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Extracellular Signal-Regulated Kinase and c-Jun NH_2-Terminal Kinase Activities Are Continuously and Differentially Increased in Aorta of Hypertensive Rats

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We first examined the activities of extracellular signal-regulated kinases (ERKs) and c-Jun NH_2-terminal kinases (JNKs) in the aorta of hypertensive rats. In Dahl salt-sensitive (DS) rats, chronic hypertension caused by a high-salt diet was followed by sustained activation of aortic p42ERK and p44ERK. p46JNK and p55JNK activities were also increased in hypertensive DS rats, but returned to control levels earlier than ERKs, suggesting that ERKs and JNKs may be independently activated in hypertensive rats. In stroke-prone spontaneously hypertensive rats (SHRSP) which spontaneously develop hypertension under a low salt-diet, aortic p42ERK and p44ERK activities were progressively increased with the development of hypertension, compared with control normotensive rats. p46JNK and p55JNK activities in SHRSP were increased, with a different time course from ERKs. Thus, we first demonstrated that ERKs and JNKs activities are chronically and differentially increased in the aorta of hypertensive rats, suggesting the involvement of these kinases in hypertensive vascular diseases.

Chronic hypertension causes vascular thickening mainly by accelerating the growth of vascular smoth muscle cells and extracellular matrix accumulation, and therefore is well known to be one of the most important risk factors responsible for the development of vascular diseases such as atherosclerosis[1-3]. However, the molecular mechanism of hypertensive vascular diseases remains to be elucidated.

Extracellular signal-regulated kinases (ERKs) belong to one subfamily of mitogen-activated protein (MAP) kinases[4-7] and are activated by various extracellular stimuli such as growth factors and other mitogens, and play a key role in cell growth and the regulation of various gene expressions[5,7]. c-Jun NH_2-terminal kinases (JNKs), or alternatively called the stress-activated protein kinases, have been recently identified as another subfamily of MAP kinases[8-11]. Unlike ERKs, JNKs are preferentially activated by stress signals rather than growth factors[12,13]. Importantly, the activation of JNKs has been shown to be associated with not only cell growth and the regulation of gene expression but also apoptosis[14], indicating the distinct biological function of JNKs from ERKs. However, previous studies on the role of ERKs and JNKs have been largely limited to cultured cells, and there is no report on the role of these kinases in hypertensive disease in vivo. To examine the in vivo role of MAP kinases in the development of hypertensive vascular disease, we determined aortic ERKs and JNKs activities in hypertensive rats. We obtained the first evidence that the activities of both ERKs and JNKs are significantly increased in the aorta of hypertensive rats in vivo.

MATERIALS AND METHODS

Experimental protocol. All procedures were in accordance with institutional guidelines for animal research. Male inbred Dahl salt-sensitive (DS) rats[15] were supplied by Eisai Co., Ltd.(Tokyo, Japan). DS rats are normotensive when fed a low-salt diet, while DS rats develop hypertension when fed a high-salt diet[15-17]. Therefore, in this study, to induce hypertension, 6-week-old DS rats were fed an 8% NaCl (high-salt) diet for 1, 5 and 9 weeks. On the other hand, control group of DS rats were fed a 0.3% NaCl (low-salt) diet for the same period. These diets were purchased from Oriental Yeast Industry (Tokyo, Japan). The diet and tap water were given ad libitum throughout the experiments. Blood pressure was measured by the tail-cuff method (TK-370A, Unicom Inc., Chiba, Japan). DS rats, treated with the high-salt or low-salt diet, were decapitated. The thoracic aorta was immediately excised, rinsed briefly with phosphate-buffered saline precooled at 4 °C, dissected from adherent fat

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and connective tissues in precooled phosphate-buffered saline (pH 7.4) containing 2.5 mmol/L Ethylenediamine-N,N,N′,N′-tetraacetic acid (EDTA), 2 mmol/L β-glycerophosphate, 10 mmol/L NaF, 1 mmol/L sodium orthovanadate (Na_3VO_4), and 1 mmol/L phenylmethylsulfonyl fluoride (PMSF) on ice, immediately frozen in liquid nitrogen and stored at − 80 °C until use. Extreme care was used to be certain that the aorta was not stretched on dissection.

In another experiments, we also examined aortic ERKs and JNKs in stroke-prone spontaneously hypertensive rats (SHRSP) which are a different hypertensive model rat from DS rat. Unlike DS rat, SHRSP spontaneously develop severe hypertension even when fed a low-salt diet[18,19]. Aortic MAP kinase activities of SHRSP at various ages were compared with those of Wistar-Kyoto rats (WKY) which are a genetic control of SHRSP and normotensive[18]. Both SHRSP and WKY were purchased from Japan SLC (Shizuoka, Japan), and were fed regular rat chow throughout the experiments. SHRSP and WKY were decapitated at 5, 14 and 24 weeks of age. Aorta, liver, lung, spleen and stomach were immediately excised, frozen in liquid nitrogen and stored at −80 °C in the same manner as DS rats described above.

Preparation of tissue protein extracts. For protein kinase assay, each tissue was homogenized on ice with polytron homogenizer (PCU-11, kinematica AG, Littau/Luzern, Switzerland) in lysis buffer (20 mmol/L Hepes (pH 7.2), 25 mmol/L NaCl, 2 mmol/L EGTA, 0.2 mmol/L dithiothreitol (DTT), 60 mg/mL aprotinin, 2 mg/mL leupeptin, 1 mmol/L PMSF, 50 mmol/L NaF, 1 mmol/L Na_3VO_4, 25 mmol/L β-glycerophosphate and 0.1 % Triton X-100). After incubation at 4 °C for 30 minutes, the homogenates were sonicated (SONIFIER 250, Branson Ultrasonics Co., Danbury) on ice for 1 min, and centrifuged at 10,000 × g at 4 °C for 30 minutes. The protein concentrations of the supernatants were measured with protein assay kit (PIERCE, Rockford) and stored at −80 °C until use.

Measurement of ERK activity. The assay of ERK activity was performed by using in-gel kinase method[20,21]. In brief, the samples of aortic protein extracts (10 μg) or other tissue protein extracts (each 50 μg), prepared as described above, were boiled for 5 minutes in Laemmli's sample buffer containing 100 mmol/L Na_3VO_4, and electrophoresed on SDS polyacrylamide (12 %) gels polymerized in the presence of 0.5 mg/ml of myelin basic protein as a substrate. After electrophoresis, the gels were incubated with 50 mmol/L Tris-HCl (pH 8.0) containing 20 % isopropanol, and then washed with 50 mmol/L Tris-HCl (pH 8.0) containing 5 mmol/L β-mercaptoethanol. After denaturation with 50 mmol/L Tris-HCl (pH 8.0) containing 6 mol/L guanidine-HCl and 5 mmol/L β-mercaptoethanol, the kinases in the gels were renatured by incubation in 50 mmol/L Tris-HCl (pH 8.0) containing 0.04 % Tween-40 and 5 mmol/L β-mercaptoethanol, and equilibrated with kinase buffer (40 mmol/L Hepes (pH 7.5), 0.1 mmol/L EGTA, 20 mmol/L $MgCl_2$, and 2 mmol/L DTT). For the kinase reaction, the gels were incubated in kinase buffer with 25 mmol/L adenosine triphosphate (ATP) and 25 μCi (γ-^{32}P)ATP. The reaction was terminated by immersing the gels in 5 % trichloroacetic acid and 1 % sodium pyrophosphate, followed by extensively washing with the same solution several times. The gels were then dried, subjected to autoradiography, and the density of autoradiograms were analyzed with a bioimaging analyzer (BAS-2000, Fuji Photo Film Co., Tokyo, Japan).

Measurement of JNK activity. The assay of JNK activity was performed by using in-gel kinase method[10,20]. JNK activity was estimated as the ability to phosphorylate c-Jun as the substrate. The GST-c-Jun(1-79) plasmid, provided by Dr. Hibi (Osaka University Medical School), was expressed as GST-fusion protein in E.coli[22], and the expressed GST-c-Jun(1-79) protein was purified using glutathione-sepharose 4B (Pharmacia Biotech Inc., Uppsala, Sweden), according to manufacturer's instructions. Except that aortic protein extracts (40 μg) and other tissue protein extracts (each 50 μg) were electrophoresed on SDS polyacrylamide (12 %) gels containing 0.2 mg/ml of GST-c-Jun(1-79), the method was the same as in-gel kinase assay of ERK described above.

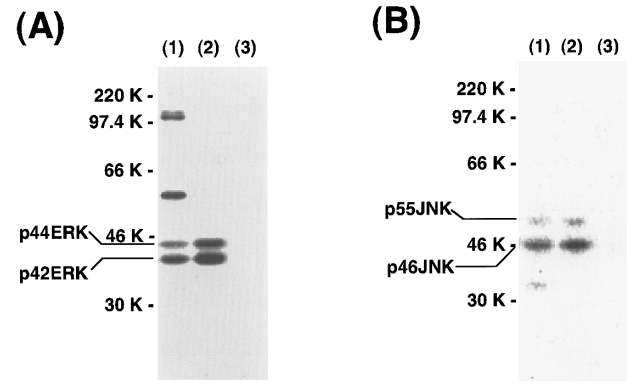

FIG. 1. Identification of ERKs (A) and JNKs (B) in DS rat aortic extracts. The positions of molecular mass markers are indicated in kilodaltons. (A) Lane (1) shows in-gel kinase assay for ERK of aortic protein extracts was carried out without immunoprecipitation (lane 1), and after immunoprecipitation with 1 μg of both anti-p44ERK IgG and anti-p42ERK IgG (lane 2), or normal rabbit IgG (lane 3). The sample treated with normal rabbit IgG as control was devoid of any band (lane 3). (B) In-gel kinase assay for JNK of aortic protein extracts was performed without immunoprecipitation (lane 1), and after immunoprecipitation with 0.5 μg of anti-JNK IgG (c-17) recognizing not only p46JNK but also p55JNK (lane 2), and normal rabbit IgG (lane 3). The sample treated with normal rabbit IgG as control was devoid of any band (lane 3). The detailed method of immunoprecipitation is described in Materials and Methods.

Identification of ERKs and JNKs by immunoprecipitation. To confirm that ERK and JNK in whole aortic extracts can be specifically measured by in-gel kinase method, we performed in-gel kinase assay after immunoprecipitation of aortic extracts. All antibodies used were purchased from Santa Cruz Biotechnology, Inc.(California), and were as follows: polyclonal rabbit anti-p44ERK (ERK-1) immunoglobulin G (IgG) (c-16); polyclonal rabbit anti-p42ERK (ERK-2) IgG (c-14); polyclonal rabbit anti-JNK IgG (c-17) recognizing both p46JNK and p55JNK. Aortic extracts (100 μg of protein) were preabsorbed with 10 μl of recombinant protein A-Agarose (50 %, vol./vol.) (Upstate biotechnology, Lake Placid) at 4 °C for 2 hours. After centrifugation at 10,000 × g at 4 °C for 15 minutes, the supernatants were incubated with 1 μg of each antibody or normal rabbit IgG at 4 °C for 2 hours, and were added 20 μl of recombinant protein A-Agarose (50 %, vol./vol.), followed by incubation at 4 °C for 12 hours. After centrifugation at 800 × g for 10 minutes, the pellets were washed four times with lysis buffer containing 0.5 mol/L NaCl. Finally, the pellets were suspended with 25 μl of lysis buffer. The immunoprecipitates were boiled for 5 minutes in Laemmli's sample buffer containing 100 mmol/L Na_3VO_4, centrifuged, and the resulting supernatants were subjected to in-gel kinase assay of ERK or JNK, as described above.

Statistical analysis. All data are expressed as mean±SEM. Statistical significance was determined with an unpaired Student's *t*-test. Differences were considered statistically significant at a value of $P < 0.05$.

RESULTS

Identification of ERKs and JNKs

As shown by in-gel assay of ERKs in Fig. 1-(A), four protein kinases, corresponding to molecular mass of 100, 60, 44 and 42 kDa, were detected in whole aortic

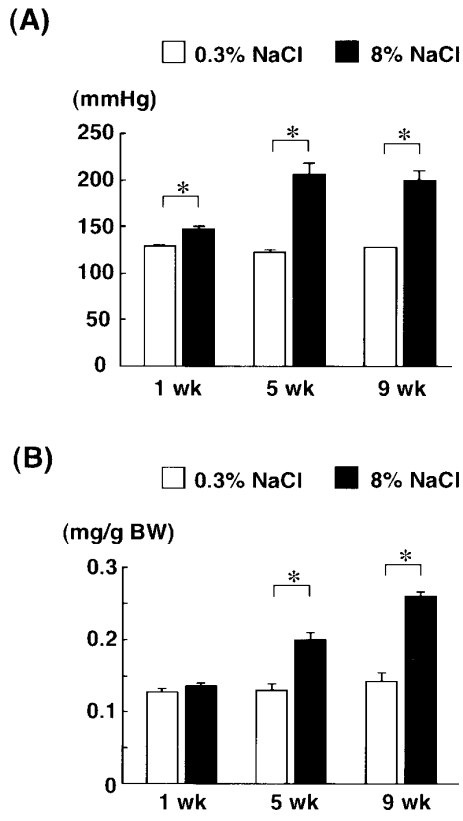

FIG. 2. Blood pressure (A) and aortic weight (B) of DS rats fed a 0.3% NaCl (low-salt) or an 8% NaCl (high-salt) diet for 1, 5 and 9 weeks. Aortic weight was corrected for body weight (BW). Each bar represents mean±SEM (n=6). *P<0.01.

extracts not subjected to immunoprecipitation. In-gel kinase assay of aortic extracts immunoprecipitated with anti-ERK antibodies confirmed that the 44-kDa kinase band corresponded to p44ERK (ERK-1) and the 42-kDa kinase band corresponded to p42ERK (ERK-2). As shown by in-gel kinase assay of JNKs in Fig. 1-(B), three protein kinases with molecular mass of 55, 46 and 34 kDa were detected in aortic extracts not subjected to immunoprecipitation. In-gel kinase assay after immunoprecipitation with anti-JNK IgG confirmed that the 46-kDa kinase band was p46JNK (JNK-1) and the 55-kDa kinase band was p55JNK (JNK-2). Thus, in the present study, the use of in-gel kinase method allowed us to successfully measure ERKs (p44ERK and p42ERK) and JNKs (p46JNK and p55JNK) in crude aortic extracts.

Blood Pressure, Aortic Weight, and Aortic MAP Kinases of DS Rats Fed a High-Salt Diet

Fig. 2-(A) shows blood pressure of DS rats fed an 8% NaCl (high-salt) for 1, 5 and 9 weeks. DS rats fed a 0.3% NaCl (low-salt) diet were normotensive throughout the experiments. Blood pressure of DS rats was already slightly but significantly increased at 1 week after start of a high-salt treatment, reached the peak levels (209±5 mmHg) after 5-week treatment, and remained a similar high blood pressure after 9-week treatment. As shown in Fig. 2-(B), aortic weight in DS rats fed an 8% NaCl significantly and progressively increased with the development of hypertension, compared with normotensive control.

As shown in Fig. 3, aortic p42ERK and p44ERK activities of DS rats tended to be increased at 1 week after start of high-salt diet, although not statistically significant. Five-week treatment with a high-salt diet increased aortic p42ERK and p44ERK activities of DS rats by 2.7 and 2.5-fold, respectively, compared with control DS rats. The similar increase in aortic p42ERK and p44ERK activities was observed in DS rats treated with a high-salt diet for 9 weeks.

As shown in Fig. 4, p46JNK was the major isoform in rat aorta. Aortic p46JNK in DS rats was already increased by 1.9-fold by 1 week of a high-salt diet, but

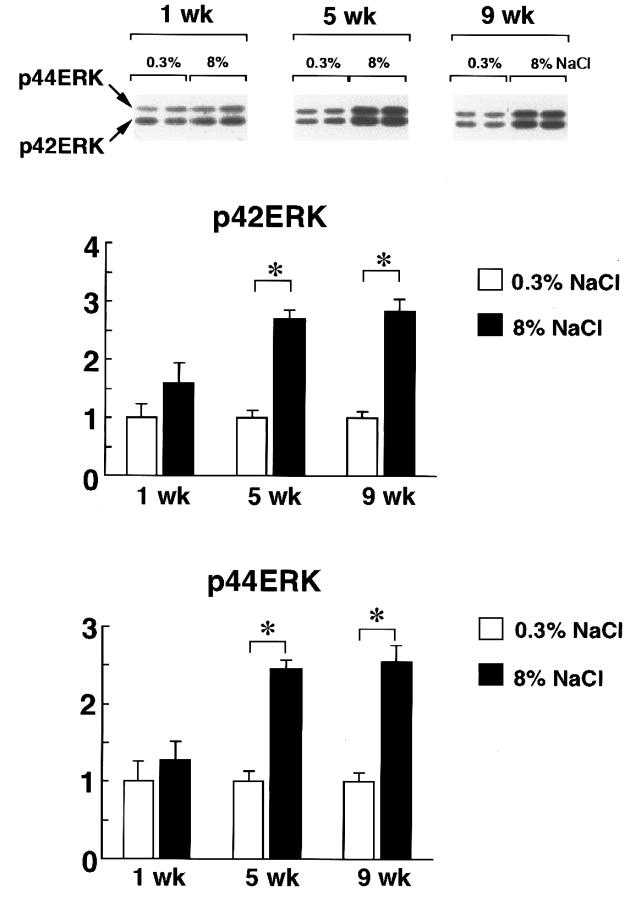

FIG. 3. Aortic p44ERK and p42ERK activities of DS rats fed a 0.3% NaCl (low-salt) or an 8% NaCl (high-salt) diet for 1, 5 and 9 weeks. Representative autoradiograms of aortic ERK activities were shown in the upper panel. In both p44ERK and p42ERK activities, the mean value of DS rats fed a low-salt diet at each age is represented as 1. Each bar represents mean±SE (n=6). *P<0.01.

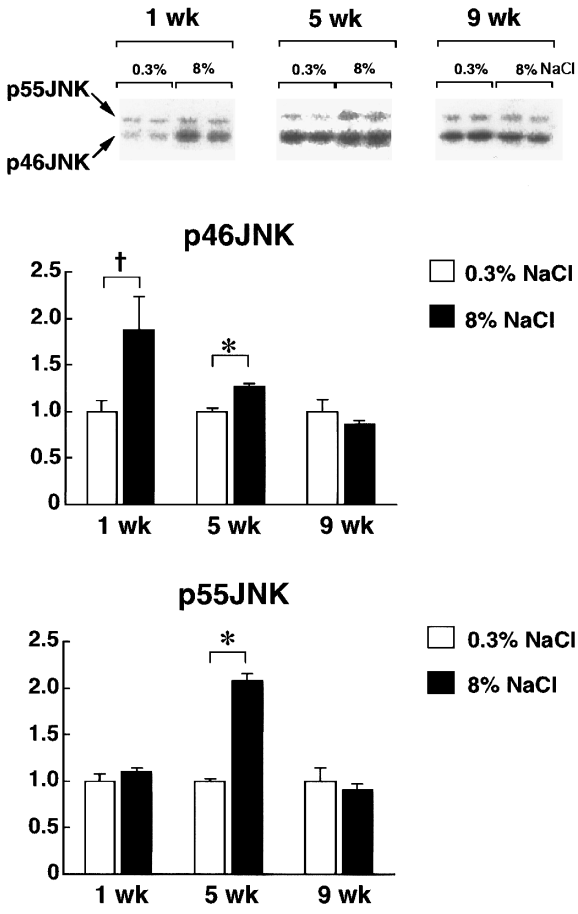

FIG. 4. Aortic p46JNK and p55JNK activities of DS rats fed a 0.3% NaCl (low-salt) or an 8% NaCl (high-salt) diet for 1, 5 and 9 weeks. Representative autoradiograms of aortic JNK activities were shown in the upper panel. In both p46JNK and p55JNK activities, the mean value of DS rats fed a low-salt diet at each age is represented as 1. Each bar represents mean±SEM (n=6). †$P<0.05$, *$P<0.01$.

thereafter decreased and returned to control levels after 9-week treatment. On the other hand, aortic p55JNK activity (the minor isoform) was not increased by 1-week treatment with a high-salt diet, but increased by 2.1-fold after 5 weeks of a high-salt diet. Thus, aortic p46JNK and p55JNK activities of DS rats were increased by a high-salt diet, with a different time course from ERKs.

Blood Pressure, Aortic Weight, and Aortic MAP Kinases of SHRSP and WKY

As shown in Fig. 5-(A), there was no significant difference in blood pressure between SHRSP and WKY at 5 weeks of age. However, blood pressure of SHRSP significantly increased at 14 weeks of age, compared with the age-matched WKY (177±5 vs. 116±3 mmHg; $P<0.01$), and further increased at 24 weeks of age (236±4 vs. 132±4 mmHg; $P<0.01$). The elevation of blood pressure in SHRSP was accompanied by the progressive increase in aortic weight, compared with WKY.

As shown in Fig. 5-(B), aortic ERK activities were not different between SHRSP and WKY at 5 weeks of age. However, p42ERK and p44ERK activities were increased by 1.8 and 1.5-fold, respectively, in 14-week-old SHRSP compared with the same-aged WKY, and were further increased by 3.0 and 4.2-fold, respectively, in 24-week-old SHRSP. As shown in Fig. 5-(C), aortic p46JNK and p55JNK activities in SHRSP were not significantly increased at 5 weeks of age compared with WKY, but significantly increased 1.3 to 1.8-fold at 14 and 24 weeks of age compared with WKY.

On the other hand, as shown in Fig. 6, there was no significant difference in the activity of ERKs or JNKs from liver, stomach, spleen and lung, between 24-week-old SHRSP and WKY rats, except for the decrease in p55JNK activity in liver of SHRSP.

DISCUSSION

Previous data concerning the activities and functions of MAP kinases have largely come from in vitro studies using cultured cells under non-physiological conditions. The in vivo role of MAP kinases remains to be elucidated. Furthermore, it is still unknown whether or not MAP kinases are activated in vascular diseases in vivo. In the present study, we first examined vascular MAP kinase activities in hypertensive rats in vivo. Hypertension in DS rats, induced by a high-salt diet, was followed by the increased activity of aortic p42ERK and p44ERK, suggesting the involvement of hypertension in the activation of ERKs. To examine whether the chronic increase in aortic ERKs was a specific event to hypertensive DS rats, we also examined aoritc ERKs in SHRSP, which is a different type of hypertensive model and spontaneously develops hypertension under a low-salt diet, unlike DS rats. As in the case of DS rats, the development of hypertension in SHRSP was accompanied by the progressive increase in aortic p42ERK and p44ERK activities. These observations first demonstrate that chronic hypertension leads to the sustained increase in aortic ERK activity in vivo. Furthermore, of note, the persistent activation of ERKs in hypertensive rats is in contrast to previous data on cultured cells, including vascular smooth muscle cells[23], in which ERKs are activated in a transient manner by extracellular stimuli, thereby suggesting the difference in the regulation of ERKs between in vivo and in vitro conditions.

Interestingly, in both DS rats and SHRSP, aortic JNK activities were increased with a different time course from ERKs. In DS rats fed a high-salt diet for 1 week, p46JNK (the major isoform of JNK) activity was already significantly increased, earlier than ERKs. These results, taken together with the in vitro evidence that JNK is stimulated by osmotic stress[9], suggest that

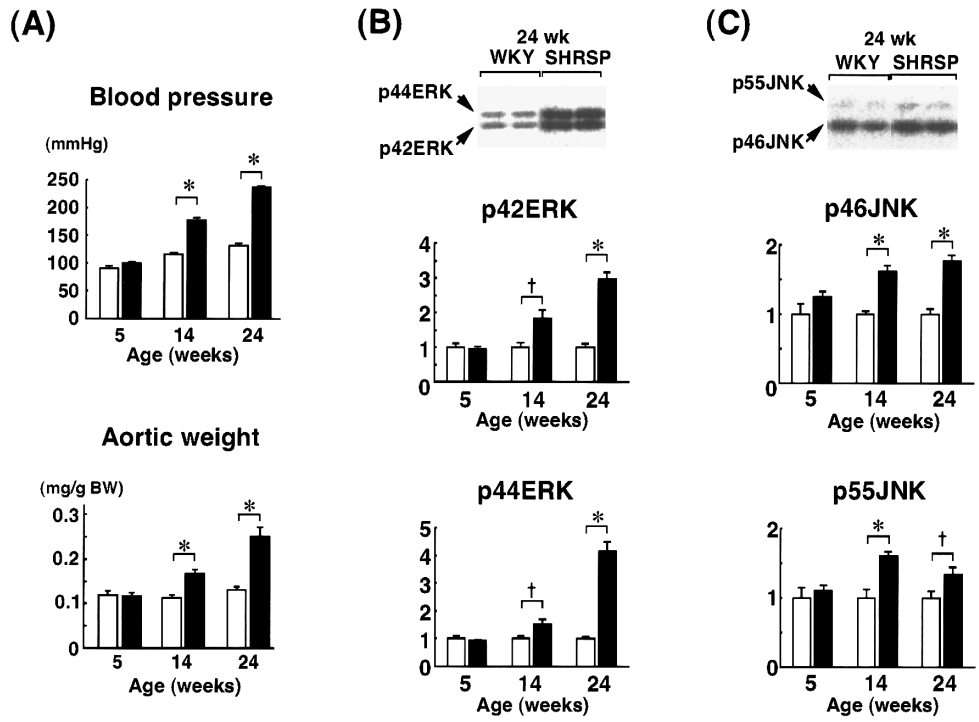

FIG. 5. Blood pressure, aortic weight, and aortic ERK and JNK activities of WKY (□) and SHRSP (■). Aortic weight was corrected for body weight (BW). Representative autoradiograms of aortic ERK and JNK from 24-week-old rats are shown in the upper panel. In both ERK and JNK activities, the mean value of WKY at each age is represented as 1. Each bar represents mean±SEM (n=4-7). †P<0.05, *P<0.01.

salt loading itself might activate aortic p46JNK in DS rats. Furthermore, unlike p46JNK, p55JNK (the minor isoform of JNK) was increased at established hypertensive phase (5 weeks of high-salt treatment), suggesting the differential regulation of the two JNK isoforms in DS rats. On the other hand, in SHRSP, the increase in both p46JNK and p55JNK was related to hypertension, but JNKs reached the peak at an earlier phase than ERKs. Thus, our present data suggest that aortic JNKs are regulated in a more complex manner than ERKs in hypertensive rats.

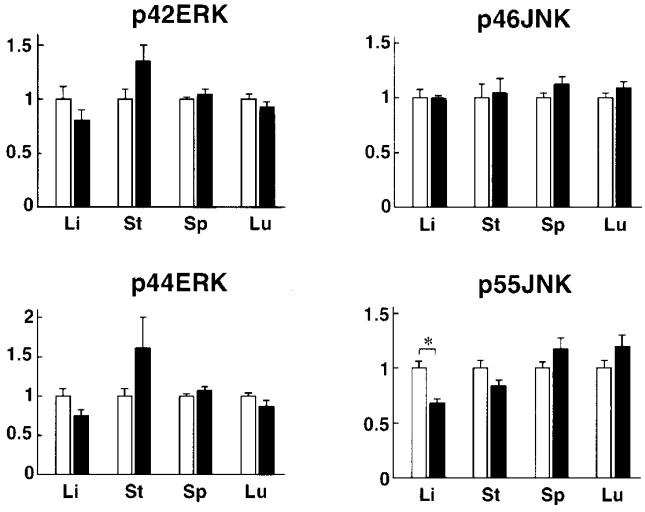

FIG. 6. ERK and JNK activities from liver (Li), stomach (St), spleen (Sp), and lung (Lu) of 24-week-old WKY (□) and SHRSP (■). In both ERK and JNK activities, the mean value of WKY is represented as 1. Each bar represents mean±SEM (n=5). *P<0.01.

To examine whether or not the increased activity of ERKs and JNKs is specific in vascular tissue, we also determined these activities in other tissues of hypertensive SHRSP. Unlike vascular tissue, we found no significant increase in the activities of ERKs or JNKs in liver, stomach, spleen and lung of hypertensive SHRSP. Furthermore, Silver et al. have previously compared vascular protein kinase C activity between hypertensive and normotensive rats, and found no significant elevation of vascular protein kinase C activity in hypertensive rats[24]. These findings support that vascular MAP kinase activities are enhanced in hypertensive rats in a specific manner.

Recent in vitro data show that the activation of ERK plays a critical role in growth factor-induced protein synthesis in rat aortic smooth muscle cells[25]. ERK phosphorylates and increases the activity of the transcriptional factor, Elk-1, which is required for enhanced transcription of the c-fos gene[26]. JNK phosphorylates c-jun on 2 critical N-terminal serines, leading

to increased Jun transactivational activity[10,26,27]. Thus, the activation of these kinases leads to increased AP-1 activity. It has been demonstrated that AP-1 can stimulate transforming growth factor-β1 (TGF-β1) expression via AP-1 consensus sequence present in the promoter region of TGF-β1 gene[28]. Furthermore, we have previously reported that the gene expression of TGF-β1 and extracellular matrix components such as collagen types I and III and fibronectin is enhanced in the aorta of hypertensive rats[29]. These findings, taken together with the fact that TGF-β1 can potently stimulate the production of extracellular matrix[30], suggest that TGF-β1 may be involved in vascular remodeling in hypertension. Thus, the increased activity of vascular ERKs and JNKs in hypertensive rats may be responsible not only for smooth muscle cell growth but also for the accumulation of extracellular matrix via the stimulation of TGF-β1 expression.

In conclusion, our present work provided the first in vivo evidence that the activity of aortic ERKs and JNKs is persistently enhanced in hypertensive rats and that the regulating mechanism is different between ERKs and JNKs. Thus, we propose that ERKs and JNKs may play an important role in the pathophysiology of hypertensive vascular disease.

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