Large Blood Pressure Variability Aggravates Arteriolosclerosis and Cortical Sclerotic Changes in the Kidney in Hypertensive Rats

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Background: It has been shown that increased short-term blood pressure (BP) variability (BPV) aggravates hypertensive cardiac remodeling in spontaneously hypertensive rats (SHRs) through a cardiac angiotensin II (angII) system. However, little was known about the renal damage induced by large BPV. Thus, histological changes in the kidney were investigated and candesartan, an angII type 1 receptor blocker (ARB), was also examined to see whether it would prevent renal damage in SHRs with large BPV.

Methods and Results: Bilateral sinoaortic denervation (SAD) was performed in SHRs to create a model of a combination of hypertension and large BPV. SAD increased BPV without changing mean BP. Seven weeks later, SAD induced patchy, wedge-shaped, focal sclerotic lesions accompanied by interstitial fibrosis and ischemic changes of glomeruli and tubules in the cortex. The pre-glomerular arterioles adjacent to the sclerotic lesions showed arteriolosclerotic changes associated with vascular smooth muscle cell proliferation and extracellular matrix deposition, leading to the luminal narrowing and occlusion. Chronic treatment with a subdepressor dose of candesartan prevented not only arteriolosclerotic changes but also cortical sclerotic lesions in SHRs with SAD without changing BPV.

Conclusions: Large BPV aggravates pre-glomerular arteriolosclerosis, which results in the cortical sclerotic changes in SHRs through a local angII-mediated mechanism. This study raised the possibility that ARB is useful for renal protection in patients who have a combination of hypertension and increased BPV. (Circ J 2014; 78: 2284–2291)

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Recenty, we have created a model of a combination of hypertension and large BPV; spontaneously hypertensive rats (SHRs) underwent bilateral sinoaortic denervation (SAD) to create exaggerated BPV. \(15,16\) In this model, the cardiac angiotensin II (angII) system has been shown to play an important role in the aggravation of hypertensive cardiac remodeling characterized by cardiac hypertrophy and myocardial fibrosis without activation of the systemic renin-angiotensin system.\(15\) Hypertensive renal damage is characterized by predominant sclerotic changes occurring in the pre-glomerular vasculatures.
Involving the glomeruli and interstitium. However, little was known about the renal damage induced by a combination of hypertension and large BPV.

Accordingly, to address this issue, we investigated histological changes in the kidney induced by the combination of hypertension and large BPV. And, the effects of a subdepressor dose of candesartan, an angII type 1 receptor blocker (ARB), were studied to determine the involvement of the local angII activation in the SAD-induced renal damage.

Methods

All experiments were conducted in accordance with the Regulation for Animal Experimentation at Kurume University. Male SHRs were purchased from SLC Inc (Shizuoka, Japan) and housed under standard conditions of humidity, room temperature and a 12:12-h dark-light cycle. SHRs were provided with free access to tap water and chow.

Study Groups

At 12 weeks old, SHRs randomly underwent SAD or a sham operation. The following 4 groups were created: (1) Sham+vehicle group receiving a sham operation and vehicle (n=8); (2) Sham+candesartan (Cand) group receiving a sham operation and candesartan (n=12); (3) SAD+vehicle group receiving SAD and vehicle (n=9); and (4) SAD+Cand group receiving SAD and candesartan (n=10). In the Sham+Cand and SAD+Cand groups, 0.1 mg kg⁻¹ day⁻¹ of candesartan (Takeda Pharmaceutical Co, Osaka, Japan) or the vehicle was orally administered every day from 7 days after the operation. This dose of candesartan was the maximum dose that did not decrease BP in SHRs throughout the experiment period, because BP was significantly lowered by a dose of candesartan that was 1.0 mg kg⁻¹ day⁻¹ or greater.

Bilateral SAD and Telemetric Hemodynamic Monitoring

SHRs underwent bilateral SAD, as previously described. Bilaterally, SHRs 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 superior laryngeal nerve were cut bilaterally. The superior cervical ganglia and cervical sympathetic trunks were resected. Then, the carotid bifurcation and the external and internal carotid arteries were stripped off the surrounding connective tissues, followed by the application of 10% phenol in absolute ethanol. Sham-operated rats underwent bilateral neck muscle preparation.

Six weeks after SAD or a sham operation, SHRs underwent implantation of a BP radiotelemeter (model TA 11 PA-C 40; Data Sciences International, Minneapolis, Minnesota) under anesthesia with intraperitoneal ketamine (50 mg/kg) and medetomidine (0.5 mg/kg). Thereafter, rats were housed individually in a hemodynamic monitoring cage. Seven days after telemetry implantation (19 weeks old), BP and heart rate were monitored for 24 h under the unrestricted, conscious condition. The BP waveform was sampled at 500 Hz in 3-s bursts every 30 s. The averages for systolic BP (SBP), diastolic BP (DBP), mean BP, and heart rate were computed for each sample period. The 24-h average, standard deviation (SD), and coefficient of the variation (CV) of the mean BP and SBP were calculated for descriptive statistics of the distribution variability. As described above, the algorithm of the telemetric monitoring system does not allow us to record pulse pressure of each beat. The difference between SBP and DBP (SBP-DBP) was defined as an indicator of pulse pressure by subtracting the average DBP from the average SBP during each sampling period.

Histological Analysis and Immunohistostaining

After BP monitoring, rats were killed with an intraperitoneal injection of overdose ketamine and xylazine. Blood was drawn from the right atrium to measure serum creatinine levels at a commercially available laboratory (SRL Co, Fukuoka, Japan). After rats were perfuse-fixed with 4% neutrally buffered paraformaldehyde at 100 mmHg, the kidney was resected and immediately embedded in paraffin. The paraffinized sections were subjected to hematoxylin-eosin staining, Mallory-Azan staining, sirius red staining and periodic acid-Schiff (PAS) stain.

Immunohistostaining was performed using a primary antibody against kidney injury molecule-1 (KIM1; R&D Systems Inc, Minneapolis, MN, USA), ED1 (Chemicon International, Temecula, CA, USA), desmin (Abcam, Cambridge, MA, USA), nephrin (Abcam), or podocin (Abcam), as previously described. NanoZoomer 2.0-HT whole slide scanner and NanoZoomer Digital Pathology software (Hamamatsu Photonics, Hamamatsu, Japan) were used for acquisition and analysis of the whole sections of the kidney. The sclerotic lesions were defined as the histological changes containing atrophic or necrotic tubules, and glomeruli and interstitial fibrosis in Mallory-Azan-stained sections. The lesion area was measured by tracing the outside border of each lesion and was summed up as the total lesion area in each section.

Vascular wall thickness, perivascular fibrosis, and vascular remodeling of the interlobular and afferent arterioles (the minimum diameter of 25–50 μm) were evaluated in the parenchymal area, which did not contain the sclerotic changes described above. Arterioles cut obliquely were excluded from the analysis. The wall thickness of each arteriole was expressed as percent wall thickness, calculated by following the formula: \(\frac{\text{medial thickness} + 2\times\text{external diameter}}{\text{external diameter}}\times100\), as described previously. The sinus red-stained area surrounding the arterioles was measured and defined as perivascular fibrosis area using Image-Pro Plus 6.2J (Media Cybernetics, Bethesda, MD, USA). Arteriolar remodeling was semi-quantitatively graded as follows: grade 0, normal (the absence of wall thickening); grade 1, wall thickening (the wall-to-lumen ration of 50% or less); grade 2, wall thickening with the luminal narrowing (the wall-to-lumen ratio greater than 50%); grade 3, wall thickening with luminal obstruction. The measurements were averaged from 3 independent whole sections for each animal. Perivascular macrophage infiltration was quantified by counting ED-1-labeled cells around the arterioles with the minimum diameter of 25–50 μm from 3 independent whole sections for each animal.

Statistical Analysis

Data are expressed as mean±SEM. Statistic analysis was performed using the software SPSS 18.0J (IBM Japan, Tokyo, Japan). One-way ANOVA followed by Scheffe’s test and the Kruskal-Wallis test were used for comparisons of variables with standard normal distribution and those with non-normal distribution, respectively. Simple linear regression analysis was performed to analyze the correlation between the SD of the mean BP and the area of cortical sclerotic lesions. P values (two-sided) of less than 0.05 were considered statistically significant. The quantitative and semi-quantitative histological analyses were performed by 2 observers in a blinded fashion. The intraobserver or interobserver variability was less than 5% in each experiment.
Results

Effects on BP, BPV and Renal Function
At 7 weeks after the operation, the averages of mean BP were similar in the Sham+vehicle, Sham+Cand, SAD+vehicle, and SAD+Cand groups (Figure 1A). SAD significantly increased the SD and CV of mean BP, indicators of BPV, in SHRs as compared with the sham-operated SHRs. A subdepressor dose of candesartan did not affect BPV in either the sham- or SAD-operated SHRs (Figures 1B,C). Similarly, SAD increased the SD and CV of SBP without affecting the average of SBP (Figures S1A–C). In contrast, SAD did not change the pulse pressure (Figure S1D). Candesartan had no effects on the average, SD, and CV of BP in the sham- and SAD-operated SHRs. Furthermore, candesartan did not reduce pulse pressure after SAD. Serum creatinine levels were not different among the 4 groups (Figure 1D). SAD did not affect the average, SD, and CV of the heart rate in SHRs (data not shown).

Renal Parenchymal Lesions
Microscopic examination demonstrated that SAD induced wedge-shaped, focal cortical lesions, characterized by interstitial fibrosis and sclerotic changes of the glomeruli and tubules (Figure 2A). These sclerotic changes were distributed in a patchy fashion in the renal cortex of the SAD+vehicle group (Figure 2B). Candesartan prevented the formation of cortical sclerotic fibrous lesions (Figure 2C). The area of the cortical sclerotic lesions was significantly associated with the SD of the mean BP, the extent of BPV (Figure 2D).

Vascular Lesions Adjacent to the Sclerotic Lesions
SHRs receiving a sham operation and vehicle exhibited mild wall thickening of the arcuate arteries and the interlobular and afferent arterioles. SAD induced characteristic arteriolsclerotic changes in the interlobular and afferent arterioles adjacent to the sclerotic lesions (Figure 3A). Although mild vascular wall thickening without the luminal narrowing (grade 1) was observed in all 4 groups, the number of arterioles with the luminal narrowing (grade 2) was significantly greater in the SAD+vehicle group than in the other groups. The occluded arterioles (grade 3) were found only in the SAD+vehicle group. The SAD-induced arteriolar remodeling was characterized by vascular smooth muscle cell (VSMC) proliferation and extracellular matrix deposition. These vascular lesions showed a patchy distribution in the cortex, especially in the juxtamedullary layer (Figure 3B). As shown in Figures 3B and C, the SAD-induced arteriolar remodeling was prevented by candesartan.
Vascular Changes in the Non-Sclerotic Region

Effects of SAD on vascular changes were investigated in the renal cortex without the sclerotic changes. Mild vascular wall thickening was observed both at the arteriolar (Figure 4) and small arterial levels in the Sham+vehicle group. SAD induced mild perivascular fibrosis around the interlobular and afferent arterioles, while not affecting vascular wall thickness. Candesartan prevented the SAD-induced perivascular fibrosis. The ED-1-labeled macrophages were scarcely found in the perivascular area of the sham-operated SHRs, and SAD did not alter the number of perivascular macrophages (data not shown).

Glomerular Changes in the Non-Sclerotic Region

PAS staining demonstrated the increase in the mesangial matrix in the Sham+vehicle and SAD+vehicle groups (Figure 5A). The mesangial matrix deposition was prevented by candesartan, irrespective of the presence or absence of SAD. There was no difference among the 4 groups in the expression and distribution of desmin, a marker of glomerular damage, and podocin and nephrin, markers of podocyte damage (data not shown).

Tubular Damage

Effects of SAD were investigated on the tubular damage in SHRs. Immunoreactivity against KIM1, a marker of early ischemic tubular damage, was scarcely found in the proximal tubules of Sham+vehicle rats (Figure 5B). SAD increased the KIM1-stained tubules with a patchy distribution. Candesartan prevented the SAD-induced KIM1 expression. Also, we measured the urine levels of tubular damage markers. However, there were no significant differences in the levels of KIM1, liver-type fatty acid binding protein (L-FABP) and neutrophil gelatinase-associated lipocalin (NGAL) between Sham+vehicle and SAD+vehicle groups (data not shown).

Discussion

The present study demonstrated that SAD induced patchy focal sclerotic changes associated with glomerular and tubular atrophy, and interstitial fibrosis in the renal cortex (Figure 2). The interlobular and afferent arterioles adjacent to the sclerotic lesions showed arteriolar remodeling characterized by VSMC proliferation and extracellular matrix deposition, leading to the luminal narrowing and occlusion (Figure 3). A subpressor dose of candesartan prevented the arteriolar remodeling and cortical sclerotic changes without affecting BPV. These findings suggest that exaggerated BPV aggravates renal arteriolar sclerosis, which leads to ischemic fibrotic changes in the perfused area through an angII-mediated mechanism.

The significance of increased BPV has been highlighted in the development and aggravation of hypertensive organ damage. However, little was known whether large BPV aggravates hypertensive changes in the kidney. The key factor was the lack of adequate animal models representing a combination of hypertension and large BPV. Thus, we used SHRs with...
chronic SAD because they have been established as the model with a combination of hypertension and large BPV.\textsuperscript{15,16,18,19} In this model, BPV is increased by approximately 1.5- to 2-fold without the changes in the averages of SBP and mean BP and the pulse pressure. It is known that the sympathetic nerve system and humoral factors, such as angII, are transiently activated at the time of SAD and thereafter the activation wanes within a couple of weeks;\textsuperscript{28,29} which might lead to acute organ damage. To rule out the effect of acute neurohumoral hyperactivation at the time of SAD, we evaluated the kidneys of SHRs 2 weeks after SAD, and there were no apparent histological changes (data not shown). Thus, the renal lesions were not likely to be caused by acute injury at the time of SAD. The most likely explanation is a hemodynamic insult. We have shown that the activation of the local angII system participates in the aggravation of hypertensive cardiac remodeling, because SAD did not change the circulating levels of norepinephrine, active renin, and aldosterone and the cardiac norepinephrine content in this model.\textsuperscript{14,18} Thus, we hypothesized that the local angII system might be involved in renal damage induced by the combination of hypertension and large BPV, as well. To determine the direct effects of the angII type 1 receptor blocking, independently of its BP-lowering effect, the maximum dose of candesartan that did not reduce the mean BP was used in this study.

The novel finding of this study was that large BPV induced arteriolar proliferative remodeling, which was associated with the luminal narrowing and occlusion and the patchy, wedge-

\begin{figure}
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\includegraphics[width=\textwidth]{figure3}
\caption{(A) Representative microphotographs demonstrating the arteriolar remodeling in sinoaortic denervation (SAD)+vehicle rats. Grade 0: normal (the absent of wall thickening); grade 1, wall thickening (the wall-to-lumen ratio of <50%); grade 2, wall thickening with the luminal narrowing (the wall-to-lumen ratio >50%); grade 3, wall thickening with the luminal obstruction. (B) Representative photographs of the whole kidney showing the distribution of the arteriolar remodeling (Grades 1–3). (C) Pooled data showing the number of vessels with arteriolar remodeling (/section). Bar=1×SEM. Sham+vehicle, n=8; SAD+vehicle, n=12; Sham+candesartan, n=7; SAD+candesartan, n=8.}
\end{figure}
Large Blood Pressure Variability-Induced Renal Damages

shaped, focal sclerotic lesions consisting of the glomerular and tubular atrophy and surrounding interstitial fibrosis in the kidney of SHRs (Figures 2,3). Moreover, ischemic damage of the proximal tubules was suggested by the SAD-induced KIM1 induction (Figure 5B). It is noteworthy that the area of the cortical sclerotic lesions was significantly associated with the

Figure 4. Arteriolar remodeling in the non-sclerotic regions. (A) Representative microphotographs of sirius red-stained sections showing the perivascular fibrosis. Pooled data of arteriolar wall thickness (B) and perivascular fibrosis area (C). Bar=1×SEM. Sham+vehicle, n=8; sinoaortic denervation (SAD)+vehicle, n=12; Sham+candesartan, n=7; SAD+candesartan, n=8.

Figure 5. Representative microphotographs of the periodic acid-Schiff-stained sections (A) and immunohistostaining against kidney injury molecule-1 (B) demonstrating the effects of sinoaortic denervation (SAD) and candesartan on the glomerular and tubular changes.

extent of BPV (Figure 2D). This finding might suggest the causal relationship between the increased BPV and the sclerotic lesion formation in the hypertensive rat kidney. The arteriolar proliferative remodeling with stenotic changes was exclusively found in the areas adjacent to the sclerotic lesions. In contrast, the narrowing or occlusion of the pre-glomerular arterioles was not found in the non-sclerotic region (Figure 4). Thus, it is suggested that the large BPV-induced stenotic changes of the pre-glomerular arterioles would induce the regional ischemia, resulting in the glomerular and tubular atrophy and interstitial fibrosis in the perfused area.

It is interesting to note that the large BPV-induced arteriolar proliferative remodeling and sclerotic lesions were distributed in a patchy fashion in the cortex, especially in the juxtamedullary layer (Figures 2,3). Patchy sympathetic dysfunction might be a possible explanation. To investigate this possibility, immunoblotting using an anti-tyrosine hydroxylase antibody was performed for visualization of the sympathetic fibers in the kidney. The distribution of the SAD-induced lesions was not associated with that of the sympathetic fiber (data not shown). Thus, this mechanism was not likely the explanation. Currently, we do not know the mechanism whereby the SAD-induced cortical lesions showed a patchy distribution. In the renal cortex, the juxtamedullary layer is supposed to be vulnerable to arteriolar and glomerular sclerosis from analogy to hypertensive renal damage. Thus, this might be a possible explanation. In this regard, "strain vessel hypothesis" proposed by Ito et al 38 might be one of the attractive explanations; Generic vessels, such as the interlobular and afferent arterioles to the majority of the nephrons (ie, the superficial cortical nephrons), become smaller gradually as it branches off, and the hemodynamic strain onto the vessel walls also declines gradually. In contrast, in the "strain vessels", such as the interlobular and afferent arterioles to the juxtamedullary nephrons, small vessels branch off directly from large vessels. Thus, the strain vessels are directly exposed to high BP levels, leading to an accelerated progression of hypertensive damage. By a similar mechanism, BPV exaggeration might induce a greater hemodynamic change in the strain vessels than in the generic vessels, and then would result in arteriolar remodeling with the stenotic change in the strain vessels, but not in the generic vessels. This might be a reason for the patchy distribution of the large BPV-induced sclerotic lesions.

It is considered that SAD-operated SHRs represent a combination of hypertensive renal lesions related to BP elevation itself (mesangial cell proliferation) and large BPV superimposed on hypertension (arteriolar proliferative remodeling and cortical sclerotic lesion formation). It has long been considered that the mechanism of renal protection provided by angiotensin-converting enzyme inhibitors or ARBs is mainly dependent on lowering glomerular capillary pressure caused by efferent arteriole dilatation.60–62 In addition, candesartan prevented mesangial matrix deposition which is induced by hypertension and is independent of BPV increase (Figure 5A). The present study provided a novel insight into the mechanism of renal protection by ARB which targets the pre-glomerular arterioles, because candesartan prevented large BPV-induced arteriolosclerosis and sclerotic changes.

Study Limitations
This study suggested that the renal angII-mediated system plays a role in the large BPV-induced arteriolar remodeling and cortical sclerotic lesion formation. Currently, there are no available data regarding the molecular mechanisms, because the size of the affected lesions has been too small to detect the mRNA and protein expression changes on the basis of the conventional methods using the whole tissue samples. Therefore, it awaits future technical innovation to enable the separate preparation and analysis of the tiny samples of small cell groups or single cells obtained from the tissue sections, such as the technique using a laser-microdissection system.63 Second, the SAD-induced arteriolar remodeling and sclerotic lesion formation did not accompany apparent inflammatory changes in the kidney, whereas chronic inflammatory changes manifested by macrophage infiltration were evident in the SAD-induced perivascular and interstitial fibrosis in the heart.15 The molecular mechanism of the phenotype difference between the kidney and heart should be addressed in a future study. Next, vascular tone was not evaluated in the present study. It is known that BPV is affected by vascular tone levels mediated by angII and other endogenous vasoconstrictors.64 However, the issue of mechanisms for BPV after SAD is beyond the scope of our study. Third, given candesartan was orally administered, the involvement of the effects of candesartan through the central nervous system cannot be ruled out. This issue should be investigated in a future study. Thus, urine secretion levels of albumin, β2-microglobulin, and N-acetyl-β-D-glucosaminidase, as well as KIM1, L-FABP, and NGAL were not changed by SAD at 7 weeks after operation (data not shown). A patchy distribution of the SAD-induced renal damage might not be enough to increase the urine levels of these markers. Another possibility was the short observation period used in the current study. Thus, further studies with longer observation periods might be desirable for determining the effects of glomerular and tubular damage on these urine markers in this model. In addition, because we were not able to evaluate the pulse pressure of each beat due to technical reasons, the SBP-DBP (the difference between the average SBP and the average DBP during each sampling period, ie, 3 s) was regarded as an indicator of pulse pressure. Thus, there is the limitation of interpreting the data on the effects of SAD on pulse pressure. Finally, it is interesting to determine the effects of the subdepressor dose of other ARBs or other antihypertensive drugs, such as angiotensin-converting enzyme inhibitors, aldosterone receptor blockers, calcium channel blockers, and diuretics, on BPV and BPV-induced aggravation of hypertensive damage. Future studies should be performed to address this important issue.

Conclusions
Increased BPV aggravated renal arteriolosclerosis, which resulted in ischemic changes and sclerotic lesions in the renal cortex of hypertensive rats through the mechanism mediated by the renal angII system activation. The present study raised the possibility that ARB is useful for renal protection in patients having a combination of hypertension and increased BPV.

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Disclosures
None.
References


Supplementary Files

Supplementary File 1

**Figure S1.** Pooled data of the effects of sinoaortic denervation (SAD) and candesartan on the average (A), standard deviation (SD) (B), and coefficient of variation (CV) (C) of systolic blood pressure (SBP), and the average of SBP-DBP in spontaneously hypertensive rats (SHRs).

Please find supplementary file(s); http://dx.doi.org/10.1253/circj.CJ-14-0027