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Different activation of vascular mitogen-activated protein kinases in spontaneously and DOCA-salt hypertensive rats

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Abstract

Regulation mechanisms of the activity of vascular mitogen-activated protein (MAP) kinases, enzymes believed to be involved in the pathway for cell proliferation, may be altered in hypertension. To examine whether vascular MAP kinase activation mechanisms are altered in hypertension, we measured the activity of MAP kinases in rat aorta strips from spontaneously hypertensive rats (SHR) and from deoxycorticosterone acetate (DOCA)-salt hypertensive rats, and examined whether vascular angiotensin and endothelin systems are responsible for the alteration of MAP kinase activation in these hypertensive models. Endothelium-denuded aorta strips were incubated at 37°C in medium. MAP kinase activity after incubation was increased in rat aorta strips. The MAP kinase activation was greater in 9- and 15-week-old SHR aorta strips than in age-matched Wistar Kyoto rats (WKY) aorta strips. Similarly, MAP kinase activation was enhanced in aorta strips from DOCA-salt hypertensive rats. In aorta strips from these kinds of rats, 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 MAP kinase activation. The losartan-induced, but not BQ123-induced, inhibition of MAP kinase activation was enhanced in 15-week-old SHR aorta strips, whereas the BQ123-induced, but not losartan-induced, inhibition of MAP kinase activation was enhanced in DOCA-salt hypertensive rat aorta strips. Angiotensin II-induced MAP kinase activation was enhanced in 15-week-old SHR aorta strips, whereas it was depressed in DOCA-salt hypertensive rat aorta strips. These results indicate that MAP kinase activation function is enhanced in aorta strips from both kinds of hypertensive rats. It appears that the enhancement of MAP kinase activation results partly from enhanced vascular angiotensin system in SHR and from enhanced vascular endothelin system in DOCA-salt hypertensive rats.

Keywords: MAP (mitogen-activated protein) kinase; Angiotensin; Endothelin; Aorta; Spontaneously hypertensive rat (SHR); DOCA-salt hypertensive rat

1. Introduction

The increase in vascular wall mass is considered to be a fundamental pathogenic factor for hypertension. Although these vascular structural remodelings are thought to occur secondarily to hypertension in both genetically and non-genetically hypertensive animals (Kubo, 1978, 1979; Folkow et al., 1982; Owens and Schwartz, 1982), exact mechanisms of the vascular structural remodeling in hypertension is unknown.

Mitogen-activated protein (MAP) kinases are members of a family of serine/threonine-specific protein kinases (Kosako et al., 1992). MAP kinases play an important role in mediating signals from growth factor receptors to ribosomes and nucleus (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). Thus, these enzymes are believed to be involved in the pathway for cell proliferation and, thus, in vascular structural remodelings.

In previous studies (Kubo et al., 1998, 1999b), we demonstrated that, in rat aorta strips, endothelium removal resulted in an activation of MAP kinase activity and the MAP kinase activation was found in the media portion of the vasculature. The MAP kinase activation was inhibited by both the angiotensin receptor antgonist, losartan, and the endothelin receptor antagonist, cyclo (D-α-aspartyl-L-prolyl-D-valyl-L-leucyl-D-tryptophyl) (BQ123). These findings suggest that, in rat aorta strips, endogenous angiotensin II and endothelins are tonically released to cause MAP kinase-stimulating effects in medial smooth muscle.

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In aorta strips from prehypertensive 4-week-old spontaneously hypertensive rats (SHR), a genetically hypertensive animal model, the endothelium removal-induced MAP kinase activation was enhanced as compared with that from age-matched Wistar Kyoto rats (WKY) (Kubo et al., 1999a), suggesting that vascular structural remodeling function may be enhanced in SHR by nature. Losartan-induced, but not BQ123-induced, inhibition of the MAP kinase activation was greater in SHR than in WKY aorta strips, suggesting that the enhancement of MAP kinase activation results, at least in part, from enhanced function of the vascular angiotensin system in prehypertensive SHR. However, these findings do not necessarily mean that the enhancement of MAP kinase activation via the vascular angiotensin system occurs in SHR also at hypertensive stages and that the vascular angiotensin system indeed contributes to vascular structural remodelings in SHR with hypertension.

To investigate whether vascular structural remodeling function is also enhanced via the vascular angiotensin system in the vasculature of SHR at hypertensive stages, we first examined whether MAP kinase activation function is altered in aorta strips from 9- and 15-week-old hypertensive SHR, as compared with that of age-matched WKY, and then examined whether vascular angiotensin and endothelin systems are responsible for the altered MAP kinase activation in the vasculature of SHR with hypertension. For comparison, we also examined whether vascular MAP kinase activation function is altered in deoxycorticosterone acetate (DOCA)-salt hypertensive rats, a non-genetically hypertensive animal model.

2. Materials and methods

Male 9- and 15-week-old SHR and age-matched WKY (Charles River, Japan) were used in this study. In some experiments, male Wistar rats initially weighing 100–120 g were used to produce DOCA-salt hypertension. They were kept under alternating 12-h periods of dark and light, and given standard rat chow and tap water ad libitum, unless otherwise noted. One day before the experiments, systolic blood pressure was measured indirectly by tail plethysmography.

The animals were killed with overdoses of ether. The thoracic aorta was removed and incubated at 4°C in Tyrode solution (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 3×4 mm each). Complete removal of the endothelium was confirmed immunohistochemically (Kubo et al., 1998).

2.1. Induction of DOCA-salt hypertension

Rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.). The left kidney was removed, with care being taken to avoid adrenalectomy. Animals were given standard rat chow and a 1% w/v NaCl solution as drinking water. DOCA was injected subcutaneously twice a week at a dose of 40 mg/kg. Sham-operated controls consisted of rats, in which the left kidney was removed and solvent (Olive oil) was injected subcutaneously instead of DOCA. The control rats were maintained on a standard diet and tap water. They were used in experiments 4 weeks after surgery.

2.2. Tissue incubation and preparation of tissue extracts

The aorta strips were placed into plates (three to four strips in each plate) containing 1 ml of Dulbecco's modi-

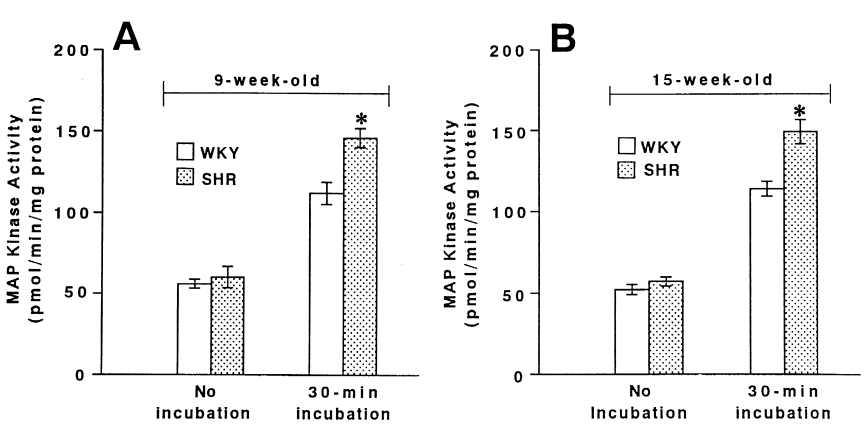

Fig. 1. MAP kinase activity levels before incubation (no incubation) and 30 min after incubation in endothelium-denuded aorta strips from 9-week-old (A) and 15-week-old (B) SHR and age-matched WKY. Aorta strips were preincubated at 37°C for 5 min, and then, 30-min incubation was started. Values are means ± S.E.M. from six experiments using different animals. * $P < 0.05$, compared with respective WKY.

Table 1
Systolic blood pressure in 9- and 15-week-old SHR, age-matched WKY, DOCA-salt hypertensive rats, and sham-operated rats

Rats	*n*	Systolic blood pressure (mm Hg)
9-week-old SHR	6	160 ± 3 *
9-week-old WKY	6	116 ± 3
15-week-old SHR	31	174 ± 2 *
15-week-old WKY	31	127 ± 1
DOCA-salt hypertensive rats	28	188 ± 2 *
Sham-operated rats	28	121 ± 1

Values are means ± S.E.M.
* Significantly different from respective controls ($P < 0.05$).

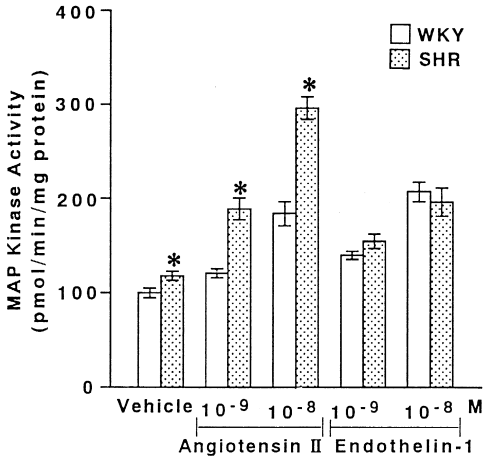

Fig. 3. Effects of angiotensin II and endothelin-1 on MAP kinase activity in endothelium-denuded aorta strips from 15-week-old SHR and age-matched WKY. Aorta strips were preincubated at 37°C for 5 min, after which, a 10-min incubation was started. Saline (vehicle), angiotensin II and endothelin-1 were added into the medium at the beginning of the 10-min incubation. Values are means ± S.E.M. from five experiments using different animals. * $P < 0.05$, compared with respective WKY.

fied 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, followed by a 10-min incubation or a 30-min incubation started at 37°C in a moist tissue incubator, containing an atmosphere of 95% air and 5% CO_2. Drugs were added into DMEM at the beginning of the incubation. Drugs were dissolved in physiological saline (0.9% NaCl) and added into DMEM in a volume of 10 μl. The reaction was terminated by chilling the plates on ice and washing twice with ice-cold phosphate-buffered saline.

The tissues were homogenised 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 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 was 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 tissues, 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 (Sigma, St. Louis, MO), HEPES buffer (Wako, Osaka, Japan), endothelin-1 human, BQ123 (Research Biochemicals International, Natick, MA) and 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 followed by Dunnett's test for post hoc

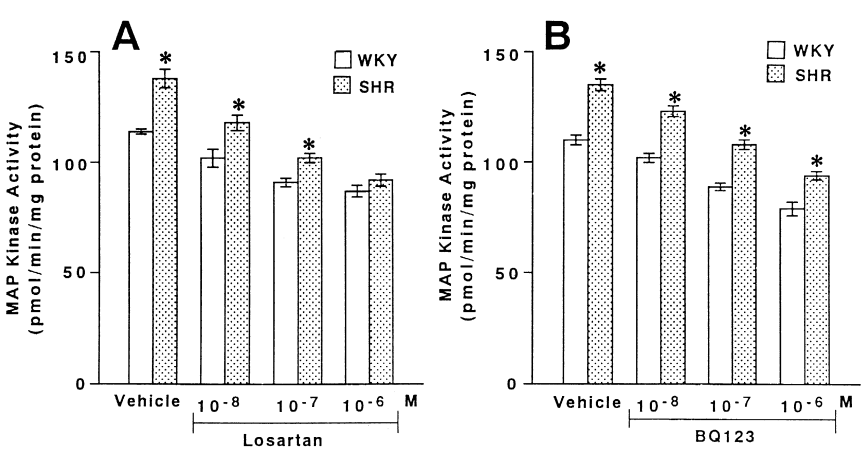

Fig. 2. Effects of losartan (A) and BQ123 (B) on the increase in MAP kinase activity after 30-min incubation in endothelium-denuded aorta strips from 15-week-old SHR and age-matched WKY. Aorta strips were preincubated at 37°C for 5 min, after which, a 30-min incubation was started. Saline (vehicle), losartan and BQ123 were added into the medium at the beginning of the 30-min incubation. Values are means ± S.E.M. from four experiments using different animals. * $P < 0.05$, compared with respective WKY.

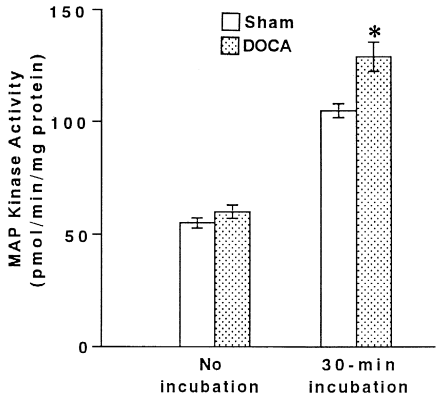

Fig. 4. MAP kinase activity levels before incubation (no incubation) and 30 min after incubation in endothelium-denuded aorta strips from DOCA-salt hypertensive (DOCA) and sham-operated (sham) rats. Aorta strips were preincubated at 37°C for 5 min, and then, 30-min incubation was started. Values are means ± S.E.M. from eight experiments using different animals. $^{*}P < 0.05$, compared with respective sham-operated rats.

analysis for intergroup comparisons. Differences were considered significant at $P < 0.05$.

3. Results

3.1. Endothelium removal-induced MAP kinase activation in aorta strips from WKY and SHR

Following a 5-min preincubation at 37°C in DMEM, 30-min incubation of endothelium-denuded aorta strips was started. In aorta strips from 9- and 15-week-old SHR, and from age-matched WKY, MAP kinase activity was increased after 30-min incubation (Fig. 1A and B). The MAP kinase activation was greater in aorta strips from SHR at both weeks of age than in those from age-matched WKY. MAP kinase activity before incubation was almost the same in aorta strips from both WKY and SHR at respective ages. Systolic blood pressure was higher in SHR than in WKY at 9 and 15 weeks of age (Table 1).

In aorta strips from 15-week-old SHR and age-matched WKY, the angiotensin receptor antagonist, losartan (10^{-8}–10^{-6} M; Fig. 2A), and the endothelin receptor antagonist, BQ123 (10^{-8}–10^{-6} M; Fig. 2B), added into DMEM at the beginning of the 30-min incubation, caused a concentration-dependent inhibition of the MAP kinase activation after endothelium removal. Although MAP kinase activity was greater in vehicle-treated SHR aorta strips than in WKY strips, the enzyme activity was almost the same in losartan (10^{-6} M)-treated aorta strips from both strains. In contrast, the enzyme activity was still greater in BQ123 (10^{-8}–10^{-6} M)-treated SHR aorta strips than in WKY strips.

When angiotensin II (10^{-9} and 10^{-8} M) or endothelin-1 (10^{-9} and 10^{-8} M) was added into DMEM at the beginning of the 10-min incubation, they caused concentration-dependent increases of MAP kinase activity in aorta strips from 15-week-old SHR and age-matched WKY (Fig. 3). MAP kinase activity was greater in vehicle-treated SHR aorta strips than in WKY strips. MAP kinase activity was also greater in angiotensin II-treated SHR aorta strips than in WKY strips, whereas the enzyme activity was almost the same in the endothelin-1-treated aorta strips from both strains.

3.2. Endothelium removal-induced MAP kinase activation in aorta strips from sham-operated and DOCA-salt hypertensive rats

In aorta strips from sham-operated and DOCA-salt hypertensive rats, MAP kinase activity was increased after 30-min incubation (Fig. 4). The MAP kinase activation was greater in DOCA-salt hypertensive rat aorta strips than in sham-operated rat strips. MAP kinase activity before

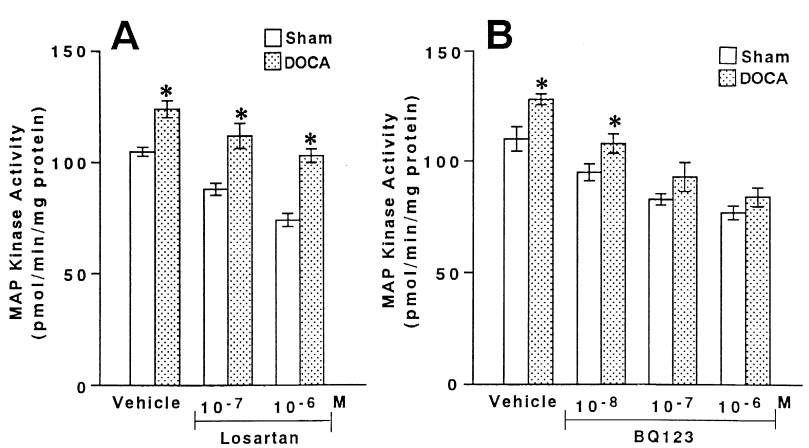

Fig. 5. Effects of losartan (A) and BQ123 (B) on the increase in MAP kinase activity after 30-min incubation in endothelium-denuded aorta strips from DOCA-salt hypertensive (DOCA) and sham-operated (sham) rats. Aorta strips were preincubated at 37°C for 5 min, after which, a 30-min incubation was started. Saline (vehicle), losartan and BQ123 were added into the medium at the beginning of the 30-min incubation. Values are means ± S.E.M. from five experiments using different animals. $^{*}P < 0.05$, compared with respective sham-operated rats.

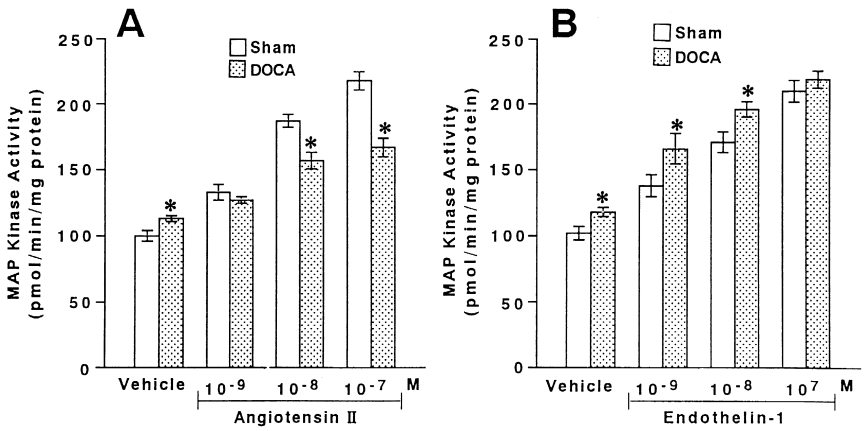

Fig. 6. Effects of angiotensin II (A) and endothelin-1 (B) on MAP kinase activity in endothelium-denuded aorta strips from DOCA-salt hypertensive (DOCA) and sham-operated (sham) rats. Aorta strips were preincubated at 37°C for 5 min, after which, a 10-min incubation was started. Saline (vehicle), angiotensin II and endothelin-1 were added into the medium at the beginning of the 10-min incubation. Values are means ± S.E.M. from five experiments using different animals. * $P < 0.05$, compared with respective sham-operated rats.

incubation was almost the same in aorta strips from both kinds of animals. Systolic blood pressure was higher in DOCA-salt hypertensive rats than in sham-operated rats (Table 1).

In aorta strips from sham-operated and DOCA-salt hypertensive rats, losartan (10^{-7} and 10^{-6} M) and BQ123 (10^{-8}–10^{-6} M), added into DMEM at the beginning of the 30-min incubation, caused a concentration-dependent inhibition of the MAP kinase activation after endothelium removal (Fig. 5A and B). Although MAP kinase activity was greater in vehicle-treated DOCA-salt hypertensive rat aorta strips than in sham-operated rat strips, the enzyme activity was not significantly different in BQ123 (10^{-7} and 10^{-6} M)-treated aorta strips from both kinds of rats. In contrast, the enzyme activity was still greater in losartan (10^{-7} and 10^{-6} M)-treated DOCA-salt hypertensive rat aorta strips than in sham-operated rat strips.

When angiotensin II (10^{-9}–10^{-7} M) or endothelin-1 (10^{-9}–10^{-7} M) was added into DMEM at the beginning of the 10-min incubation, they caused concentration-dependent increases in MAP kinase activity in aorta strips from sham-operated and DOCA-salt hypertensive rats (Fig. 6A and B). MAP kinase activity was greater in vehicle-treated DOCA-salt hypertensive rat aorta strips than in sham-operated rat strips. MAP kinase activity was also greater in endothelin-1 (10^{-9} and 10^{-8} M)-treated DOCA-salt hypertensive rat aorta strips than in sham-operated rat strips, whereas the enzyme activity was smaller in angiotensin II (10^{-8} and 10^{-7} M)-treated DOCA-salt hypertensive rat aorta strips than in sham-operated rat strips.

4. Discussion

In the present study, endothelium removal caused an increase of MAP kinase activity in rat aorta strips, which confirmed the findings of previous studies (Kubo et al., 1998, 1999b). The increase in MAP kinase activity was greater in 9- and 15-week-old SHR aorta strips than in age-matched WKY aorta strips. The increase in MAP kinase activity was also greater in DOCA-salt hypertensive rat aorta strips than in sham-operated rat aorta strips. These findings suggest that MAP kinase activation function is enhanced in the vasculature of both SHR and DOCA-salt hypertensive rats with hypertension.

Previously, we demonstrated that MAP kinase activation after endothelium removal in rat aorta strips was inhibited by either the angiotensin receptor antagonist, losartan, or by the endothelin receptor antagonist, BQ123 (Kubo et al., 1998), suggesting the involvement of vascular angiotensin and endothelin systems in the MAP kinase activation. In the present study, losartan and BQ123 similarly inhibited the MAP kinase activation in aorta strips from 15-week-old SHR and age-matched WKY. MAP kinase activity was greater in vehicle-treated 15-week-old SHR aorta strips than in age-matched WKY strips, but the enzyme activity was almost the same in losartan (10^{-6} M)-treated aorta strips from both WKY and SHR. In contrast, MAP kinase activity was still greater in BQ123 (10^{-8}–10^{-6} M)-treated SHR aorta strips than in WKY strips. The dose 10^{-6} M is a maximally effective dose of BQ123 for MAP kinase inhibition in aorta strips (Kubo et al., 1998). Thus, the results of the present study suggest that angiotensin system function, rather than endothelin system function, is enhanced in aorta strips from SHR with hypertension, and that this enhanced angiotensin system function contributes to the enhanced MAP kinase activation in SHR aorta strips.

MAP kinase activity was again greater in vehicle-treated DOCA-salt hypertensive rat aorta strips than in sham-operated rat strips, but the enzyme activity was almost the same in BQ123 (10^{-7} and 10^{-6} M)-treated aorta strips from both kinds of rats. In contrast, MAP kinase activity

was still greater in losartan (10^{-7} and 10^{-6} M)-treated DOCA-salt hypertensive rat aorta strips than in sham-operated rat strips. These findings suggest that endothelin system function, rather than angiotensin system function, is enhanced in aorta strips from DOCA-salt hypertensive rats, and that this enhanced endothelin system function contributes to the enhanced MAP kinase activation in aorta strips from DOCA-salt hypertensive rats.

In the present study, angiotensin II and endothelin-1 produced concentration-dependent increases in MAP kinase activity in aorta strips from 15-week-old SHR and age-matched WKY. MAP kinase activity was greater in angiotensin II-treated SHR aorta strips than in WKY strips, while the enzyme activity was almost the same in the endothelin-1-treated aorta strips from both strains. Thus, it seems likely that reactivity to angiotensin II for MAP kinase activation is specifically enhanced in SHR aorta strips. From these findings, it could be speculated that the enhanced reactivity to angiotensin II may contribute, at least in part, to the enhanced MAP kinase activation in SHR aorta strips. The results of the present study are compatible with those of Touyz et al. (1994) showing that, in cultured mesenteric vascular smooth muscle cells from SHR aged 9 and 17 weeks, cystosolic free-Ca^{2+} concentration responses to angiotensin II, but not to endothelin-1, are enhanced. Furthermore, it has been reported that angiotensin II-induced proliferation of aortic myocytes is considerably enhanced in SHR cells, and this abnormality may be linked to an increased number of angiotensin II receptors (Schiffrin et al., 1984; Paquet et al., 1990; Bunkenburg et al., 1992).

Increased activities of renin and angiotensin converting enzyme (Asaad and Antonaccio, 1982; Okunishi et al., 1991), and increased angiotensin II content (Morishita et al., 1992), have been shown in vascular smooth muscles or vascular smooth muscle cells from adult SHR compared with those of age-matched WKY. Thus, it could be considered that, in addition to the enhanced angiotensin reactivity, enhanced angiotensin production function is also related to the enhancement of the vascular angiotensin system in SHR.

In contrast, MAP kinase activity was greater in endothelin-1 (10^{-9} and 10^{-8} M)-treated DOCA-salt hypertensive rat aorta strips than in sham-operated rat strips, while the enzyme activity was smaller in angiotensin II (10^{-8} and 10^{-7} M)-treated DOCA-salt hypertensive rat aorta strips than in sham-operated rat strips. Thus, it seems likely that the reactivity to endothelin-1 is enhanced in DOCA-salt hypertensive rat aorta strips. It could be considered that the enhanced reactivity to endothelin-1 may contribute, at least in part, to the enhanced MAP kinase activation in DOCA-salt hypertensive rat aorta strips.

Enhanced endothelin-1 gene expression (Lariviere et al., 1993a; Li et al., 1994) and enhanced endothelin immunoreactivity (Lariviere et al., 1993b) have been shown in blood vessels of DOCA-salt hypertensive rats. Thus, in addition to the enhanced endothelin reactivity, enhanced endothelin production function may also contribute to the enhanced MAP kinase activation in DOCA-salt hypertensive rat aorta strips.

It is well known that plasma renin activity is depressed in DOCA-salt hypertensive rats. Previously, we demonstrated that renin plays the determining role in the regulation of angiotensin production in the vasculature and the major source of the renin is renin in circulation (Kubo et al., 1999b). Thus, not only reactivity to angiotensin II, but also angiotensin production, may be reduced in DOCA-salt hypertensive rat aorta strips. It could be considered that enhanced endothelin system function surpassed the decreased angiotensin system function and, thus, MAP kinase activity was enhanced in DOCA-salt hypertensive rats. The mechanism of the reduced reactivity to angiotensin II in DOCA-salt hypertensive rat aorta strips is unclear and remains to be settled.

Previously, we have demonstrated that endothelium removal-induced MAP kinase activation is enhanced via the vascular angiotensin system in aorta strips from prehypertensive 4-week-old SHR (Kubo et al., 1999a). In the present study, similar enhancement was also found in aorta strips from hypertensive 9- and 15-week-old SHR, although the extent of the enhancement was somewhat small in aorta strips from 4-week-old SHR. Since MAP kinases are believed to be involved in the pathway for cell proliferation and, thus, for vascular structural remodelings, these findings are compatible with the idea that vascular structural remodeling function is enhanced via the vascular angiotensin system in SHR at both prehypertensive and hypertensive stages, and that this may contribute to the development and maintenance of hypertension in SHR. Indeed, angiotensin converting enzyme inhibitors and AT_1 receptor antagonists are reported to inhibit vascular hypertrophy in SHR (Soltis, 1993). In addition, these renin–angiotensin system inhibitors produce antihypertensive effects in SHR but not in normotensive rats (Antonaccio and Cushman, 1981; Li and Jackson, 1987; Timmermans et al., 1991). Similarly, the results of the present study suggest that vascular structural remodeling function is enhanced via the vascular endothelin system in DOCA-salt hypertensive rats. In this connection, it has been reported that endothelin receptor antagonists blunt the development of hypertrophy and remodeling of vascular structure, and produce antihypertensive effects in DOCA-salt hypertensive rats (Li et al., 1994). In contrast, chronic treatment with an angiotensin AT_1 receptor antagonist is reported to produce no antihypertensive effect in these hypertensive rats (Fujita et al., 1997). Nevertheless, it should be noted that the vessel aorta used in this study is a conduit vessel and, thus, its contribution to hypertension is minimal.

In summary, the results of the present study demonstrate that endothelium removal-induced MAP kinase activation is enhanced in aorta strips from hypertensive SHR and DOCA-salt hypertensive rats and, thus, suggest that

vascular structural remodeling function may be enhanced in both hypertensive models. It appears that the enhancement of MAP kinase activation results, at least in part, from enhanced function of the vascular angiotensin system in SHR and from enhanced function of the vascular endothelin system in DOCA-salt hypertensive rats. The results of the present study would provide further important information for elucidating the ethiology of hypertension in SHR.

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