Endothelin Antagonism Suppresses Plasma and Cardiac Endothelin-1 Levels in SHRSPs at the Typical Hypertensive Stage

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Endothelin-1 (ET-1) has been implicated in hypertension, heart failure, atherosclerosis, and pulmonary hypertension. In all these conditions, plasma immunoreactive ET-1 levels are elevated, and tissue ET-1 expression is increased. Clinical trials have demonstrated potentially important benefits of ET antagonism among patients with essential hypertension, pulmonary hypertension, and heart failure. It is unknown whether ET antagonism affects the production of ET-1 in stroke-prone spontaneously hypertensive rat (SHRSP) heart at the typical hypertensive stage. The objective of this study was to investigate the effects of ET blockade on the expression levels of plasma and cardiac ET-1 in SHRSPs. SHRSPs were treated for 3 months with SB209670 (ET_A/ET_B dual receptor antagonist) or with saline (vehicle) commencing at the prehypertensive stage (age 6 weeks). Plasma and left ventricular ET-1 peptide levels were measured using enzyme-linked immunoabsorbent assay. Compared with age-matched control Wistar-Kyoto rats, peptide levels of ET-1 were significantly upregulated in vehicle-treated SHRSP heart; this upregulation was reversed by long-term ET antagonism. Plasma ET-1 levels were also significantly increased in vehicle-treated SHRSPs and were normalized by ET antagonism. mRNA expression of preproET-1, which is the source of ET-1 peptide production, was significantly increased in vehicle-treated SHRSP heart and was normalized by ET antagonism. Marked cardiac hypertrophy and fibrosis at the histologic level in SHRSPs were ameliorated by ET antagonism, and left ventricular hypertrophy as seen on echocardiography in SHRSPs was suppressed by ET blockade. After ET antagonism, systolic blood pressures were reduced in SHRSPs; diastolic blood pressures were unchanged. The reversal effect of the upregulated ET system in SHRSP heart by ET antagonism might be independent of blood pressure change. By suppressing the upregulated ET system, ET antagonism might be beneficial in arresting cardiac remodeling. Exp Biol Med 231:919–924, 2006

Key words: SHRSP; heart; endothelin-1; endothelin antagonism

The stroke-prone spontaneously hypertensive rat (SHRSP) is a rat model of genetic hypertension. SHRSP is regarded as a useful animal model for investigation of the mechanism of pathologic cardiac hypertrophy and remodeling *in vivo*. Significant vascular and cardiac hypertrophy in this model has been previously documented (1), and classic genetic crosses can be performed to test the cosegregation of each hypertrophy phenotype with blood pressure (BP) (2). In addition, pharmacologic interventions may be used in a controlled fashion to manipulate BP and angiotensin II, one of the most important growth factors for vascular smooth muscle cells and cardiac myocytes (3, 4). Treatment of mature SHRSPs with the angiotenson-converting enzyme inhibitor perindopril for 4 weeks resulted in significant reduction in angiotensin II concentration and in significant regression of cardiac hypertrophy (1). SHRSPs develop not only prominent left ventricular (LV) hypertrophy and fibrosis but also enhanced expression of cardiac remodeling–associated genes, such as transforming growth factor–β_1 (TGFβ_1) and collagen mRNA (5, 6). Few studies have focused on endothelin (ET) expression in SHRSP heart, although some work has been done on spontaneous hypertensive rats (SHRs).

ETs are vasoconstrictive peptides and have been implicated in a wide variety of cardiovascular diseases. There are three distinct isoforms of ET (ET-1, ET-2, and

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ET-3), which mediate their biologic actions by interacting with at least two receptors, ET_A and ET_B (7, 8). ET-1 is a pleiotropic peptide produced primarily by endothelial cells, and its synthesis is stimulated by major signals of cardiovascular stress, such as vasoactive agents (angiotensin II, norepinephrine, vasopressin, and bradykinin), cytokines (tumor necrosis factor-α and TGFβ), and other factors, including thrombin and mechanical stress. Because of the potent vasoconstricting and mitogenic effects of ET-1 and its involvement in various cardiovascular diseases, blockade of ET receptor has received considerable attention in the prevention of cardiovascular diseases (9, 10). ET-1 has been implicated in hypertension, heart failure, atherosclerosis, and pulmonary hypertension. In all these conditions, plasma immunoreactive ET-1 levels are elevated, and tissue ET-1 expression is increased. Clinical trials have demonstrated potentially important benefits of ET antagonists among patients with essential hypertension, pulmonary hypertension, and heart failure (10, 11).

This study was designed to investigate the expression of ET-1 in plasma and in cardiac tissues in SHRSPs at the typical hypertensive stage. It also evaluated the effects of dual ET antagonism on these alterations.

Materials and Methods

Animals and Drug Treatment. The experimental design was approved by the Hokkaido University School of Medicine Animal Care and Use Committee and by the University of Tsukuba Animal Care. Male SHRSPs were inbred in our laboratory (current generation, F57) and were maintained under constant temperature and lighting conditions, with free access to food and water. At age 6 weeks, which is the prehypertensive stage in this rat model, SHRSPs were randomly divided into two groups. One group of 15 animals was administered an ET_A/ET_B dual receptor antagonist, SB209670 (1 mg/day/rat), subcutaneously using an osmotic pump (Model 2ML4; DURECT Corporation, Cupertino, CA), which was implanted in the back. Another group of 15 animals was implanted with an osmotic pump containing saline as vehicle. The treatment period was 12 weeks, and the animals were evaluated at age 18 weeks, when they were in the typical hypertensive stage. Age-matched normotensive Wistar-Kyoto (WKY) rats ($n = 15$) served as a control group. Body weight and BP were measured every 4 weeks. During the last few days of the treatment period, echocardiographic measurement was performed in the experimental animals. On the day of experiment, the animals were deeply anesthetized with ketamine chloride (100 mg/kg, ip) and were exsanguinated. The heart was excised, and whole-heart and LV weights were measured. Some LV portions were embedded in optimum cutting temperature (OCT) compound (Sakura Finetechnical, Tokyo, Japan) and immediately frozen in liquid nitrogen. The remaining portions were preserved at –80°C without OCT compound. For paraffin sections, the heart was post-fixed in 4% paraformaldehyde overnight and was routinely processed for paraffin embedding.

Enzyme Immunoassay for Plasma and Cardiac ET-1 Levels. ET-1 concentrations in plasma and in LV tissue extracts were obtained using an ET-1 enzyme immunoassay kit (Immuno-Biological Laboratories, Fujioka, Japan). This kit is a solid-phase sandwich enzyme-linked immunoabsorbent assay (ELISA) using 2 kinds of high-specific antibodies. Tetraethyl benzidine is used as a coloring agent (chromogen), the strength of which is in proportion to the quantities of ET-1. In plasma and in LV tissue extracts, an equal volume of 0.1% trifluoroacetic acid (TFA) in water was added. The sample was then centrifuged at 3000 *g* for 15 mins at 4°C for clarification, and the supernatant was saved. After a 200-mg C18 Sep-Pak column (Part 235D1; Waters Inc., Milford, MA) was washed with 4×1 ml of 60% acetonitrile in 0.1% TFA followed by 4×5 ml of 0.1% TFA in water, the supernatant was applied to the column and was washed with 4×5 ml of 0.1% TFA in water. The sample was eluted slowly by applying 3×1 ml of 60% acetonitrile in 0.1% TFA in water, and the eluant was collected in a plastic tube. The eluant was then evaporated to dryness using a centrifugal concentrator under vacuum. The sample was reconstituted with assay buffer and was measured immediately according to the manufacturer's instructions.

Quantitative Real-Time Polymerase Chain Reaction (RT-PCR). Total LV tissue RNA was isolated by acid guanidinium thiocyanate-phenol-chloroform extraction with Isogen (Nippon Gene Ltd., Toyama, Japan). Briefly, the LV tissue was homogenized in Isogen (100 mg tissue/1 ml Isogen) with a Polytron tissue homogenizer (Model PT10SK/35; Kinematica, Lucerne, Switzerland). Precipitated RNA was extracted with chloroform, precipitated with isopropanol, and washed with 75% (vol/vol) ethanol. Resulting RNA was resolved in diethyl pyrocarbonate–treated water, treated with DNase I (Takara, Shiga, Japan) and was extracted again with Isogen to eliminate the genomic DNA. The RNA concentration was determined spectrophotometrically at 260 nm. Total tissue RNA was primed with 0.05 μg of oligo $d(pT)_{12–18}$ and reverse transcribed by omniscript reverse transcriptase using a first-strand cDNA synthesis kit (Qiagen, Tokyo, Japan). The reaction was performed at 37°C for 60 mins.

mRNA expression levels of preproET-1 in the heart were analyzed by quantitative RT-PCR with TaqMan probe using an ABI Prism 7700 Sequence Detector (Perkin-Elmer Applied Biosystems, Foster City, CA) as previously described (12). The gene-specific primers and TaqMan probes were synthesized using Primer Express v. 1.5 software (Perkin-Elmer Applied Biosystems) according to the published cDNA sequences for preproET-1 (13) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (14) mRNA. The sequences of the oligonucleotides were as follows:

preproET-1 forward:
5′-TCTACTTCTGCCACCTGGACAT-3′
preproET-1 reverse:
5′-GAAGGGCTTCCTAGTCCATACG-3′
preproET-1 probe: 5′-CATCTGGGTCAACACTCC-3′
GAPDH forward:
5′-GTGCCAAAAGGGTCATCATCTC-3′
GAPDH reverse:
5′-GGTTCACACCCATCACAAACATG-3′
GAPDH probe: 5′-TTCCGCTGATGCCCC-3′

The expression of GAPDH mRNA was used as an internal control. The PCR mixture (25 μl total volume) consisted of 450 n*M* of forward and reverse primers for preproET-1 and GAPDH, 200 n*M* of FAM-labeled primer probes, and TaqMan Universal PCR Master Mix (all from Perkin-Elmer Applied Biosystems). Each PCR amplification was performed in triplicate, using the following profile: 1 cycle of 95°C for 10 mins and 40 cycles of 94°C for 15 secs and 60°C for 1 min. For the standard curve in the quantitative RT-PCR, serial dilutions of rat heart cDNA were performed within the range of various concentrations (1×, 2×, 4×, 8×, and 16×). A no-template (water) reaction mixture was prepared as a negative control.

Statistical Analysis. Values are means ± SD. Statistical assessment of the data was performed using one-way analysis of variance, with multiple comparisons using the Fisher protected least significant difference test. Nonparametric data were analyzed using the Mann-Whitney *U* test or Wilcoxon signed rank test. $P < 0.05$ was considered significant.

Results

Plasma ET-1 Level. Plasma ET-1 levels were significantly increased in vehicle-treated SHRSPs compared with the genetic control WKY rats. After ET antagonism, plasma ET-1 levels were significantly decreased (Fig. 1).

Cardiac ET-1 Level. Cardiac ET-1 levels were significantly increased in vehicle-treated SHRSPs compared with the WKY rats. ET antagonism significantly decreased this upregulation (Fig. 2A).

Cardiac PreproET-1 mRNA Level. mRNA expression levels of cardiac preproET-1 were significantly upregulated in vehicle-treated SHRSPs. They were normalized by ET antagonism (Fig. 2B).

Changes in BP. Systolic BPs in experimental animals were 143 ± 5, 244 ± 7, and 211 ± 16 mm Hg for the WKY, vehicle-treated SHRSP, and SB209670-treated SHRSP groups, respectively ($P < 0.05$). Diastolic BPs were unchanged in the experimental animals.

Discussion

This study demonstrates that the gene and peptide expression levels of ET-1 are significantly increased in SHRSP heart at the typical hypertensive stage and that these upregulations are reversed by dual ET antagonism. Increased plasma ET-1 levels are also normalized by ET antagonism.

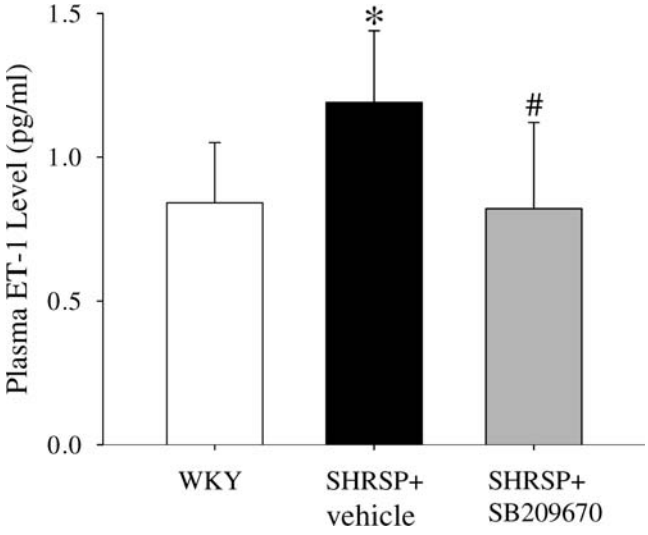

Figure 1. Plasma ET-1 levels (determined using ELISA) in the WKY, vehicle-treated SHRSP, and SB209670-treated SHRSP groups. Data are means ± SD ($n = 14$). *$P < 0.01$ compared with the corresponding values obtained in the WKY group. #$P < 0.01$ compared with the corresponding values obtained in the vehicle-treated SHRSP group.

Based on the development in SHRSPs of cerebrovascular disease that is pathologically similar to human disease (15), SHRSPs are unique among a large number of hypertensive rat models. Previous studies have documented significant vascular and cardiac hypertrophy in SHRSPs (1, 16). Furthermore, SHRSPs develop not only prominent LV hypertrophy and fibrosis but also increased expression of cardiac remodeling–associated genes such as TGFβ1 and collagen mRNA (5, 6). Therefore, SHRSP is a good model for studying the mechanisms of pathologic cardiac hypertrophy and remodeling *in vivo*.

ET-1, the most powerful endothelium-derived vasoconstrictor, is generated as preproET-1, which is enzymatically cleaved to form an intermediate big ET-1, and big ET-1 is further processed to form the biologically active mature ET-1 by a specific phosphoramidon-sensitive metalloprotease called ET-converting enzyme (7, 17). In the present study, ET antagonism suppressed ET-1 levels in plasma and in heart tissue in SHRSPs. The interactions among the different components of the ET system are complex and depend on a variety of factors. After ET antagonism, unbound ET-1 levels seem to be higher, but dual ET antagonism suppresses ET-1 levels in plasma and in cardiac tissues. Although complex feedback regulatory mechanisms persist among different components of the ET system, other vasoregulatory molecules such nitric oxide take part in the regulation of ET-1.

ET-1 binding has been shown to be upregulated in SHRSP heart, suggesting that ET-1 may have a pathophys-

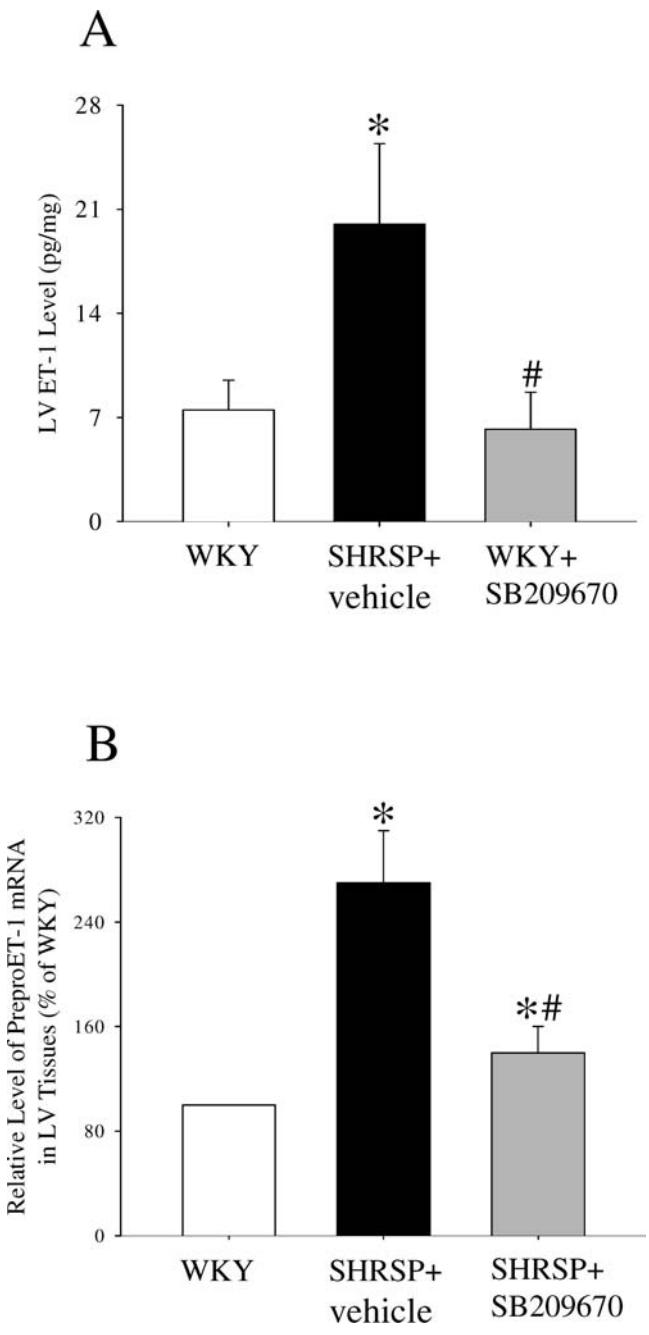

Figure 2. ET-1 peptide levels determined using ELISA (A) and mRNA expression of preproET-1 in LV tissues examined using RT-PCR (B) in the WKY, vehicle-treated SHRSP, and SB209670-treated SHRSP groups. Data are means ± SD ($n = 13$). *$P < 0.01$ compared with the corresponding values obtained in the WKY group. #$P < 0.01$ compared with the corresponding values obtained in the vehicle-treated SHRSP group.

iologic role in this animal model of genetic hypertension (18). ET-1 may directly or indirectly be involved in cardiac remodeling in SHRSP heart. ET-1 is also produced by cardiomyocytes (19). ET-1 acts as a key autocrine/paracrine mediator to trigger the hypertrophic signaling pathways by activation of extracellular signal-regulated kinase in myocardium via activation of ET_A/ET_B dual receptor (20). ET receptor blockade attenuates ventricular hypertrophy (21). Therefore, treatment with ET antagonists appears to be an attractive alternative to attenuate ventricular hypertrophy. ET antagonism reduced cardiac hypertrophy in the present study (data not shown). ET antagonism could also ameliorate echocardiographic alterations in SHRSP heart at the typical hypertensive stage (authors' unpublished observation, 2005). Diastolic dysfunction and cardiac hypertrophy are obvious on echocardiography in vehicle-treated SHRSPs and are greatly improved by ET antagonism. ET-1 may directly induce hypertrophic changes in SHRSPs, and ET blockade may regress these changes. The finding of attenuated cardiac hypertrophy accompanied by reduced cardiac ET-1 indicates that autocrine/paracrine ET-1 secretion pathways could be operative in the proliferative process blunted by ET antagonism. Decreases in regional ET-1 concentrations may result from ET-1 production decreases or from increased ET-1 clearance. The production of ET-1 begins with the cleavage of the translational product preproET-1. Our results show inhibition of ET antagonism on preproET-1 mRNA at the transcription levels. Therefore, decreases in ET-1 production may play a major role in regional ET-1 changes. Secretion and synthesis of ET-1 may be inhibited after ET antagonists reduce tissue ET-1 levels, which attenuates the progression of cardiomyocyte hypertrophy.

ET antagonism commencing at the prehypertensive stage suppressed cardiac angiotensin II levels in SHRSP heart (authors' unpublished observation, 2005). Moreover, ET antagonism downregulated increased $TGF\beta_1$ levels in SHRSP heart. Complex interactions may persist concerning the hypertrophy regression effects of ET antagonism in SHRSP heart. ET antagonism not only reduces cardiac hypertrophy but also regresses perivascular fibrosis in SHRSP heart. In salt-loaded SHRSPs, increases in renal ET-1 were associated with increases in $TGF\beta_1$, basic fibroblast growth factor, Type I procollagen expression, and matrix metalloproteinase-2 activity, which in turn were normalized by a selective ET_A antagonist (22), indicating that ET-1 is involved in renal fibrosis by stimulating growth factors and by inducing inflammation. The reversal of cardiac remodeling by ET antagonism may involve several growth factors and cytokines regulated by ET-1 in SHRSP heart at the typical hypertensive stage.

Various stages of hypertension may be differentially regulated in SHRs (23). This concept can be applied to SHRSPs. Cardiac ET-1 may act as an initiating hypertrophic factor during the early stage of pressure overload, but other factors, such as the renin-angiotensin system, may take over during the late stage of pressure overload (24). Our study was performed using young animals (i.e., age 18 weeks at the end of treatment) in which the development of hypertrophy is at its early stages compared with that associated with the severe malignant hypertensive stage in SHRSP heart. We speculate that early ET-1 production acts as a triggering factor to cardiac remodeling in SHRSPs.

Cardiac ET-1 levels were significantly higher at age 8 weeks in SHRs compared with WKY rats (25), and increased cardiac ET-1 activity has an important role in triggering cardiac hypertrophy at the development phase of hypertension in SHRs (26). This concept can also be applied to SHRSPs. The early reversal of upregulated ET-1 levels in SHRSP heart would prevent the alterations of other factors regulated by ET-1, contributing to further cardiac remodeling and the development of malignant hypertension in SHRSPs.

Normalization of cardiac ET-1 levels may be dependent on BP change or may be independent of BP in SHRSPs. In the present study, systolic BPs were slightly but significantly reduced in SHRSPs after ET antagonism, while diastolic BPs were unchanged.

Future studies should be directed at elucidating the mechanism of the suppression of ET-1 levels in SHRSP heart. Detailed investigations of other hypertrophy-related signaling pathways are needed.

Based on the results of the present study, we cannot conclusively comment about how mixed ET blockade in SHRSPs reduces plasma ET-1 concentrations. A previous study demonstrated that long-term (3 month) administration of BQ-123 (selective ET_A receptor antagonist) or SB209670 (used in the present study) comparably improved long-term survival and hemodynamic parameters in rats with chronic heart failure (27). In that study, SB209670 was used at a dosage of 1 mg/day/rat (27). The same dosage and treatment duration were used in the present study. Selective ET_B antagonists (28) and mixed blockade (29) have been shown to impair clearance of ET-1. Although clinical studies have shown ET-1 plasma concentrations to be variable among hypertensive patients (30), mixed blockade results in a rise in ET-1 concentration (31) rather than a fall as seen in the present study. Selective ET_A blockade can result in a decrease in ET-1 concentration (32), perhaps by displacement of ET-1 to clearing ET_B receptors. Therefore, we speculate that the dosage of SB209670 used in the present study (1 mg/rat/day) may result in predominantly ET_A rather than mixed ET receptor blockade, but more studies will be required to clarify this issue.

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