incubation in endothelium-denuded aortic strips of rats. Saline (vehicle), pepstatin A, captopril and losartan were added into DMEM at the beginning of the 30-min incubation. Values are mean ± S.E.M. from five experiments. * $P < 0.05$, compared with vehicle.

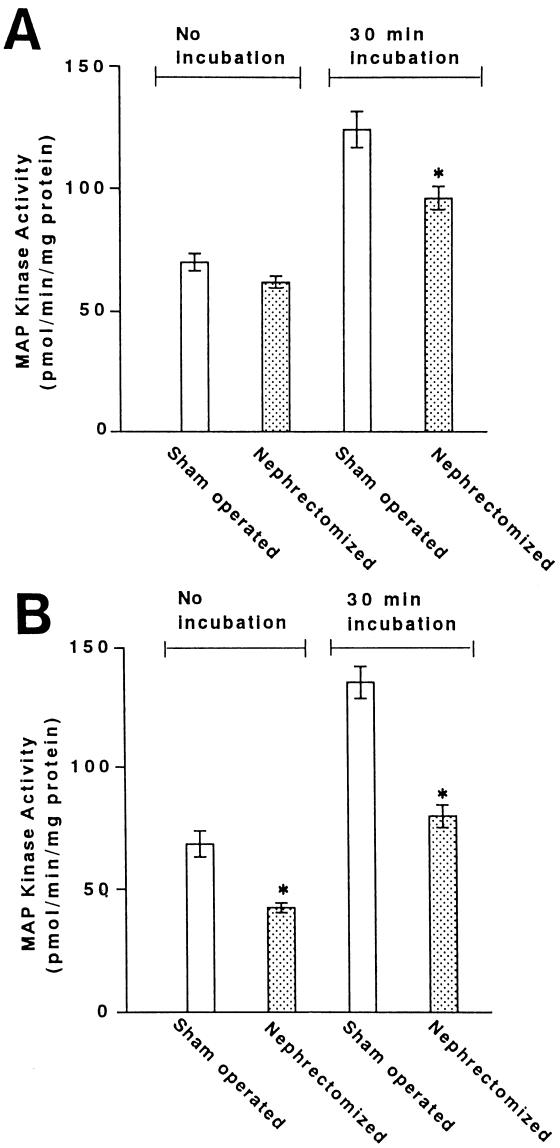

Fig. 6. MAP kinase activity levels at no incubation and after 30-min incubation in endothelium-denuded aortic strips from sham-operated and nephrectomized rats. To prepare nephrectomized rats, rats were bilaterally nephrectomized 24 h (A) or 36 h (B) before sacrifice. Values are mean ± S.E.M. from five experiments. * $P < 0.05$, compared with respective sham operated.

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 adeno-

sine-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 microliters 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 a solution of orthophosphoric acid containing carmosine red. Then, 30 μl of terminated reaction mixture were 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). Plasma renin activity was measured by radioimmunoassay (Haber et al., 1969).

Drugs used were angiotensin I acetate salt, angiotensin II acetate salt, porcine renin (Sigma, St. Louis, MO), captopril (Research Biochemicals International, Natick, MA), pepstatin A (Chemicon International, California), HEPES buffer (Wako, Osaka, Japan), leupeptin hemisulfate, aprotinin (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. 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 comparison. Differences were considered significant at $P < 0.05$.

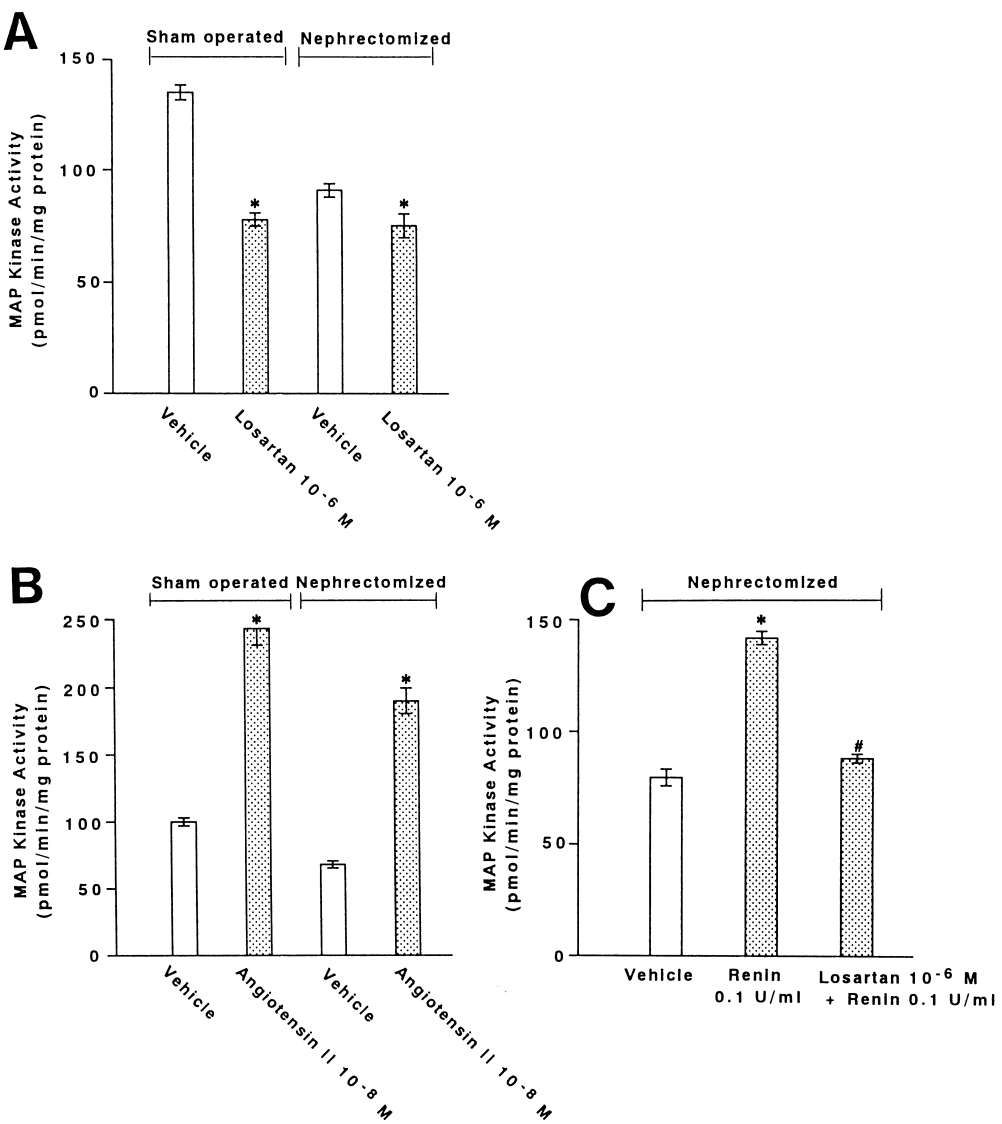

Fig. 7. Effects of losartan (A), angiotensin II (B) and renin (C) on MAP kinase activity in aortic strips from 36-h-sham-operated and 36-h-nephrectomized rats. Aortic strips were preincubated for 5 min in 37°C DMEM and then a 10-min (in B,C) or 30-min (in A) incubation was started. Drugs were added at the beginning of the 10-min or 30-min incubation. In C, losartan was added into DMEM at the beginning of the 5-min preincubation. Values are mean ± SEM from five experiments. $* P < 0.05$, compared with respective vehicle. $^{\#}P < 0.05$, compared with renin alone.

3. Results

3.1. Effects of angiotensin II, angiotensin I and renin on MAP kinase activity

Following a 5-min preincubation at 37°C in DMEM, incubation of endothelium-denuded aortic strips was started at 37°C in a moist tissue culture incubator containing an atmosphere of 95% air and 5% CO_2. MAP kinase activity was increased time-dependently (Kubo et al., 1998). When angiotensin II (10^{-9} to 10^{-6} M) was added into DMEM at the beginning of the 10-min incubation, it caused a concentration-dependent increase in MAP kinase activity in endothelium-denuded aortic strips (Fig. 1A). Angiotensin I (10^{-9} to 10^{-6} M) also caused a concentration-dependent increase in MAP kinase activity in the strips (Fig. 1B). The angiotensin I-induced but not the angiotensin II-induced increase in MAP kinase activity was inhibited by the angiotensin-converting enzyme inhibitor captopril (10^{-6} M)(Fig. 2).

Renin (0.1 U/ml) caused an increase in MAP kinase activity in endothelium-denuded aortic strips (Fig. 3A). The renin inhibitor pepstatin A (10^{-5} M) inhibited the renin-induced increase in MAP kinase activity (Fig. 3A), whereas the renin inhibitor did not affect the angiotensin I- and angiotensin II-induced increase in MAP kinase activity (Fig. 3B).

3.2. Effects of pepstatin A and captopril on MAP kinase activation after endothelium removal

The renin inhibitor pepstatin A (10^{-8} to 10^{-5} M) inhibited the increase of MAP kinase activity in endothelium-denuded aortic strips after 30-min incubation in a concentration-dependent manner (Fig. 4A). Maximal inhibition of the MAP kinase activation was obtained at 10^{-5} M. The angiotensin-converting enzyme inhibitor captopril (10^{-8} to 10^{-5} M) also inhibited the increase of MAP kinase activity in endothelium-denuded aortic strips after 30 min incubation in a concentration-dependent manner (Fig. 4B). Maximal inhibition of the MAP kinase activation was obtained at 10^{-6} M. The inhibitions of the endothelium removal-induced MAP kinase activation, induced by captopril (10^{-6} M), pepstatin A (10^{-5} M) and the angiotensin receptor inhibitor losartan (10^{-6} M) were almost the same (Fig. 5). The vehicle DMSO itself did not affect the MAP kinase activation after endothelium removal (data not shown).

3.3. Endothelium removal-induced MAP kinase activation in aortic strips from nephrectomized rats

In endothelium-denuded aortic strips from nephrectomized rats (24 and 36 h), MAP kinase activity before incubation was smaller than that of sham-operated rats (Fig. 6). MAP kinase activity in both sham-operated and nephrectomized rat aortic strips was increased after 30 min incubation, but the MAP kinase activities after 30 min incubation were smaller by $23 \pm 4\%$ and by $41 \pm 4\%$ in the aortic strips from 24-h- and 36-h-nephrectomized rats, respectively, than those from respective sham-operated rats ($P < 0.05$)(Fig. 6). The angiotensin receptor antagonist losartan (10^{-6} M) caused an inhibition of the endothelium removal-induced MAP kinase activation in sham-operated and nephrectomized rat aortic strips, but the degree of the inhibition was smaller in nephrectomized rat aortic strips ($-17 \pm 3\%$) than that of sham-operated rat aortic strips ($-48 \pm 5\%$, $P < 0.05$)(Fig. 7 A). Angiotensin II (10^{-8} M) produced an increase in MAP kinase activity in endothelium-denuded aortic strips from both nephrectomized rats and sham-operated rats (Fig. 7B). In nephrectomized rat aortic strips, addition of renin again caused an increase in MAP kinase activity and the renin-induced increase in MAP kinase activity was inhibited by losartan (10^{-6} M)(Fig. 7C). Plasma renin activity was decreased 3, 24, and 36 h after nephrectomy (Table 1).

Table 1
Plasma renin activity [ng Ang I/(ml h)] in nephrectomized rats

Control	After nephrectomy		
	3 h	24 h	36 h
5.6 ± 0.8	1.6 ± 0.4[a]	< 0.1[a]	< 0.1[a]

Values are mean ± S.E.M. ($n = 5$).
[a] Significantly different from control ($P < 0.05$).

4. Discussion

In a previous study, we demonstrated that in rat aortic strips, endothelium removal caused an increase of MAP kinase activity and the increase in MAP kinase activity was inhibited by the angiotensin receptor antagonist losartan, suggesting that the increase in MAP kinase activity is mediated through angiotensin II (Kubo et al., 1998). In addition, the increase in MAP kinase activity was found in the media portion of aortic strips but not in the adventitia portion of the strips, suggesting that the MAP kinase activation occurs mainly in the media of the strips.

Angiotensin II is well known to be generated in the endothelium, but it is unclear whether this peptide is also generated in the media of the vasculature, and thus, it is uncertain whether the angiotensin II responsible for the MAP kinase activation is indeed due to angiotensin II generated in the media portion or due to angiotensin II previously produced in the endothelium and straged in the media portion of the vasculature. In the present study,

however, the increase in MAP kinase activity after endothelium removal was inhibited either by the renin inhibitor pepstatin A or by the angiotensin-converting enzyme inhibitor captopril. Pepstatin A indeed inhibited renin-induced MAP kinase activation but did not inhibit angiotensin I- and angiotensin II-induced MAP kinase activation, suggesting that pepstatin A inhibits renin activity specifically in this preparation. Captopril inhibited angiotensin I-induced but not angiotensin II-induced MAP kinase activation, suggesting that captopril inhibits the activity of angiotensin-converting enzyme specifically in this preparation. Therefore, the results of the present study demonstrate that angiotensin II generated via renin and angiotensin-converting enzyme mediates the increase in MAP kinase activity after endothelium removal. Since in the previous study (Kubo et al., 1998), we demonstrated that the MAP kinase activation after endothelium removal was found only in the media portion but not in the adventitia portion of the vasculature, the results of the present study suggest the presence of a functional local renin–angiotensin system in the media of the vasculature.

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 the endothelium removal-induced MAP kinase activation. In addition, renin application onto endothelium-denuded aortic strips caused a marked increase of MAP kinase activity and this effect was inhibited by pepstatin A. These findings suggest that renin is an important factor in determining angiotensin generation also in the media portion of the vasculature.

It has been demonstrated that renin is synthesized in vascular smooth muscle cells (Re et al., 1982) and renin mRNA is expressed in vascular tissues (Samani et al., 1988), suggesting a role of locally produced renin in vascular angiotensin generation. On the other hand, renin of kidney origin has been demonstrated to be taken up into the vascular wall (Loudon et al., 1983; Mizuno et al., 1988) and the renin taken up has been suggested to play a role in vascular angiotensin generation (Kato et al., 1993). In the present study, endothelium removal also produced MAP kinase activation in aortic strips from nephrectomized rats, but the extent of the increase of MAP kinases is much smaller in aortic strips from nephrectomized rats than that of sham-operated rats. The angiotensin receptor antagonist losartan inhibited the endothelium removal-induced MAP kinase activation in aortic strips from nephrectomized rats, but the extent of the losartan-induced inhibition of MAP kinase activation was much smaller in aortic strips from nephrectomized rats than that of sham-operated rats. Angiotensin II produced similar increases in MAP kinase activity in aortic strips from both sham-operated and nephrectomized rats, suggesting that the sensitivity to angiotensin II is normal in nephrectomized rat aortic strips. In addition, renin application into the medium produced an increase of MAP kinase activity also in nephrectomized rat aortic strips, indicating that angiotensin II generation components other than renin, for examples, angiotensinogen and angiotensin-converting enzyme, are intact or at least not seriously damaged in the nephrectomized rat aortic strips. Finally, nephrectomy caused a marked decrease of plasma renin. These findings suggest that in rats, nephrectomy causes a marked renin-dependent decrease of angiotensin II generation in the media portion of the vasculature. Therefore, it could be considered that renin of kidney origin is the major source of functional renin in the media portion of the vasculature. The results of the present study confirmed and extended the results of the study of Kato et al. (1993) showing that angiotensin release from isolated perfused rat hind legs is decreased in nephrectomized rats. The results of the present study with nephrectomized rat aortic strips are again compatible with the idea that renin is an important factor in determining angiotensin II generation also in the media of the vasculature.

In nephrectomized rat aortic strips, losartan caused only a small but still significant inhibition of MAP kinase activation, suggesting that a small amount of angiotensin II is still produced in the nephrectomized rat vessels. This might be because a small amount of renin of plasma origin remained in the vasculature even 36 h after nephrectomy. In connection with this, in 24 h-nephrectomized rats, plasma renin activity was markedly decreased but endothelium removal-induced MAP kinase activation in aortic strips was only partially decreased, suggesting that activity of renin taken up in the vasculature declines much more slowly than plasma renin. Alternatively, a small amount of renin might be produced locally within the vasculature. More studies will be needed to determine this question.

It has been demonstrated that endothelial injury by balloon catheterization causes myointimal thickening in the rat carotid and the rat aorta (Powell et al., 1989, 1990; Daemen et al., 1991; Janiak et al., 1992). From the results of the present study, it can be considered that the endothelial injury causes an increase of angiotensin II generation and thus MAP kinase activation in the media, and this MAP kinase activation next participates in the myointima formation. Indeed, chronic treatment with angiotensin-converting enzyme inhibitors and the angiotensin receptor antagonist losartan have been shown to reduce the neointima formation (Powell et al., 1989, 1990; Capron et al., 1991; Daemen et al., 1991; Osterrieder et al., 1991; Janiak et al., 1992).

In summary, this study provides evidence suggesting that an apparently fully intact and functional renin–angiotensin system is present also in the media of the vasculature and this system serves to increase MAP kinase activity. It appears that also in the local renin–angiotensin system within the media, renin plays the determining role in the regulation of angiotensin production and the major source of the renin is renin of kidney origin but not renin locally produced in the vasculature.

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