Renin-Angiotensin-Aldosterone System

Renin-Angiotensin Inhibition Reverses Advanced Cardiac Remodeling in Aging Spontaneously Hypertensive Rats

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Background: Many experiments using young hypertensive animal models support the evidence that angiotensin-converting enzyme inhibitor or angiotensin receptor type 1 blocker attenuates the progression of cardiac hypertrophy. However, it is still unclear whether inhibiting the renin-angiotensin system can reverse age-related cardiac hypertrophy. To clarify the role of renin-angiotensin system inhibition in naturally advanced myocardial hypertrophy we treated spontaneously hypertensive, aging rats with an angiotensin-converting enzyme inhibitor or an angiotensin receptor type 1 blocker.

Methods: We used osmotic pumps to deliver the blood-pressure reducers temocaprilat, olmesartan, hydralazine, or saline for 4 weeks.

Results: Heart and body weights were significantly reduced in animals treated with temocaprilat or olmesartan compared with animals treated with hydralazine or saline. Histologic myocyte size and cardiac fibrosis were significantly attenuated by temocaprilat or olmesartan. Real-time polymerase chain reaction (PCR) revealed that temocaprilat or olmesartan suppressed expression of cardiac transforming growth factor-β1 and fibroblast growth factor-2 mRNA, a marker of cardiac fibrosis. Cardiac and systemic oxidative stress assessed by 8-isoprostane levels was significantly reduced in animals treated with temocaprilat or olmesartan compared with hydralazine-treated or saline-treated rats. Renin-angiotensin system inhibition reduced cardiac expression of NAD(P)H oxidative components p22phox, p47phox, and gp91phox.


Key Words: Aging SHR, hypertrophy, ACE inhibition, angiotensin antagonists, oxidative stress.

Cardiac hypertrophy is an important complication of hypertension and is predictive of a high incidence of clinical events attributable to cardiovascular disease. Regression of cardiac hypertrophy may improve the prognosis in hypertensive patients. Previous studies have shown that lowering blood pressure (BP) is an important factor, but not the sole factor, for the development and reversal of cardiac hypertrophy. The spontaneously hypertensive rat (SHR) provides an animal model of high BP that is similar to essential hypertension in humans. Several studies have used SHRs to investigate the effects of angiotensin-converting enzyme inhibitors (ACEI) and angiotensin receptor type 1 blockers (ARB) on cardiac hypertrophy. In those studies, ACEI and ARB attenuated the progression of cardiac hypertrophy compared with other antihypertensive drugs in <15-week-old SHRs. It was reported that left-ventricular weight was comparable between <20-week-old SHRs and normotensive Wistar-Kyoto (WKY) rats and that left-ventricular weight in SHRs gradually increased with age. Linz et al reported that ACEI attenuated the progression of hypertrophy in 15-month-old SHRs. To translate research evidence from animals to humans, many opportunities to improve the study design are available for preclinical research.
ever, the effects of renin-angiotensin system (RAS) inhibition beyond BP reduction were not fully investigated in their study, as additional antihypertensive drugs were not included for control or hypertensive complications were not discussed.

A primary contribution of the RAS to the development of cardiovascular dysfunction is the generation of oxidative stress. The main enzymatic sources of superoxide production in the vascular wall and cardiomyocyte are \( \text{NAD(P)H} \) oxidase, xanthine oxidase, and endothelial nitric oxide (NO) synthase. The \( \text{NAD(P)H} \) oxidase plays a central role in angiotensin II–mediated superoxide production, which is blocked by ARB and ACEI. Several reports indicate that cardiac hypertrophy is partly due to the accumulation of reactive oxygen species, and inhibition of oxidative stress may attenuate cardiac hypertrophy.

The purpose of the present study was to clarify whether RAS inhibition improves age-related cardiac hypertrophy. We treated aging SHRs with ACEI and ARB. Furthermore, we assessed cardiac \( \text{NAD(P)H} \) oxidase production to elucidate whether RAS inhibition has any additional benefits independent of BP reduction. We also investigated the impact of oxidative stress on age-related cardiac hypertrophy.

**Methods**

**Experimental Protocol**

The study was approved by an institutional review committee and was conducted in accordance with the National Institutes of Health (NIH) “Guide for the Care and Use of Laboratory Animals.” Male SHRs (Oriental Yeast Co., Tokyo, Japan) had free access to normal chow and tap water. At the age of 50 weeks, the rats were anesthetized with intraperitoneal ketamine (80 mg/kg) and xylazine (10 mg/kg). Osmotic mini-pumps (ALZET model 2ML4; DURECT Co., Cupertino, CA) were implanted through a right inguinal vein. Drugs were infused through the catheter that was placed in the inferior vena cava through the right inguinal vein. Drugs were infused through the mini-pumps during the 4-week experiment. A pilot study was performed to determine antihypertensive drug doses for equivalent BP reduction. Experimental animals were divided into four groups. Each group (\( n = 6 \)) was treated as follows: ACEI (temocaprilat, 1 mg/kg/d), ARB (olmesartan, 0.2 mg/kg/d), hydralazine (0.15 mg/kg/d), or 0.9% saline. To assess age-related differences in cardiac phenotypes, we included six 10-week-old (young control) WKYs and SHRs and six 50-week-old (aging control) SHRs.

**Hemodynamic Measurements and Echocardiography**

Blood pressure and heart rate were measured with a tail-cuff system (BP-98; Softron, Tokyo, Japan) 1 day before pump implantation and 1, 3, 7, 14, 21, and 28 days after implantation. After the hemodynamic measurement on day 28, rats were anesthetized with intraperitoneal pentobarbital sodium (50 mg/kg, heart rate ~300 beats/min to avoid excess effects of anesthesia on cardiac function). An echocardiography system equipped with a 12-MHz transducer (Power Vision 6000; Toshiba, Tokyo, Japan) was used to obtain short-axis cardiac views and M-mode traces of the left ventricle. Left-ventricular (LV) end-diastolic diameter, LV systolic diameter, end-diastolic septum thickness, and posterior wall were measured according to American Society of Echocardiography guidelines. The LV mass was calculated using standard formulas: LV mass = \( 1.04 \times [(\text{LVDD} + \text{IVS} + \text{PW})^3 – \text{LVDD}^3] \).

**Tissue Samples**

After the hemodynamic and echocardiographic procedures, additional pentobarbital sodium (50 mg/kg) was administered, and blood and urine were collected from the vena cava and bladder, respectively. The rats were then intravenously perfused with heparinized saline (1000 USP/kg). Their hearts were excised and weighed before further analysis. To evaluate cardiac hypertrophy, we calculated the ratio of heart weight to body weight (HW/BW) in each treatment group.

**Histologic Analysis**

Hearts were immersed in 10% formalin, embedded in paraffin, and sectioned on a microtome. Every third 5-μm transverse section through the left ventricle was stained with hematoxylin-eosin and every fourth section with eosin and Masson’s trichrome. Images of the stained sections were captured digitally (Biozero; KEYENCE Co., Osaka, Japan). To evaluate myocardial hypertrophy, the cardiomyocyte cross-sectional area (>100 cells/section) in each hematoxylin and eosin-stained section was measured using Scion Image Beta 4.03 (Scion Co., Frederick, MD), and data were averaged across sections. The area of cardiac fibrosis was assessed in Masson’s trichrome-stained sections by quantifying the blue pixel content with Image J 1.34 (NIH, Bethesda, MD), and the data were averaged. For both measurements, area and pixel number were determined by the observer using a blinded protocol.

**Quantitative Real-Time Reverse Transcriptase-Polymerase Chain Reaction**

The left ventricle was isolated from cardiac tissue and quickly stored in an RNA stabilization reagent (RNALater; Qiagen GmbH, Hilden, Germany). RNA was purified using an RNA isolation protocol (SV Total RNA Isolation System; Promega Inc., Madison, WI). We used a TaqMan Gold reverse transcriptase–polymerase chain reaction (RT-PCR) kit, according to the manufacturer’s instructions (Applied Biosystems, Foster City, CA). Quantitative PCR was performed using real-time detection technology and analyzed on a Sequence Detection System (ABI PRISM 7900HT; Applied Biosystems) with specific primers and fluorescent probes for \( \text{NAD(P)H} \) components.
p22phox, p47phox, and gp91phox, transforming growth factor-β1 (TGF-β1), and fibroblast growth factor-2 (FGF-2) mRNA (TaqMan Gene Expression Assays; Applied Biosystems). Levels of mRNA were compared at various time points after normalization to concurrent 18s rRNA amplification.

Left Ventricular and Systemic Oxidative Stress

Urinary 8-isoprostane serves as a noninvasive index of oxidative stress. It is even possible to use 8-isoprostane to assess oxidative stress or damage to specific target organs. Therefore, we assessed 8-isoprostane in the left ventricle as the index of oxidative stress of the left ventricle. We assessed the total 8-isoprostane in LV tissue and urine with an EIA kit (Cayman Chemical Co., Ann Arbor, MI). Briefly, LV tissue was homogenized on ice in 100 mol/L phosphate buffer, at pH 7.4, containing 0.005% butylated hydroxy toluene at a ratio of 10 μL buffer/mg tissue. An equal volume of 15% KOH was added, and the homogenate was incubated at 40°C for 1 h. The sample was centrifuged, and the supernatant was neutralized with 1 mol/L KH₂PO₄. After purification, we assayed the tissue and urine at two dilutions, in duplicate, with the enzyme immunoassay kit and read at an absorbance of 405 nm. The 8-isoprostane level in LV tissue was expressed as picograms per milligram of protein. Urinary 8-isoprostane was expressed as nanogram per millimole per liter of urinary creatinine assessed by the Jaffe reaction kit (Cayman Chemical Co.).

In Situ Detection of Superoxide

Dihydroethidium (DHE), which is relatively specific for superoxide, is an oxidative fluorescent dye that undergoes a two-electron oxidation to form the DNA-binding fluorophore ethidium bromide or a structurally similar product. The DHE (Calbiochem, Darmstadt, Germany) staining for superoxide was carried out as previously described. Briefly, transmural myocardium tissue from each group was harvested, placed in cold saline, and embedded in optimal cutting temperature compound (Sakura Finetek, Carpiniteria, CA), and stained with 5 μmol/L DHE at 37°C for 30 min. Images of ethidium-stained tissue were obtained with an imaging system (Biozero; KEYENCE Co.) with a 595-nm filter. Generation of superoxide was demonstrated by red fluorescent labeling.

Statistical Analysis

Data are expressed as mean ± standard error of mean and were assessed with STATVIEW software (v.5; Abacus Concept Inc., Berkeley, CA). Group differences at specific time points were assessed by one-factor ANOVA and Fisher’s tests. Two-factor ANOVAs for repeated measures were followed by Fisher’s tests for testing serial changes. Statistical significance was set at P < .05.

Results

Blood Pressure in Aging Spontaneously Hypertensive Rats

Body weight and BP in aging SHR controls were higher than in young controls (Table 1). There were no significant differences of the heart rate data among the five groups; however, heart rate treated with hydralazine showed a higher tendency compared with other groups. As expected from the pilot study, each antihypertensive drug reduced BP equivalently in aging SHRs (Fig. 1).

Cardiac Hypertrophy and Fibrosis in Aging Spontaneously Hypertensive Rats

Age-related increases in HW/BW were observed in aging controls compared with young controls (Table 1). After 4 weeks of treatment, HW/BW in SHRs with RAS inhibition therapy was lower than in saline-treated SHRs (Table 1). Hydralazine treatment did not lower HW/BW compared with saline treatment.

Echocardiographic analysis showed that LV wall thickness and LV mass index were reduced in SHRs with RAS inhibition therapy compared with saline controls (Table 1). End-diastolic wall thickness of the septum treated with hydralazine was reduced compared with sham operation and LV end-diastolic diameter showed a higher tendency compared with other groups.

Histologic analysis of LV tissue was performed after 4 weeks of drug treatment (Fig. 2A, B). The cardiomyocyte cross-sectional area was smaller in SHRs with RAS inhibition therapy compared with hydralazine-treated and saline-treated animals. Compared with 10-week-old WKYs or SHRs, 50-week-old SHRs had extensive interstitial and perivascular fibrosis that was stained blue with Masson’s trichrome. Myocardial fibrosis was attenuated in 50-week-old SHRs that received RAS inhibition therapy compared with those that were treated with hydralazine or saline.

Expression of Hypertrophic Markers in Aging Spontaneously Hypertensive Rats

We measured expression of TGF-β1 and FGF-2 as markers of cardiac hypertrophy and fibrosis (Fig. 2C, D). Age-related differences were observed in TGF-β1 and FGF-2 expression in young and aging SHR controls. The RAS inhibition therapy significantly decreased TGF-β1 and FGF-2 expression in 50-week-old SHRs compared with saline controls. Only SHRs that were treated with a combination of olmesartan and temocaprilat had significantly different TGF-β1 and FGF-2 expression than hydralazine-treated animals.
### Table 1.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Young WKY</th>
<th>Young SHR</th>
<th>Aging SHR</th>
<th>Sham</th>
<th>Hydralazine</th>
<th>ACEI</th>
<th>ARB</th>
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<tr>
<td>Body weight (g)</td>
<td>337±11</td>
<td>335±4</td>
<td>336±4</td>
<td>331±4</td>
<td>433±10</td>
<td>426±11</td>
<td>419±10</td>
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<td>SBP (mm Hg)</td>
<td>132±4</td>
<td>124±5</td>
<td>128±5</td>
<td>135±6</td>
<td>147±13</td>
<td>134±9</td>
<td>140±10</td>
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<tr>
<td>HR (beats/min)</td>
<td>234±23</td>
<td>235±23</td>
<td>240±24</td>
<td>235±23</td>
<td>429±12</td>
<td>423±11</td>
<td>402±11</td>
</tr>
<tr>
<td>HW/BW (mg/g)</td>
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<td>2.9±0.2</td>
<td>3.0±0.2</td>
<td>3.0±0.2</td>
<td>3.0±0.2</td>
<td>2.9±0.2</td>
<td>2.9±0.2</td>
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<tr>
<td>LVDd (mm)</td>
<td>6.1±0.2</td>
<td>6.2±0.2</td>
<td>6.2±0.2</td>
<td>6.2±0.2</td>
<td>6.2±0.2</td>
<td>6.2±0.2</td>
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</tr>
<tr>
<td>LVSd (mm)</td>
<td>3.7±0.1</td>
<td>4.0±0.1</td>
<td>4.0±0.1</td>
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<td>4.0±0.1</td>
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<tr>
<td>PW (mm)</td>
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<td>1.9±0.1</td>
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### Hemodynamic measurements and echocardiography

The intensity of DHE staining in vascular endothelial cells and myocytes of 50-week-old SHRs was significantly enhanced compared with 10-week-old WKYs or SHRs, and in those treated with RAS inhibition it was significantly reduced compared with hydralazine and saline treatment (Fig. 3).

The LV and urine levels of 8-isoprostane were measured as a marker of oxidative stress in the heart and body (Fig. 4A, B). Cardiac and urinary 8-isoprostane levels were greater in 50-week-old SHRs than 10-week-old WKYs or SHRs. The RAS inhibition therapy significantly decreased cardiac and urinary 8-isoprostane levels in 50-week-old SHRs compared with hydralazine-treated or saline-treated SHRs.

The NAD(P)H oxidase is involved in angiotensin II–mediated superoxide production. We measured the cardiac expression of NAD(P)H oxidase components p22phox, p47phox, and gp91phox (Fig. 4C, D, E). The cardiac expression of these components was lower in 10-week-old WKYs or SHRs than in 50-week-old SHRs. The cardiac expression of these components was lower in aging SHRs with RAS inhibition therapy than in those treated with hydralazine and saline. No significant differences were observed in 8-isoprostane levels and expression of NAD(P)H components in SHRs in the three RAS inhibition therapy groups.

### Discussion

In the present study, we demonstrated that RAS inhibition therapy with ACEI and ARB attenuated cardiac hypertrophy in 50-week-old SHRs. Aging SHRs had cardiac hypertrophy and fibrosis related to higher oxidative stress and growth factor production than young SHRs. In contrast to young SHRs, aging SHRs had obvious LV hypertrophy and cardiac fibrosis. These conditions have an

### Oxidative Stress in Aging Spontaneously Hypertensive Rats

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### FIG. 1.

Blood pressure of 50-week-old spontaneously hypertensive rats (SHRs) before treatment to 4 weeks of treatment. The SHRs were treated with (○) saline, (●) hydralazine, (◆) angiotensin-converting enzyme inhibitor, and (●) angiotensin receptor type 1 blocker. *P < .05 vs other treatments.
important role in the pathogenesis of diastolic dysfunction in hypertensive heart disease. The RAS inhibition therapy not only reduced heart weight but also improved fibrosis in the left ventricle and reduced TGF-β1 and FGF expression. These data support a previous report that ramipril treatment significantly extended the lives of 15- and 21-month-old SHRs. The pharmacologic profiles of ACEI and ARB are substantially different. For instance, ARB inhibits the action of angiotensin II generated not only by angiotensin-converting enzyme but also through

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**FIG. 2.** Histologic semiquantitative evaluation and mRNA of transforming growth factor-β1 (TGF-beta1) and fibroblast growth factor-2 (FGF-2). (A) The cardiomyocyte cross-sectional area in each group. (B) The myocardial fibrosis in each group. (C) The level of TGF-β1 mRNA in left ventricle in each group. (D) The level of FGF-2 mRNA in each group. *P < .05 v young WKY or SHR; †P < .05 v sham; #P < .05 v hydralazine. ACEI = angiotensin-converting enzyme inhibitor; ARB = angiotensin receptor type 1 blocker; SHR = spontaneously hypertensive rat; WKY = Wistar-Kyoto rat.

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**FIG. 3.** Dihydroethidium staining of left ventricular tissue. Representative images of the left ventricle stained with the oxidative fluorescent dye DHE. (A) Young WKY; (B) young SHR; (C) sham; (D) hydralazine; (E) angiotensin-converting enzyme inhibitor; (F) angiotensin receptor type 1 blocker. Original magnification, ×200.
alternative pathways. On the other hand, ACEI blocks the breakdown of bradykinin. Recent studies suggest that the efficacy of combined treatment with ACEI and ARB may exceed that of each drug alone. The subpressor dose of combined ARB and ACEI blocked the progression of ventricular fibrosis and hypertrophy more than ACEI alone at advanced stages of hypertensive diastolic heart failure in Dahl salt-sensitive rats.

From echocardiographic analysis, wall thickness reduction was shown in aging SHRs treated by hydralazine as well as RAS inhibition; however, treatment with hydralazine showed larger tendency of LV end-diastolic diameter compared with RAS inhibition. The wall thickness reduction might be caused by lowering BP, and cardiac enlargement might be caused by hydralazine-induced tachycardia with stimulation of the sympathetic nervous system, even if statistical significance was not observed. Thus, the improvement in cardiac hypertrophy evaluated as LV mass index by hydralazine was less than with RAS inhibition therapy despite equivalent BP reduction by the therapies. Accumulating these results, the anticardiac remodeling effects of RAS inhibition did not depend on BP reduction or heart rate.

Cardiac oxidative stress, as measured by the production of cardiac 8-isoprostane and DHE staining, was higher in 10-week-old SHR compared with age-matched WKY and increased in 50-week-old SHR. These results are consistent with previous reports in other animal models that superoxide levels are higher in SHR than WKY and increase with age. Potential sources of superoxide are endothelial NO synthase, xanthine oxidase, and NAD(P)H oxidase. Among them, NAD(P)H oxidase is the main source of angiotensin II–mediated superoxide production. In this study, RAS inhibition therapy reduced cardiac oxidative stress and reduced expression of NAD(P)H components. Because the same depressor dose of hydralazine failed to attenuate cardiac oxidative stress, the difference in antihypertrophic effects between hydralazine and RAS inhibition therapy may be due to antioxidative effects of RAS inhibition therapy.

A reversal of existing remodeling is one of the very important findings in the present study. A previous report

**FIG. 4.** Oxidative stress measured by urinary and ventricular 8-isoprostane and mRNA of p22phox, p47phox, and gp91phox. (A) The levels of urinary 8-isoprostane indexed to creatinine in each group. (B) The levels of 8-isoprostane in the left ventricle (LV) indexed to protein in each group. (C) The level of p22phox mRNA in the left ventricle in each group. (D) The level of p47phox mRNA in the left ventricle in each group. (E) The level of gp91phox mRNA in the left ventricle in each group. *P < .05 v young WKY or SHR; †P < .05 v sham; #P < .05 v hydralazine. Abbreviations as in Fig. 2.
showed that ARB improved matrix metalloprotease (MMP) and tissue inhibitor of MMP (TIMP) balance.\textsuperscript{27} Another report showed that oxidative stress activated MMPs and changed TIMP to oxidized TIMP, and then extracellular matrix and collagen in the left ventricle might increase.\textsuperscript{28} This report also revealed that RAS inhibition with an ACE inhibitor or an ARB decreased fibrosis with reduction in oxidative stress and MMP activity. To reverse existing fibrosis, this balance of the MMP/TIMP family might be quite important, and RAS inhibition might have a reductive balance in the MMP/TIMP family, and then finally reduce fibrosis in the left ventricle. Furthermore, Tamura et al\textsuperscript{29} also suggested that RAS inhibition could reverse cardiac myocyte hypertrophy in 22-month-old hypertensive rats with histologic \(\alpha\)-actin reduction. This might be one of the possibilities to reduce cardiac myocyte size in the present study.

**Study Limitations**

The present study included several limitations. First, to clarify influences of long-term hypertension, further studies using an aging WKY would be required. Second, further investigation might be also needed to clarify the differences and agreements of ACEI and ARB to improve cardiac hypertrophy.

**Perspectives**

Because the SHR is regarded as a model of human essential hypertension, aging SHRs in this study were comparable to patients who develop cardiac hypertrophy as a result of a long hypertensive state. Thus, the efficacy of RAS inhibition therapy in the present study can be useful for interpreting the results of studies of cardiac hypertrophy in humans.

In the present study we did not assess complications of cardiac hypertrophy such as heart failure and arrhythmia, which affect the prognosis for hypertension. Thus, further investigation is required to determine whether RAS inhibition therapy attenuates these complications in aging rodents compared with other hypertensive drugs.

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