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Vascular mitogen-activated protein kinase activity is enhanced via angiotensin system in spontaneously hypertensive rats

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Abstract

The vascular structural remodeling function may be altered in genetically hypertensive animals, spontaneously hypertensive rats (SHR). To examine this possibility, we measured the activity of mitogen-activated protein (MAP) kinases, enzymes believed to be involved in the pathway for cell proliferation, in rat aorta strips, and examined whether the endothelium removal-induced MAP kinase activation function is altered in SHR and whether vascular angiotensin and endothelin systems are responsible for the alteration of MAP kinase activation in SHR. Male 4-week-old SHR and age-matched Wistar Kyoto rats (WKY) supplied by Charles River Japan were used. Endothelium-denuded aorta strips were incubated at 37°C in medium. MAP kinase activity after incubation was time-dependently increased in strips from SHR and WKY. MAP kinase activation was greater in SHR than in WKY aorta strips. Similarly, MAP kinase activation was enhanced in aorta strips from 4-week-old SHR and stroke prone SHR supplied by the Diseases Model Cooperative Research Association (Kyoto, Japan). In aorta strips from SHR and WKY, the angiotensin receptor antagonist, losartan, and the endothelin receptor antagonist, cyclo (D-α-aspartyl-L-prolyl-D-valyl-L-leucyl-D-tryptophyl)(BQ123), caused concentration-dependent inhibition of MAP kinase activation. The losartan-induced but not BQ123-induced inhibition of MAP kinase activation was greater in SHR than in WKY aorta strips. Angiotensin II caused a concentration-dependent increase in MAP kinase activity and the angiotensin II-induced MAP kinase activation was greater in SHR than in WKY aorta strips. These results indicate that endothelium removal-induced MAP kinase activation is enhanced in aorta strips from young SHR, suggesting that vascular structural remodeling function may be enhanced in SHR. It appears that the enhancement of MAP kinase activation results, at least in part, from enhanced function of vascular angiotensin system in SHR.

Keywords: Mitogen-activated protein kinase; Angiotensin; Endothelin; Aorta; Spontaneously hypertensive rat (SHR)

1. Introduction

Vascular hypertrophy is considered to be a fundamental pathogenic factor for both hypertension and atherosclerosis. Indeed, vascular structural alterations have been shown to contribute to the maintenance of hypertension in spontaneously hypertensive rats (SHR), a rat model for essential hypertension in the human (Kubo, 1978, 1979; Folkow et al., 1982; Owens and Schwartz, 1982). Although these vascular structural remodelings are known to occur secondarily to hypertension in both genetically and non-genetically hypertensive animals (Kubo, 1978; Folkow et al., 1982; Owens and Schwartz, 1982), it is also possible that vascular structural remodeling function is enhanced in the genetically hypertensive animal, SHR, and that this enhanced vascular structural remodeling function contributes to the development of hypertension in SHR.

Endothelial and vascular smooth muscle cell integrity appears to be a crucial factor for maintenance of the structural properties 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). Vasoactive substances like angiotensin II and endothelins are suggested to be related to these pathophysiological responses. For example, chronic treatment with angiotensin converting enzyme inhibitors or the angiotensin AT1 receptor antagonist, losartan, has been shown to reduce neointima formation (Powell et al., 1989; Capron et al., 1991; Daemen et al., 1991; Dzau et al., 1991; Osterrieder et al., 1991; Janiak et al., 1992). Balloon angioplasty is reported to increase angiotensin AT1 receptor expression

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(Viswanathan et al., 1992) and endothelin ETA receptor expression (Viswanathan et al., 1997) in rat arteries.

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 also 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 remodeling. In previous studies (Kubo et al., 1998; Kubo et al., 1999), we demonstrated that, in rat aorta strips, endothelium removal resulted in activation of MAP kinase activity. MAP kinase activation was found in the media portion but not in the adventitia portion. MAP kinase activation was inhibited by both the angiotensin receptor antagonist, losartan, and the endothelin receptor antagonist, cyclo (D-α-aspartyl-L-prolyl-D-valyl-L-leucyl-D-tryptophyl) (BQ-123). 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.

To investigate whether vascular structural remodeling function is enhanced in the vasculature of the genetically hypertensive animals, SHR, we measured the activity of MAP kinases in endothelium-denuded aortic strips from SHR and their controls, Wistar Kyoto rats (WKY), and examined whether MAP kinase activation is altered in SHR. We also examined whether vascular angiotensin and endothelin systems are responsible for the altered MAP kinase activation in the SHR vasculature.

2. Materials and methods

Male 4-week-old SHR and age-matched Wistar Kyoto rats (WKY) (Charles River Japan) were used in this study. In some experiments, we used 4-week-old SHR (SHR/Izm), stroke-prone SHR (SHRsp/Izm) and WKY (WKY/Izm) supplied by the Diseases Model Cooperative Research Association (Japan). They were kept under alternating 12-h periods of dark and light, and given standard rat chow and tap water ad libitum. 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. Tissue incubation

The aorta 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 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.

2.2. Preparation of tissue extracts

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.

2.3. Assay for MAP kinase activity

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. Briefly, MAP kinase activity was measured based on 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 microliters of samples, 10 μl of substrate in a buffer containing HEPES and 0.125 mM sodium orthovanadate, and 5 μl of 1.2 mM magnesium [^{32}P]ATP (200 μCi/ml) were mixed in tubes and incubated for 30 min in a 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 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, leupeptin hemisulfate, aprotinin (Sigma, St. Louis), HEPES buffer (Wako, Osaka, Japan), endothelin-1 human, BQ123 (Research Biochemicals International, Natick, MA) and Dulbecco's modified Eagle's medium (DMEM) (Dainihon Pharmaceuticals, Osaka, Japan). Losartan was generously supplied by Dupont-Merck Pharmaceuticals (Wilmington, DE).

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

3. Results

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

Following a 5-min preincubation at 37°C in DMEM, 10-min or 30-min incubation of endothelium-denuded aorta strips was started. In aorta strips from 4-week-old SHR and age-matched WKY, MAP kinase activity after incubation was time-dependently increased (Fig. 1). The MAP kinase activation was greater in SHR aorta strips after both 10-min and 30-min incubations than in WKY strips. MAP kinase activity before incubation was almost the same in aorta strips from both strains of animals. There was no significant difference in systolic blood pressure between the two strains (Table 1).

Table 1
Systolic blood pressure in 4-week-old spontaneously hypertensive rats (SHR), age-matched Wistar Kyoto rats (WKY), SHR/Izm, stroke-prone SHR/Izm (SHRsp/Izm), WKY/Izm and Wistar rats. values are means ± S.E.M.

Rats	*n*	Systolic blood pressure (mm Hg)
SHR	10	114 ± 2
WKY	10	111 ± 2
SHR/Izm	5	112 ± 3
SHRsp/Izm	5	117 ± 3
WKY/Izm	5	108 ± 4
Wistar rats	5	110 ± 2

To examine whether the enhancement of MAP kinase activation observed in aorta strips from 4-week-old SHR also occurs in aorta strips from other strains of SHR, we also tested strips from SHR/Izm and SHRsp/Izm rats. MAP kinase activity before incubation was almost the same in strips from 4-week-old SHR/Izm, 4-week-old SHRsp/Izm, age-matched WKY/Izm and Wistar rats (Fig. 2). MAP kinase activation after 30-min incubation was again greater in aorta strips from SHR/Izm and SHRsp/Izm than in strips from either WKY/Izm or Wistar rats. Systolic blood pressure was almost the same for these four strains (Table 1).

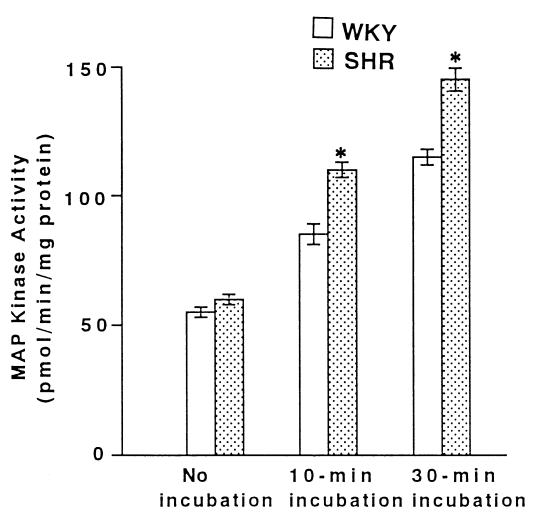

Fig. 1. MAP kinase activity levels before incubation (no incubation) and after incubation in endothelium-denuded aorta strips from 4-week-old SHR and age-matched WKY. Aorta strips were preincubated at 37°C for 5 min and then, 10-min or 30-min incubation was started. Values are means ± S.E.M. from 10 experiments using different animals. $^*P < 0.05$, compared with respective WKY.

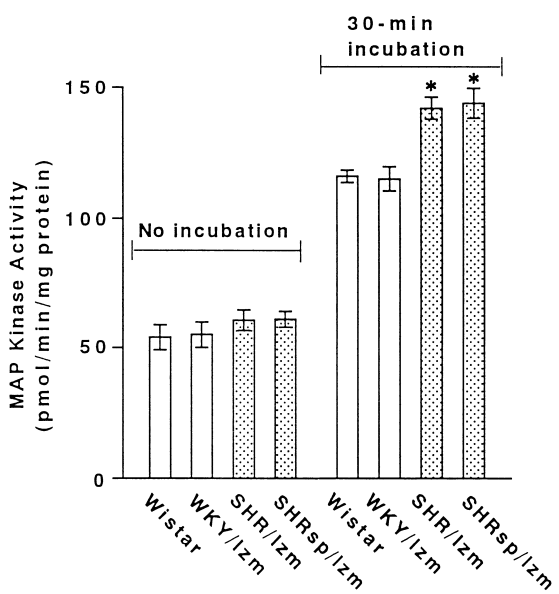

Fig. 2. MAP kinase activity levels before incubation (no incubation) and after incubation in endothelium-denuded aorta strips from 4-week-old Wistar rats (Wistar), WKY/Izm, SHR/Izm and SHRsp/Izm. Aorta strips were preincubated at 37°C for 5 min, after which a 30-min incubation was started. Values are means ± S.E.M. from five experiments using different animals. $^*P < 0.05$, compared with 30-min incubation Wistar rats and WKY/Izm.

3.2. Effects of losartan and BQ123 on the endothelium removal-induced MAP kinase activation in aorta strips from WKY and SHR

In aorta strips from 4-week-old SHR and age-matched WKY, the angiotensin receptor antagonist, losartan (10^{-9}–10^{-6} M) (Fig. 3A), and the endothelin receptor antagonist, BQ123 (10^{-8}–10^{-5} M) (Fig. 3B), 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^{-7} and 10^{-6} M)-treated aorta strips from both strains. In contrast, the enzyme activity was still greater in BQ123 (10^{-8}–10^{-5} M)-treated SHR aorta strips than in WKY strips.

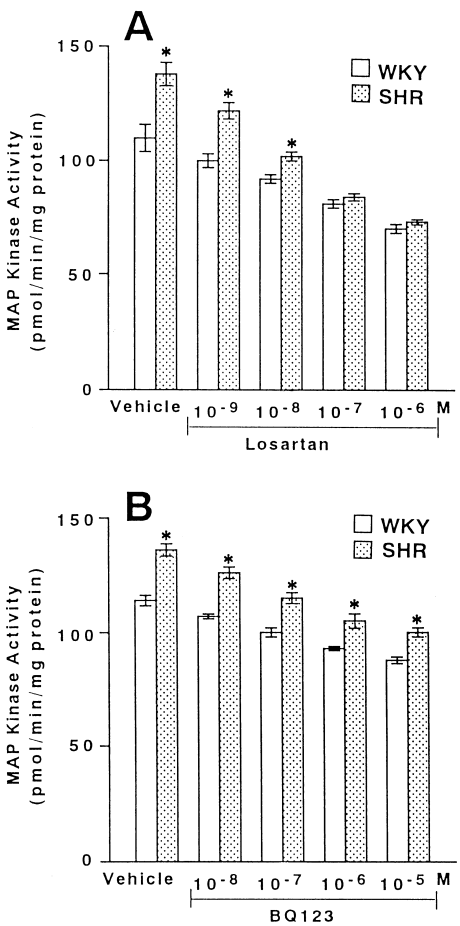

Fig. 3. Effects of losartan (A) and BQ123 (B) on the increase in MAP kinase activity after 30-min incubation in endothelium-denuded aorta strips from 4-week-old SHR and age-matched WKY. Aorta strips were preincubated at 37°C for 5 min, after which a 30-min incubation was started. Saline (vehicle), losartan 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.

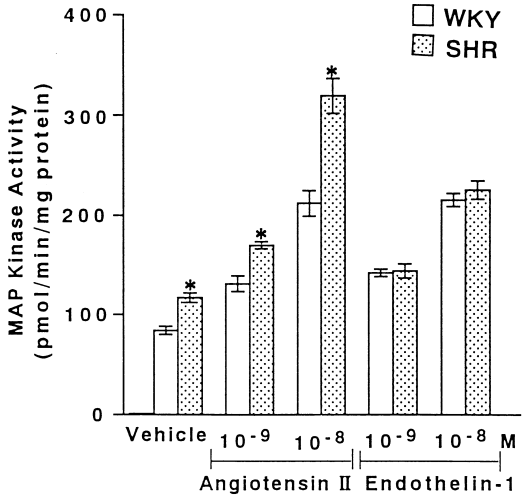

Fig. 4. Effects of angiotensin II and endothelin-1 on MAP kinase activity in endothelium-denuded aorta strips from 4-week-old SHR and age-matched WKY. Aorta strips were preincubated at 37°C for 5 min, after which a 10-min incubation was started. Saline (vehicle), angiotensin II and endothelin-1 were added into the medium at the beginning of the 10-min incubation. Values are means ± S.E.M. from eight experiments using different animals. $^{*}P < 0.05$, compared with respective WKY.

3.3. Effects of angiotensin II and endothelin-1 on MAP kinase activity in endothelium-denuded aorta strips from WKY and SHR

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 in MAP kinase activity in aorta strips from 4-week-old SHR and age-matched WKY (Fig. 4). 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.

4. Discussion

In previous studies (Kubo et al., 1998), we have demonstrated that, in rat aorta strips, endothelium removal results in activation of MAP kinase activity in the medial layer. In the present study, also in aorta strips from 4-week-old SHR and age-matched WKY, endothelium removal caused an increase of MAP kinase activity. The increase in MAP kinase activity was greater in aorta strips from SHR than in those of WKY. There was no difference in systolic blood pressure between animals of both strains. These findings suggest that MAP kinase activation function is enhanced in the vasculature of prehypertensive SHR. Fur-

thermore, in the present study, MAP kinase activation was also enhanced in 4-week-old SHR/Izm and 4-week-old SHRsp/Izm, other strains of SHR, as compared with either age-matched WKY/Izm or age-matched Wistar rats. Thus, it seems unlikely that the enhancement of MAP kinase activation observed in SHR aorta strips results merely from strain differences. The results of the present study, therefore, suggest that the endothelium removal-induced MAP kinase activation is naturally enhanced in SHR aorta strips.

The vascular wall has been suggested to contain a local renin–angiotensin system (Campbell and Habener, 1986; Cassis et al., 1988; Dzau and Gibbons, 1988; Samani et al., 1988; Inagami et al., 1991; Naftilan et al., 1991; Fishel et al., 1995) and an endothelin system (Sung et al., 1994). It has been suggested that both the vascular renin–angiotensin system and the endothelin system participate in vascular smooth muscle proliferation and neointima formation after balloon angioplasty (Dzau et al., 1991; Viswanathan et al., 1992; Viswanathan et al., 1997). Previously, we demonstrated that MAP kinase activation after endothelium removal from rat aorta strips was inhibited by either the angiotensin receptor antagonist, losartan, or the endothelin receptor antagonist, BQ123 (Kubo et al., 1998), suggesting the involvement of vascular angiotensin and endothelin systems in MAP kinase activation. In the present study, losartan and BQ123 also inhibited the endothelium removal-induced MAP kinase activation in aorta strips from 4-week-old SHR and age-matched WKY in a concentration-dependent manner. 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^{-7} and 10^{-6} M)-treated aorta strips from both WKY and SHR. In contrast, MAP kinase activity was still greater in BQ123 (10^{-8}–10^{-5} M)-treated SHR aorta strips than in WKY strips. The 10^{-5} M dose is a maximally effective dose of BQ123 for MAP kinase inhibition in aorta strips (Kubo et al., 1998), suggesting that the endothelin system is not mainly involved in the enhancement of MAP kinase activation observed in endothelium-denuded aorta strips from SHR. Thus, the results of the present study suggest that angiotensin system function rather than endothelin system function is enhanced in aorta strips from SHR, and that this enhanced angiotensin system function contributes to the enhancement of MAP kinase activation in aorta strips from SHR.

In the present study, angiotensin II and endothelin-1 produced concentration-dependent increases in MAP kinase activity in aorta strips from WKY and SHR. 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 than in WKY aorta strips, while the enzyme activity was almost the same in the endothelin-1-treated aortic strips from both strains. Thus, it seems likely that sensitivity to angiotensin II rather than to endothelin-1 for MAP kinase activation is enhanced in SHR aorta strips. From the results of the present study, it could be thought that the enhancement of endothelium removal-induced MAP kinase activation in SHR aorta strips may be, at least in part, due to the enhanced sensitivity to angiotensin II. In this connection, it has been demonstrated 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). The results of the present study are also compatible with those of Touyz et al. (1994) showing that, in cultured mesenteric vascular smooth muscle cells from SHR aged 9 and 17 weeks, cytosolic free-Ca^{2+} concentration responses to angiotensin II but not to endothelin-1 are enhanced.

In addition to enhanced angiotensin reactivity, 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. To assess local angiotensin system function exactly, both angiotensin production function and angiotensin reactivity in tissues must be estimated totally. The results of the present study with losartan reflect the total function of both angiotensin release and angiotensin reactivity. Many of the studies referred to above were performed in SHR with established hypertension. Sustained hypertension itself may cause various changes in vascular smooth muscle function secondarily, including changes in vascular renin–angiotensin system function (Li et al., 1997). In the present study, we used prehypertensive SHR. Accordingly, the results of the present study would provide important evidence suggesting that the total function of the local angiotensin system is naturally enhanced in the vasculature of SHR.

Since MAP kinases are believed to be involved in the pathway for cell proliferation and thus for vascular structural remodeling, the results of the present study are compatible with the idea that vascular structural remodeling function is enhanced via the local angiotensin system in the genetically hypertensive rat, SHR, and this may contribute to the development of hypertension in SHR. Indeed, angiotensin converting enzyme inhibitors and angiotensin AT1 receptor antagonists are reported to inhibit vascular hypertrophy in SHR (Soltis, 1993). In addition, these renin–angiotensin inhibitors produce antihypertensive effects in SHR but not in normotensive rats (Antonaccio and Cushman, 1981; Li and Jackson, 1987; Timmermans et al., 1991).

In summary, the results of the present study demonstrate that endothelium removal-induced MAP kinase activation is enhanced in aortic strips from prehypertensive SHR, and thus suggest that vascular structural remodeling function may be enhanced in SHR by nature. It appears that the enhancement of MAP kinase activation results, at

least in part, from enhanced function of the vascular angiotensin system in SHR. Since these alterations were found in prehypertensive SHR, the results of the present study would provide important information for elucidating the ethiology of hypertension in the genetically hypertensive animals, SHR.

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