Exaggerated Blood Pressure Variability Superimposed on Hypertension Aggravates Cardiac Remodeling in Rats via Angiotensin II System-Mediated Chronic Inflammation

Hiroshi Kudo, Hisashi Kai, Hidemi Kajimoto, Mitsuhsa Koga, Narimasa Takayama, Takahiro Mori, Ayami Ikeda, Suguru Yasuoka, Takahiro Anegawa, Hiroharu Mifune, Seiya Kato, Yoshitaka Hirooka, Tsutomu Imaizumi

Abstract—Hypertensive patients with large blood pressure variability (BPV) have aggravated end-organ damage. However, the pathogenesis remains unknown. We investigated whether exaggerated BPV aggravates hypertensive cardiac remodeling and function by activating inflammation and angiotensin II–mediated mechanisms. A model of exaggerated BPV superimposed on chronic hypertension was created by performing bilateral sinoaortic denervation (SAD) in spontaneously hypertensive rats (SHRs). SAD increased BPV to a similar extent in Wistar Kyoto rats and SHRs without significant changes in mean blood pressure. SAD aggravated left ventricular and myocyte hypertrophy and myocardial fibrosis to a greater extent and impaired left ventricular systolic function in SHRs. SAD induced monocyte chemoattractant protein-1, transforming growth factor-β, and angiotensinogen mRNA upregulations and macrophage infiltration of the heart in SHRs. The effects of SAD on cardiac remodeling and inflammation were much smaller in Wistar Kyoto rats compared with SHRs. Circulating levels of norepinephrine, the active form of renin, and inflammatory cytokines were not affected by SAD in Wistar Kyoto rats and SHRs. A subdepressor dose of candesartan abolished the SAD-induced left ventricular/myocyte hypertrophy, myocardial fibrosis, macrophage infiltration, and inductions of monocyte chemoattractant protein-1, transforming growth factor-β, and angiotensinogen and subsequently prevented systolic dysfunction in SHRs with SAD. These findings suggest that exaggerated BPV induces chronic myocardial inflammation and thereby aggravates cardiac remodeling and systolic function in hypertensive hearts. The cardiac angiotensin II system may play a role in the pathogenesis of cardiac remodeling and dysfunction induced by a combination of hypertension and exaggerated BPV. (Hypertension. 2009;54:832-838.)

Key Words: blood pressure variability ■ hypertension ■ inflammation ■ angiotensin II ■ cardiac hypertrophy

The goal of hypertension treatment is not only to reduce blood pressure (BP) levels but also to prevent cardiovascular events. Among hypertensive patients, patients with large BP variability (BPV) have more advanced end-organ damage, such as left ventricular (LV) hypertrophy and carotid atherosclerosis.1–7 Recent studies have shown that exaggerated BPV is a risk factor for cardiovascular events in hypertensive patients, independent of diurnal BP changes.11 An exaggerated BPV is a characteristic feature of hypertension, especially in the elderly and in patients with carotid atherosclerosis.12–14 However, little is known about the mechanism underlying the aggravation of end-organ damage induced by a combination of hypertension and large BPV aggravates.

Our recent studies have shown that perivascular inflammation plays a pivotal role in hypertensive cardiac remodeling, especially in myocardial fibrosis (for review see References 15 and 16): in Wistar Kyoto rats (WKYs) with suprarenal aortic constriction, BP elevation induces perivascular inflammation characterized by monocyte chemoattractant protein-1 (MCP-1) induction and macrophage infiltration, which triggers transforming growth factor-β (TGF-β) induction and reactive myocardial fibrosis in the later phase.17–20 In addition, we have shown that a subdepressor dose of candesartan not only prevents MCP-1 induction and macrophage infiltration but also ameliorates reactive myocardial fibrosis in pressure-overloaded hearts.21 These findings suggest that the cardiac angiotensin II (angII) system triggers the fibroinflammatory process, independent of its pressor effect. Accordingly, in the present study, we investigated whether the angII system–mediated inflammation would be involved in the mechanism underlying the large BPV-induced...
aggravation of hypertensive remodeling. For this purpose, we created a rat model representing chronic hypertension with exaggerated BPV by performing bilateral sinoaortic denervation (SAD)\textsuperscript{22–24} in spontaneously hypertensive rats (SHRs).

**Methods**

The study protocol was reviewed and approved by the Kurume University Animal Care and Treatment Committee. Male WKYs and SHRs were purchased from SLC, Inc, and housed under standard conditions of humidity, room temperature, and a 12:12-hour dark-light cycle. They were provided with free access to tap water and chow. The Extended Methods section is available in the online Data Supplement (please see http://hyper.ahajournals.org).

**Experiment 1**

Experiment 1 included the following 4 groups: WKY/\textsuperscript{H11001} sham-operation (sham) group, WKY/\textsuperscript{H11001} SAD group, SHR/\textsuperscript{H11001} sham group, and SHR/\textsuperscript{H11001} SAD group. At 12 weeks, WKYs and SHRs randomly underwent the bilateral SAD or sham operation. Seven weeks after the SAD operation (19 weeks), rats (n/\textsuperscript{H11005}15/group) were subjected to BP monitoring, echocardiography, morphometry, immunohistostaining, and RT-PCR analysis, as described below.

**Bilateral SAD Telemetric Hemodynamic Monitoring**

At 12 weeks, rats underwent the bilateral SAD according to the procedure described previously by Krieger\textsuperscript{22} and van Vliet et al.\textsuperscript{23,24} At 18 weeks (6 weeks after the SAD operation), rats underwent the implantation of a BP radiotelemeter (model TA 11 PA-C 40, Data Sciences International). Seven days after the telemeter implantation (19 weeks), 24-hour BP and heart rate were monitored in unrestricted, conscious rats. The BP waveform was sampled at 500 Hz in 3-second bursts every 30 seconds (ie, 2880 samples per day). The averages of systolic BP, diastolic BP, mean BP (mBP), and heart rate were computed for each sample period. The 24-hour average, SD, and coefficient of the variance in mBP and heart rate were calculated for descriptive statistics of the distribution variability.

**Echocardiographic Studies**

After hemodynamic monitoring, transthoracic echocardiographic measurements of LV end-diastolic dimension, LV mass, and LV fractional shortening (n/\textsuperscript{H11005}10 per group) were performed using a commercially available echocardiographic machine equipped with a 7.5-MHz transducer (SDD 5500, Aloca).\textsuperscript{17}

**Morphometric Analysis and Immunohistostaining**

On the next day of the echocardiographic study, rats (n/\textsuperscript{H11005}10 per group) were euthanized with an overdose of pentobarbital. Blood was drawn from the right atrium for the measurement of serum norepinephrine, the active form of renin (active renin), interleukin-1β, and tumor necrosis factor-α at a commercially available laboratory (SRL Co). The rats were perfused fixed with 4% neutrally buffered paraformaldehyde at 100 mm Hg; then, the LV was isolated, weighed, and immediately embedded in paraffin.\textsuperscript{17}

The shortest transverse myocyte diameter was measured in 50 nucleated transverse sections of the myocytes in 3 hematoxylin and eosin–stained sections of each rat.\textsuperscript{25} The percentage area of myocardial fibrosis was calculated in 3 Mallory-Azan–stained sections of each rat.\textsuperscript{26} The ED1-labeled macrophages were counted at ×200 magnification in 3 cross-sections of each rat.\textsuperscript{19}
Real-Time RT-PCR Analysis
After hemodynamic monitoring, rats \(n=5\) per group were killed with an overdose of pentobarbital and then perfused with ice-cold saline for 5 minutes. Unfixed LV was isolated and snap frozen in liquid nitrogen until use. Total RNA was extracted and reverse transcribed, as described previously.\(^{27}\) Equal amounts of the resulting cDNA were subjected to real-time PCR for rat MCP-1, rat TGF-\(\beta\), and rat angiotensinogen.\(^{17,28}\) The expression level of the target gene was normalized by the GAPDH level in each sample.

Experiment 2
SHRs were randomly assigned into the following 4 groups: SHR+SAD+candesartan (Cand) group receiving SAD and candesartan; SHR+SAD+vehicle group receiving SAD and vehicle; SHR+Cand group receiving sham operation and candesartan; and SHR+sham group receiving sham operation and vehicle. At 12 weeks, SAD or sham operation was performed in SHRs. In the SHR+SAD+Cand group and SHR+Cand group, 0.1 mg/kg of candesartan was orally administered every day from 7 days after the operation. This dose of candesartan was the maximum dose that did not reduce BP during the observation period (data not shown). At 19 weeks, rats \(n=15\) per group) were subjected to BP monitoring, echocardiography, morphometry, immunohistostaining, or RT-PCR analysis.

Statistical Analysis
Data are expressed as mean±SD. The quantitative histological analysis was performed by 2 observers in a blinded manner. The interobserver or intraobserver variability was <5% in each experiment. Two-factor factorial ANOVA followed by Scheffe F test was performed for the comparison. A \(P<0.05\) was considered statistically significant.

Results

Experiment 1
Figure 1A shows the representative 24-hour telemetric recordings of mBP. At 7 weeks after the operation (19 weeks), mBP levels were \(\approx 100\) mm Hg in WKY+sham and 140 mm Hg in SHR+sham groups. The average of mBP was not changed by SAD in either WKYs or SHRs. SAD exaggerated BPV (Figure 1A and Table 1); SAD significantly increased parameters of BPV, namely, the SD and the coefficient of the variance of mBP, in WKYs and SHRs to a similar extent. Pulse pressure was not different among the 4 groups (data not shown). SAD did not affect the average, the SD, and the coefficient of the variance of heart rate in WKYs and SHRs (Table 1).

Effects of SAD on Hypertensive Cardiac Remodeling
In WKYs, SAD induced concentric LV hypertrophy (Figure 1B) and significantly increased LV wet weight (LVW)/body weight (BW) and myocardite diameter (Figure 1C, parts a and b). SHR+sham rats showed concentric LV hypertrophy associated with significant increases in LVW/BW and myocyte diameter. In SHRs, SAD induced further LV and myocyte hypertrophy. The magnitude of the SAD-induced increase in the ratio of LVW/BW and myocyte diameter were similar between WKYs and SHRs.

There was little myocardial fibrosis in WKY+sham rats (Figure 1B and 1C, part c). In WKYs, SAD induced modest perivascular fibrosis and little interstitial fibrosis. SHR+sham rats showed mild perivascular fibrosis. In SHRs, SAD not only enhanced perivascular fibrosis but also induced patchy and massive reparative interstitial fibrosis, resulting in a great increase in the percentage area of myocardial fibrosis.

Effects on Myocardial Inflammatory Changes
At 19 weeks, ED1-positive macrophages were scarcely found in the myocardium of WKY+sham rats (Figure 2). In WKYs, SAD induced mild perivascular macrophage infiltration. Also, SHR+sham rats showed mild macrophage infiltration in the perivascular space. In contrast to WKYs, SAD caused massive perivascular and interstitial macrophage infiltration in SHRs.

Myocardial mRNA expressions of MCP-1 (a potent chemokine for monocyte/macrophage), TGF-\(\beta\) (a tissue fibrosis protein), and rat angiotensinogen were subject to real-time PCR for rat MCP-1, rat TGF-\(\beta\), and rat angiotensinogen.\(^{17,28}\) The expression level of the target gene was normalized by the GAPDH level in each sample.

\begin{table}
\centering
\caption{General Characteristics of the Experimental Groups at 19 Weeks}
\begin{tabular}{l|c|c|c|c}
\hline
\textbf{Variable} & \textbf{WKY} & \textbf{SHR} & \textbf{WKY} & \textbf{SHR} \\
\hline
mBP, mm Hg & & & & \\
\hline
Average & 102±6 & 106±9 & 140±5* & 145±10* \\
SD & 9.1±0.7 & 22.5±4.3* & 13.3±0.9 & 20.9±6.2† \\
Coefficient of variance & 8.8±0.3 & 19.8±3.1* & 8.4±0.5 & 16.6±2.6† \\
\hline
Heart rate, bpm & & & & \\
\hline
Average & 313±18 & 322±11 & 296±18 & 304±18 \\
SD & 41±2.8 & 40±4 & 40±5 & 38±5 \\
Coefficient of variance & 13±2 & 12±1 & 13±1 & 12±2 \\
\hline
BW and right ventricular wet weight & & & & \\
\hline
BW, g & 368±14 & 342±15 & 333±10 & 300±27 \\
R/W BW, mg/g & 0.07±0.01 & 0.09±0.01 & 0.10±0.02 & 0.10±0.01 \\
Circulating neurohumoral factors & & & & \\
\hline
Active renin, pg/mL & 6.2±5 & 8.5±5 & 10±11 & 9±6 \\
Interleukin-1β, pg/mL & 3.4±0.5 & 3.2±0.6 & 3.1±0.5 & 2.9±0.3 \\
Tumor necrosis factor-α, pg/mL & 20.7±1.8 & 21.8±2.8 & 16.8±2.0 & 17.8±3.1 \\
Cardiac norepinephrine & & & & \\
Nonpinephrine, pg/mg wet tissue & 297±40 & 238±79 & 231±91 & 275±61 \\
Echocardiographic data & & & & \\
\hline
LV mass index, mg/g BW & 1.96±0.20 & 2.67±0.58* & 2.94±0.65* & 3.91±0.42† \\
LVDC, mm & 8.0±0.4 & 7.0±0.8 & 7.6±0.6 & 7.3±0.7 \\
LV fractional shortening, % & 46.0±2.3 & 49.9±1.6 & 50.3±2.1 & 44.2±3.9† \\
\hline
\end{tabular}
\end{table}

Unless described, there were no significant differences. Data are mean±SD. R/W BW, the ratio of RV free wall weight to BW; Active renin, the active form of renin; LVDC, LV end-diastolic dimension.

*\(P<0.05\) vs WKY+sham group.
†\(P<0.05\) vs SHR+sham group.

Figure 1. Representative microphotographs of immunohistostainings for ED1* macrophages (arrows; A) and pooled data of the number of infiltrating ED1* macrophages (B) at 19 weeks. Bar=1×SD \(n=10\) per group). **\(P<0.01\).
mediator), and angiotensinogen were examined for the fibroinflammatory changes induced by SAD (Figure 3). Myocardial expression levels of these molecules were similar in WKY + sham and SHR + sham rats. In WKYs, SAD upregulated MCP-1 and TGF-β expressions. In SHRs, SAD induced much greater upregulations of MCP-1 and TGF-β. The angiotensinogen expression was upregulated by SAD in SHRs but not in WKYs. Circulating levels of norepinephrine, active renin, interleukin-1β, and tumor necrosis factor-α did not differ among the 4 groups (Table 1).

Effects on Echocardiographic Cardiac Function
At 19 weeks, echocardiography revealed that SHR + sham rats showed concentric hypertrophy, manifested by a significant increase in LV mass compared with WKYS (Table 1). WKY + SAD rats showed mild hypertrophy with preserved LV systolic function. In SHRs, SAD induced significant LV hypertrophy and impaired LV fractional shortening.

Experiment 2
To determine the role of angII in the large BPV-induced aggravation of hypertensive cardiac remodeling and LV dysfunction, a subdepressor dose of candesartan was administered everyday to SHRs after SAD.

Effects of Candesartan on SAD-Induced Cardiac Remodeling in SHRs
The subdepressor dose of candesartan had no effect on BPV or hypertensive cardiac remodeling in sham-operated SHRs (Table 2 and Figure 4). Although candesartan did not change SAD-induced BPV in SHRs, SAD-induced cardiac remodeling was attenuated by candesartan. Candesartan inhibited the SAD-induced LV and myocyte hypertrophy. Moreover, candesartan almost prevented the SAD-induced perivascular and interstitial fibrosis, reducing the percentage area of myocardial fibrosis to the levels of SHR + sham rats.

Effects of Candesartan on SAD-Induced Cardiac Inflammation in SHRs
In SHRs, SAD upregulated myocardial MCP-1, TGF-β, and angiotensinogen mRNA expressions, associated with enhanced macrophage infiltration (Figures 4B, part d, and 5). Candesartan inhibited not only MCP-1 induction but also macrophage infiltration induced by SAD. Candesartan also prevented SAD-induced TGF-β and angiotensinogen induction. The expression level of these molecules and macrophage infiltration did not differ between SHR + sham and SHR + sham + Cand rats.

Effects of Candesartan on SAD-Induced LV Dysfunction in SHRs
SAD significantly reduced LV fractional shortening in SHRs (Table 2). The subdepressor dose of candesartan prevented the SAD-induced LV dysfunction. Candesartan did not affect LV function in sham-operated SHRs.

Discussion
The present study demonstrated that SAD induced chronic cardiac inflammation and aggravated hypertensive cardiac remodeling and LV dysfunction in SHRs. The effect of SAD on cardiac remodeling was greater in SHRs than in WKYS. Moreover, our results suggest that the cardiac angII system plays a key role in the SAD-induced inflammatory changes and the resultant aggravation of cardiac remodeling in SHRs.

Arterial baroreflex is the neural regulatory mechanism to dampen rapid BP changes. SAD disrupts the afferent pathway.

Table 2. Effects of a Subdepressor Dose of Candesartan on BP and Echocardiographic Data

<table>
<thead>
<tr>
<th>Variable</th>
<th>SHR + Sham</th>
<th>SHR + Cand</th>
<th>SHR + SAD + Vehicle</th>
<th>SHR + SAD + Cand</th>
</tr>
</thead>
<tbody>
<tr>
<td>mBP, mm Hg</td>
<td>140±7</td>
<td>141±10</td>
<td>142±10</td>
<td>142±12</td>
</tr>
<tr>
<td>SD</td>
<td>13.4±0.9</td>
<td>14.0±2.0</td>
<td>26.4±3.6*</td>
<td>27.4±5.9*</td>
</tr>
<tr>
<td>Coefficient of variance</td>
<td>9.4±0.5</td>
<td>9.7±1.5</td>
<td>18.3±2.6*</td>
<td>19.1±3.3*</td>
</tr>
<tr>
<td>Echocardiographic data</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LV mass, mg/g BW</td>
<td>3.02±0.56</td>
<td>3.05±0.73</td>
<td>3.83±0.45*</td>
<td>3.20±0.47†</td>
</tr>
<tr>
<td>LVDD, mm</td>
<td>7.7±0.7</td>
<td>7.8±0.9</td>
<td>7.2±0.8</td>
<td>7.6±0.6</td>
</tr>
<tr>
<td>LV fractional shortening, %</td>
<td>51.1±4.5</td>
<td>50.5±4.5</td>
<td>44.5±6.8*</td>
<td>53.2±3.1†</td>
</tr>
</tbody>
</table>

Telemetric BP measurement and transthoracic echocardiography were performed in SHRs at 7 weeks after SAD or sham operation (19 weeks old). Data are mean±SD. Unless described, there were no significant differences. LVDD indicates LV end-diastolic dimension.

*P<0.05 vs SHR + sham.
†P<0.05 vs SAD + vehicle.
of the arterial baroreflex system, which results in the augmentation of BPV without changing the average of mBP (Figure 1 and Table 1). Thus, we performed SAD in SHRs to create a model of rats with hypertension and a large BPV. The SAD procedure itself is known to induce sympathetic nerve activation and may lead to other changes currently unknown. An earlier study has shown that sympathetic nerve activity is transiently increased soon after SAD, and the activation wanes within a couple of weeks. An earlier study has shown that sympathetic nerve activity is transiently increased soon after SAD, and the activation wanes within a couple of weeks. SAD did not increase the circulating and cardiac norepinephrine levels in the chronic phase (19 weeks) in the present study (Table 1). In this context, the sympathetic nerve activity is not always increased in hypertensive patients with large BPV. In our preliminary experiment, any apparent histological changes were not observed in the hearts of SHRs 2 weeks after SAD (data not shown). Thus, the effect of SAD is likely attributable to the increased BPV in the chronic phase of this model.

In the present study, SAD induced much greater cardiac remodeling, particularly myocardial fibrosis, in SHRs as compared with WKYs (Figure 1B and 1C). It may have been possible that SAD induced a greater hemodynamic change in SHRs than in WKYs and then resulted in greater cardiac remodeling in SHRs. However, it was not so, because SAD did not change the average of mBP in either WKYs or SHRs and increased the magnitude of BPV in WKYs and SHRs to the similar extent (Figure 1A and Table 1). It is worthwhile to mention that SAD not only enhanced perivascular fibrosis but also provoked reparative fibrosis in SHRs. These findings suggest that the superimposition of large BPV on hypertension may have caused massive myocyte damage and loss, which may have resulted in reparative fibrosis to replace the damaged myocardium. This mechanism may account for the deterioration of LV function in SHR/SAD rats (Table 1).

The most important finding of this study is that chronic cardiac inflammation (ie, MCP-1 upregulation and macrophage infiltration) was induced by SAD in hypertensive hearts (Figures 2 and 3). It has been shown that infiltrated macrophages produce inflammatory cytokines, angiotensin-converting enzyme, and TGF-β, which would, in turn, amplify the inflammatory process. Thus, it is suggested that chronic myocardial inflammation participates in the aggravation of cardiac hypertensive remodeling by a combination of
hypertension and exaggerated BPV. Because circulating levels of interleukin-1β, tumor necrosis factor-α, norepinephrine, and active renin were not changed (Table 1), the SAD-induced myocardial inflammation is independent of systemic inflammation, sympathetic nerve activation, and the systemic renin-angiotensin system.

Interestingly, myocardial angiotensinogen expression was upregulated only in SHR+SAD rats (Figure 3). AngII has been shown to mediate macrophage transmigration through the direct activation of the angII type 1 receptor (AT1R) and through the indirect effect via the MCP-1 induction.34–38 We have demonstrated that a rapid BP rise transiently induces MCP-1 upregulation and macrophage infiltration, which leads to the TGF-β–mediated myocardial fibrosis in rats with a suprarenal aortic constriction.17–19 Furthermore, the cardiac angII system has been shown to trigger perivascular inflammatory changes and resultant myocardial fibrosis, independent of its pressor effect.23 Therefore, it is possible that the activation of the cardiac angII system with a combination of hypertension and exaggerated BPV would have induced the aggravation of hypertensive cardiac remodeling by creating the positive feedback loop of the fibroinflammatory process. To further address this issue, we examined rats 3 weeks after SAD (15 weeks). Macrophage infiltration and expression levels of MCP-1 and angiotensinogen were significantly greater in SHR+SAD rats than in SHR+sham rats, whereas cardiac hypertrophy and myocardial fibrosis did not differ between the 2 groups (Figure S1). These findings suggest that these inflammatory changes would precede myocardial remodeling.

It was reported that a large dose of candesartan, which significantly decreased BP levels, prevented LV hypertrophy in normotensive rats with SAD.39 However, it remains unknown whether the inhibitory effect of candesartan on cardiac remodeling was attributable to the direct AT1R blocking or simply to its depressor effect. In this study, a subpressor dose of candesartan prevented the SAD-induced aggravation of myocardial remodeling and LV dysfunction in SHRs without changing the magnitude of BPV (Figure 4 and Table 2). As shown in Figure S2, in SHRs, the subpressor dose of candesartan significantly reduced the level of SAD-induced AT1R tyrosine phosphorylation, an indicator of the AT1R activity, suggesting that SAD activated the AT1R, and this dose of candesartan effectively blocked the AT1R activity. Thus, it was considered that candesartan inhibited the downstream pathway thereafter. Importantly, candesartan abolished the SAD-induced upregulation of inflammatory mediators and macrophage infiltration. Because angiotensinogen induction was inhibited in SHR+SAD+Cand rats, candesartan may have blocked the positive feedback loop linking the fibroinflammatory process and the angII system. Taken together, it is suggested that the cardiac angII system may participate in the mechanism whereby large BPV induces chronic fibroinflammatory changes and subsequently aggravates cardiac remodeling and LV dysfunction in hypertensive hearts. Recently, Zou et al40 have found that mechanical stress can activate the AT1R independently of angII. Candesartan has been shown to inactivate the angII-independent activation of the AT1R as an inverse agonist.41 Therefore, it is possible that candesartan might inhibit the SAD-induced inflammation and remodeling, at least in part, through its inverse agonistic effect.

**Limitation of this Study**

The initial step of the large BPV-induced inflammatory change remains unknown from this study. Also, we did not determine the mechanism whereby a combination of hypertension and exaggerated BPV enhances inflammation and angiotensinogen induction. In the present study, the effects of BPV itself were not directly investigated. Thus, it is possible that other unknown mechanisms might have contributed to the effects of SAD. Finally, we cannot deny the possibility that the use of anesthesia during blood sampling may limit the interpretation of measurements of circulating norepinephrine and active renin.

**Perspectives**

Exaggerated BPV provokes chronic inflammation through the cardiac angII system–mediated mechanism, which leads to the aggravation of cardiac remodeling and LV function in hypertensive hearts. Candesartan may inhibit the large, BPV-induced aggravation of hypertensive cardiac remodeling independent of its BP-lowering effect. AngII system–mediated inflammation may be a target for the prevention and treatment of exaggeration of end-organ damage in patients with a large BPV superimposed on hypertension.

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**Disclosures**

None.

**References**


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Exaggerated Blood Pressure Variability Superimposed on Hypertension Aggravates Cardiac Remodeling in Rats via Angiotensin-Mediated Chronic Inflammation

Hiroshi Kudo¹, Hisashi Kai¹, Hidemi Kajimoto², Mitsuhisa Koga², Narimasa Takayama¹, Takahiro Mori¹, Ayami Ikeda¹, Suguru Yasuoka¹, Takahiro Anegawa¹, Hiroharu Mifune³, Seiya Kato⁴, Yoshitaka Hirooka⁵, Tsutomu Imaizumi¹.

¹Department of Internal Medicine Division of Cardio-Vascular Medicine,
²Cardiovascular Research Institute and ³Institute of Animal Experimentation,
Kurume University School of Medicine, Kurume, Japan, ⁴Department of Pathology and Cell Biology, Graduate School and Faculty of Medicine,
University of Ryukyus, Nishihara, Okinawa, Japan, and ⁵Department of Cardiovascular Medicine, Kyushu University Graduate School of Medical Sciences, Fukuoka, Japan.

Corresponding author: Hisashi Kai, MD, PhD
Department of Internal Medicine Division of Cardio-Vascular Medicine,
Kurume University School of Medicine.
67 Asahimachi, Kurume 830-0011, Japan.
phone: +81-942-31-7562 FAX: +81-942-33-6509
e-mail: naikai@med.kurume-u.ac.jp
**Expanded Methods**

The study protocol was reviewed and approved by the Animal Care and Treatment Committee of Kurume University. Male WKY and SHR were purchased from SLC Inc. (Shizuoka, Japan) and housed under standard conditions of humidity, room temperature and a 12:12-hours dark-light cycles. They were provided with free access to tap water and chow.

**Experiment 1**

Experiment 1 included the following 4 groups: WKY+sham operation (sham) group, WKY+SAD group, SHR+sham group, and SHR+SAD group. At 12 weeks old (w), WKY and SHR randomly underwent bilateral SAD or sham operation. Seven weeks after SAD operation (19 w), rats (n=15/group) were subjected to BP monitoring, echocardiography, morphometry, immunohistostaining or RT-PCR analysis.

**Bilateral SAD**

At 12 w, rats underwent bilateral SAD according to the procedure as previously described by Krieger and van Vliet. Briefly, rats were anesthetized intraperitoneally with a mixture of ketamine (50 mg/kg), medetomidine (0.5 mg/kg) and atropine sulfate (0.5 mg/kg). The aortic depressor nerve and the superior laryngeal nerve were cut bilaterally at the junction with the vagi. Bilateral superior cervical ganglia and cervical sympathetic trunks were resected. Thereafter, the carotid bifurcation and the initial 5 mm-segment of the external and internal carotid arteries were bilaterally stripped of the surrounding connective tissues using fine forceps, followed by the application of 10% phenol in absolute ethanol. Sham-operated rats underwent bilateral isolation of the neck
muscle alone. Phenylephrine test was performed immediately after SAD operation by monitoring arterial pressure through the left external carotid artery. Successful SAD was defined when a bolus intravenous injection of 10 µg/kg phenylephrine induced a BP increase more than 50 mmHg and produced a reflex heart rate decrease less than 20 beats/min.⁴

**Telemetric hemodynamic monitoring and data analysis**

At 18 w (6 weeks after SAD operation), rats underwent implantation of a BP radiotelemeter (model TA 11 PA-C 40, Data Sciences International, Minneapolis, Minnesota). Briefly, the catheter tip of the telemeter was inserted into the abdominal aorta between the origin of the renal arteries and the bifurcation of the iliac arteries under anesthesia with intraperitoneal pentobarbital (30 mg/kg) and ketamine (50 mg/kg).⁴ Thereafter, rats were housed individually in a hemodynamic monitoring cage.

At 19 w (7 days after telemeter implantation), 24-hour BP and heart rate were monitored in unrestricted, conscious rats. Hemodynamic data were recorded on a personal computer using a general purpose data acquisition system (Dataquest Art system 1.1 silver, Data Sciences International). The system was set to sample the BP waveform at 500 Hz in 3-second bursts every 30 seconds (i.e. 2880 samples per day). The averages of systolic BP, diastolic BP, mBP and heart rate were computed for each sample period, and were recorded on a diskette. A 24-hour segment of data was analyzed using a commercially available software (Microsoft Excel 2004). Any corrections not were made for change in telemeter offset. The 24-hour average, standard deviation and coefficient of the variance of mBP and heart rate were calculated for descriptive statistics of the distribution variability.
**Echocardiographic studies**

After hemodynamic monitoring, transthoracic echocardiographic studies were performed using a commercially available echocardiographic machine equipped with a 7.5-MHz transducer (SDD 5500, Aloca, Tokyo, Japan). Rats (n=10/group) were anesthetized with intraperitoneal ketamine (50 mg/kg) and xylazine (10 mg/kg). LV end-diastolic dimension (LVDd), LV mass, and LV fractional shortening were measured as described previously.\(^5\)

**Morphometric analysis and immunohistostaining**

At the next day of echocardiographic study, rats (n=10/group) were euthanized with an overdose pentobarbital. Blood was drawn from the right atrium for measurement of serum concentrations of active form of renin (active renin), norepinephrine, interleukin (IL)-1\(\beta\), and tumor necrosis factor (TNF)-\(\alpha\) at a commercially available laboratory (SRL Co, Fukuoka, Japan). After rats were perfuse-fixed with 4% neutrally buffered paraformaldehyde at 100 mmHg, the heart was removed. The LV was isolated from the both atria and the right ventricular free wall, weighed, and immediately embedded in paraffin. The paraffinized sections were subjected to hematoxylin-eosin (HE) staining, Mallory-Azan staining and immunohistostaining.\(^5\)

To evaluate myocyte hypertrophy and myocardial fibrosis, 3 independent HE-stained and 3 Mallory-Azan-stained sections of each rat were scanned and analyzed using a digital image analyzer, respectively. The shortest transverse myocyte diameter was measured in 50 nucleated transverse sections of the myocytes in each HE-stained section.\(^6\) The percent area of Azan-stained myocardial fibrosis (% myocardial fibrosis area) was calculated as previously
described. Paraffinized sections were subjected to immunohistostaining with an antibody for ED-1 (Chemicon International, Temecula, California) and a commercially available detection system (DAKO, Glostrup, Denmark). The labeled cells were counted at ×200 magnification in 3 independent entire cross-sections of each animal.

**Real-time reverse-transcription (RT)-polymerase chain reaction (PCR) analysis**

After hemodynamic monitoring, rats (n=5/group) were killed with an overdose of pentobarbital and then perfused with ice-cold saline for 5 minutes. Unfixed LV was isolated and snap-frozen in liquid nitrogen until use. Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, California) followed by RNase-free DNase I (Pharmacia Biotech, Piscataway, New Jersey). Aliquots of total RNA (1 µg) were reverse-transcribed using Ready-to-Go Your Prime-First Strand Beads (Pharmacia Biotech). Equal amount of the resulting cDNA was subjected to real-time PCR using the TaqMan Universal PCR Master Mix and a Sequence Detection System model 7700 (Applied Biosystems, Forrest City, California). Primer pairs and TaqMan probes for rat MCP-1, rat TGF-β, and rat angiotensinogen were obtained from Applied Biosystems. The TaqMan Rodent GAPDH Control Reagents were used to detect GAPDH as the internal standard. Expression level of the target gene was normalized by GAPDH level in each sample.

**Experiment 2**

SHR were randomized to the following 4 groups: SHR+SAD+candesartan (Cand) group receiving SAD and candesartan; SHR+SAD+vehicle group...
receiving SAD and vehicle; SHR+Cand group receiving sham operation and candesartan; and SHR+sham group receiving sham operation and vehicle. At 12 w, SAD or sham operation was performed in SHR. In SHR+SAD+Cand group and SHR+Cand group, 0.1 mg/kg candesartan was orally administered everyday from 7 days after the operation. This dose of candesartan was the maximum dose not to reduce BP over the observation period (data not shown). At 19 w, rats (n=15/group) were subjected to BP monitoring, echocardiography, morphometry, immunohistostaining or RT-PCR analysis, as described above.

**Statistical analysis**

Data are expressed as means±SD. Quantitative histological analysis was performed by 2 observers in a blinded manner. The interobserver or intraobserver variability was <5% in each experiment. Two-factor factorial ANOVA followed by Scheffe’s F test was performed for the comparisons. A p value <0.05 was considered statistically significant.
Supplementary Data

Inflammatory changes and cardiac remodeling in SHR at 15 w

We examined whether SAD-induced cardiac inflammatory changes would precede cardiac remodeling. Inflammatory changes and cardiac remodeling were investigated at 15 w, because preliminary experiment showed that SAD did not induce any apparent histological changes at 14 w. As shown in Figure S1A, there were no differences in LVW/BW and myocardial fibrosis between SHR+sham and SHR+SAD. However, SAD significantly increased macrophage count in SHR. MCP-1 and angiotensinogen (ATN) expressions were significantly greater in SHR+SAD than in SHR+sham (Figure S1B), whereas TGF-β expression was similar in both groups. These findings suggest that MCP-1-induced macrophage infiltration precedes cardiac remodeling. It was considered that myocardial fibrosis did not develop probably because TGF-β had not yet been upregulated at this early time point. These observations are in line with our previous studies demonstrating that inflammatory changes precedes cardiac remodeling and that MCP-1-mediated macrophage infiltration in early phase is crucial for induction of myocardial TGF-β upregulation and subsequent cardiac remodeling in later phase in pressure-overloaded rats.5, 8

Effects of a subdepressor dose of candesartan on AT1R activation in SHR

We examined the tyrosine phosphorylation levels of the AT1R in SHR, because AT1R activation induces the auto-phosphorylation of the tyrosine residues of the AT1R, which plays a role in the angII-induced activation of the intracellular signaling, such as phospholipase C-γ- and JAK/STAT-mediated pathways.11-13

As shown in Figure S2, at 19 w, SAD not only increased cardiac AT1R expression but also the AT1R phosphorylation in SHR, whereas the AT1R
expression and phosphorylation did not differ between WKY+sham and WKY+SAD. The magnitude of the increase in the AT1R phosphorylation was greater than that of the increase in the expression, suggesting net increase in phosphorylated AT1R. Candesartan prevented the SAD-induced increase and phosphorylation of the AT1Rs in SHR+SAD, but not in SHR+sham. These findings indicated that SAD activates the AT1R in SHR, which is effectively blocked by a subdepressor dose of candesartan. It remains to be elucidated in which cell components the AT1R is upregulated and activated in the hearts in SHR+SAD.

References


The role of tyrosine phosphorylation in angiotensin II-mediated 
Figure S1. Inflammatory changes and cardiac remodeling in SHR at 15 w

A. Pooled data of the effects of SAD on LVW/BW, %myocardial fibrosis, and ED-1-labeled macrophage count at 15 w. Bar=1xSD (n=4 per group).

B. Pooled data of real-time RT-PCR analysis for myocardial MCP-1, TGF-β, and angiotensinogen at 15 w. Bar=1xSAD (n=5 per group).
**Figure S2. Effects of a subdepressor dose of candesartan on AT1R activation in SHR**

A. Representative photographs of immunoblots for tyrosine-phosphorylated AT1R (pAT1R), AT1R, and GAPDH at 19 w. B. Pooled data of the effects of SAD on AT1R expression and the tyrosine phosphorylation levels of AT1R at 19 w. AT1R expression was normalized by GAPDH expression level and the phosphorylated AT1R level was normalized by AT1R expression level.

**Method:** At Week 19, unfixed LV (n=4 per group) was isolated and homogenized by using FastPrep homogenizer (ThermoSavant). The tissue proteins were immunoprecipitated using an anti-AT1R monoclonal antibody (Abcam). The immunoprecipitants were separated on 10% SDS-PAGE and subjected to immunoblotting using an anti-phosphotyrosine monoclonal antibody (Cell Signaling) and the chemiluminescence detection system (Pierce Biotechnology).