Renal Sympathetic Denervation Suppresses De Novo Podocyte Injury and Albuminuria in Rats With Aortic Regurgitation

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Background—The presence of chronic kidney disease is a significant independent risk factor for poor prognosis in patients with chronic heart failure. However, the mechanisms and mediators underlying this interaction are poorly understood. In this study, we tested our hypothesis that chronic cardiac volume overload leads to de novo renal dysfunction by coactivating the sympathetic nervous system and renin-angiotensin system in the kidney. We also examined the therapeutic potential of renal denervation and renin-angiotensin system inhibition to suppress renal injury in chronic heart failure.

Methods and Results—Sprague-Dawley rats underwent aortic regurgitation and were treated for 6 months with vehicle, olmesartan (an angiotensin II receptor blocker), or hydralazine. At 6 months, albuminuria and glomerular podocyte injury were significantly increased in aortic regurgitation rats. These changes were associated with increased urinary angiotensinogen excretion, kidney angiotensin II and norepinephrine (NE) levels, and enhanced angiotensinogen and angiotensin type 1a receptor gene expression and oxidative stress in renal cortical tissues. Aortic regurgitation rats with renal denervation had decreased albuminuria and glomerular podocyte injury, which were associated with reduced kidney NE, angiotensinogen, and angiotensin II, and oxidative stress. Renal denervation combined with olmesartan prevented podocyte injury and albuminuria induced by aortic regurgitation.

Conclusions—In this chronic cardiac volume-overload animal model, activation of the sympathetic nervous system augments kidney renin-angiotensin system and oxidative stress, which act as crucial cardiorenal mediators. Renal denervation and olmesartan prevent the onset and progression of renal injury, providing new insight into the treatment of cardiorenal syndrome. (Circulation. 2012;125:1402-1413.)

Key Words: albuminuria ■ aortic valve insufficiency ■ cardio-renal syndrome ■ renin-angiotensin system ■ sympathetic nervous system

Clinical Perspective on p 1413

Microalbuminuria, a surrogate marker of renal injury, is strongly associated with increased risk of cardiovascular events in patients with diabetes mellitus, coronary artery disease, and hypertension.4–6 The prevalence of microalbuminuria was found to be significantly higher in CHF patients than in healthy individuals, even in the absence of diabetes mellitus and hypertension, and these patients had worse outcomes compared with CHF patients without microalbuminuria.7,8

The sympathetic nervous system (SNS) and renin-angiotensin system (RAS) have been suggested as possible cardiorenal mediators.9 Sympathetic nerve activity is increased in patients with CHF10,11 and may influence cardiovascular and renal prognosis. Similarly, chronic kidney disease is often accompanied by increased sympathetic nerve activity and is improved by renal denervation.12–15 It has also been documented that RAS intervention with angiotensin-converting enzyme inhibitors and angiotensin II (AngII)
receptor blockers (ARBs) protects the heart and kidney independently of their effects on blood pressure lowering.16

Therefore, the aim of this study was to clarify the mechanism by which albuminuria develops during the progression of CHF. We hypothesized that chronic volume overload induced by aortic regurgitation (AR) leads to de novo renal injury by coactivating the renal SNS and RAS. To test our hypothesis, we evaluated albuminuria and glomerular podocyte injury and measured kidney levels of norepinephrine (NE) and RAS components 6 months after surgically inducing AR. We also examined the effects of an ARB, olmesartan, and chronic renal denervation on albuminuria and cardiac status in AR rats. Our findings might offer new insight into the management of patients with CHF to prevent renal dysfunction.

Methods

Animals

All experimental procedures were performed according to the guidelines for the care and use of animals established by Kagawa University. Five-week-old male Sprague-Dawley rats (CLEA Japan Inc, Tokyo, Japan) were maintained in a pathogen-free facility under a controlled temperature (24±2°C) and humidity (55±5%) with a 12-hour light/dark cycle.

Experimental Protocols

Protocol 1

AR or sham operation was performed at 9 weeks of age (AR, n=36; sham, n=8). The AR rats were divided into 3 groups and treated with vehicle (AR; n=12), olmesartan (0.03% in chow; ∼15 mg/kg body weight per day; Daiichi-Sankyo Co, Ltd, Tokyo, Japan; n=12), or hydralazine (0.075% in chow; ∼50 mg/kg body weight per day; Wako Co, Ltd, Osaka, Japan; n=12). Preliminary studies showed that olmesartan and hydralazine at the doses described above elicited similar blood pressure reductions in AR and sham-operated rats (data not shown). Blood pressure at baseline and every month during the 6-month treatment was measured in conscious rats by tail-cuff plethysmography (BP-98A; Softron Co, Tokyo, Japan).

Figure 1. Systolic blood pressure (SBP), diastolic blood pressure (DBP), and urinary albumin-to-creatinine ratio (UACR; C) profiles in protocol 1. A, Aortic regurgitation (AR) rats and sham-operated rats have similar SBP, and treatment with olmesartan or hydralazine lowered SBP to equivalent levels. B, DBP is lower in AR rats than in sham rats. In contrast, DBP is not significantly affected by olmesartan or hydralazine. C, UACR in protocol 1. AR rats develop marked albuminuria, which is prevented by olmesartan. *P<0.05, **P<0.01, ****P<0.001 vs sham; ##P<0.01, ####P<0.001 vs AR.

Induction of AR and Renal Denervation

AR was induced as previously described.16,19 In protocol 2, the rats underwent UNX and RDX under anesthesia with sodium pentobarbital (50 mg/kg IP). Complete RDX was achieved by cutting all of these vessels with a solution of 10% phenol in ethanol.13 This method ablates the afferent and efferent renal nerves.13 After the rats were decapitated, renal tissue NE content was measured to confirm the completeness of RDX.13,20 In the present study, the kidney NE content in all rats was almost undetectable (<3 ng/g tissue), indicating that denervation was complete.

Echocardiography

Transthoracic echocardiography was performed under anesthesia with ketamine (50 mg/kg IP) and xylazine (10 mg/kg IP) with a SONOS5500 (Philips Medical Systems, Andover, MA) equipped with a 7.5-MHz transducer as previously described.21

Sample Collection

After decapitation, trunk blood was collected in chilled tubes containing an inhibitor mixture to prevent AngII degradation for...
AngII measurement or in chilled tubes containing EDTA for other measurements as previously described.\textsuperscript{13,22,23} Immediately after collection of the blood, half of the right kidney was homogenized in cold methanol and processed to measure the AngII content.\textsuperscript{24,25} and the other half of the kidney tissue was cut and fixed in 10% buffered paraformaldehyde or embedded in optimal cutting temperature compound, and remaining tissues was snap-frozen in liquid nitrogen. The left ventricle (LV) was collected, weighed, and snap-frozen in liquid nitrogen.

**Histological Examination**

Kidney tissues were fixed with 10% paraformaldehyde, embedded in paraffin, sectioned into 4-μm-thick slices, and stained with periodic acid–Schiff reagent. Immunohistochemistry for desmin was performed as previously described.\textsuperscript{26-27} Frozen, optimal cutting temperature–embedded kidney tissue was cryosectioned into 10-μm-thick sections, which were stained with 10 μmol/L dihydroethidium (DHE) solution (Invitrogen, Carlsbad, CA). DHE fluorescence intensity was measured as previously described. Images were obtained by confocal laser-scanning fluorescence microscopy (Radiance2100; Bio-Rad Laboratories, Hercules, CA).

**Laser Capture Microdissection Techniques**

To measure glomerular AGT, and nephrin and podocin mRNA levels, the glomeruli were microdissected with a laser capture microdisSECTION technique (LM-200; Arcturus Bioscience, Mountain View, CA). Glomerular mRNA was extracted with RNAqueous-Micro kits (Ambion, Austin, TX) as previously described.\textsuperscript{27}

**Real-Time Polymerase Chain Reaction Analysis**

The mRNA expression of GAPDH, AGT, renin, nephrin, podocin, p22\textsuperscript{phox} and gp91\textsuperscript{phox} was analyzed by real-time polymerase chain reaction with a LightCycler FastStart DNA Master SYBR Green I kit. Angiotensin type 1a (AT1a) receptor mRNA expression was measured with a TaqMan Gene Expression Assay (Assay ID: Rn00578456 m1; Applied Biosystems). Polymerase chain reaction was performed using previously described conditions\textsuperscript{27} with the following oligonucleotide primer sequences (sense and antisense): GAPDH, 5'-TGAACGGGAAAGTCATCTGG-3' and 5'-TCCACACCCGTG-GTCTGA-3'; AGT, 5'-TCTGGTAGAGGAGGCTGTAT-3' and 5'-TGCTAGATGATGACTCTGTG-3'; renin, 5'-TTGGTGTGGTGAAGC-AAATCTC-3' and 5'-CCACATTTTGGGGTTATCC-3'; nephrin, 5'-CAGATGGGACAACTATATGG-3' and 5'-GACACCGATCTGGCCGTTATCC-3'; podocin, 5'-CTTTCATGAGTGGTAGAAC-CCA-3' and 5'-GATGGCCTTGGACACATGAG-3'; p22\textsuperscript{phox}, 5'-TCCACTACTCTGGTGCG-3' and 5'-TCAAATGGGGAGTCCCCACTG-3'; gp91\textsuperscript{phox}, 5'-TTGGTGTAGGTAGGGGAC-3' and 5'-CTTTCTTGATCTGGTCT-3'; α-myosin heavy chain (MHC), 5'-GGCAGAGGGGCAGGGA-3' and 5'-GGCGAGCTGTTCGTGACCA-3'; and βMHC, 5'-GGCCGACTTGCCGAGTCCTCC-3' and 5'-AGGCTTACGGTCTAGGGGAG-3'. All data are expressed as the relative difference to the sham group in protocol 1 or to the UNX group in protocol 2 after normalization for GAPDH expression.

**Urinary Parameters**

Urinary albumin and creatinine concentrations were measured with assay kits for albumin (code No. AKRAL-120; Shibayagi Co, Shibukawa, Japan) and creatinine (micro CRE-test; Wako Co, Ltd), respectively.\textsuperscript{27} Urinary concentrations of AGT were measured with a Rat Total Angiotensinogen Assay Kit (code No. 27414; IBL Co, Ltd, Fujioka, Japan) as previously described.\textsuperscript{29} Creatinine clearance (CrCl) was calculated from the following equation as previously described:\textsuperscript{30}

\[
\text{CrCl} = \frac{\text{urinary creatinine (mg/dL)} \times \text{urinary volume (mL)} \times \text{plasma creatinine (mg/dL)}}{1000 \times \text{body weight (g)} \times 1/1440 \text{ (min)}}. 
\]

**Other Analytic Procedures**

Renal cortical tissue renin activity\textsuperscript{31} and plasma and renal cortical tissues NE levels\textsuperscript{32} were measured as previously described. Plasma and kidney AngII concentrations were measured by a radioimmunoassay as previously described.\textsuperscript{24,25} The degree of lipid peroxidation in plasma and renal cortical tissue was evaluated by the use of biochemical assays for thiobarbituric acid reaction substances (TBARS) as previously described.\textsuperscript{22} Collagen content in the LV tissues was determined from hydroxyproline concentrations as previously described.\textsuperscript{22}

### Table 1. Echocardiographic Data at Baseline and 6 Months After Aortic Regurgitation or Sham Operation in Protocol 1

<table>
<thead>
<tr>
<th></th>
<th>Sham (n=8)</th>
<th>AR (n=12)</th>
<th>AR+ Omiesartan (n=12)</th>
<th>AR+ Hydralazine (n=12)</th>
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<tbody>
<tr>
<td>LVEDD, mm</td>
<td>6.55±0.21</td>
<td>6.49±0.23</td>
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<td>6.57±0.22</td>
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<td>8.10±0.40</td>
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<td>10.05±0.35</td>
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<tr>
<td>LVESD, mm</td>
<td>3.59±0.16</td>
<td>3.43±0.18</td>
<td>3.67±0.15</td>
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<tr>
<td>6 mo</td>
<td>4.80±0.35</td>
<td>6.70±0.67</td>
<td>6.50±0.31</td>
<td>6.60±0.26</td>
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<tr>
<td>LV mass</td>
<td>803±67</td>
<td>731±38</td>
<td>869±47</td>
<td>857±50</td>
</tr>
<tr>
<td>6 mo</td>
<td>1452±23</td>
<td>2393±152</td>
<td>1710±122</td>
<td>2224±122</td>
</tr>
<tr>
<td>SW, mm</td>
<td>1.59±0.09</td>
<td>1.65±0.12</td>
<td>1.77±0.20</td>
<td>1.70±0.09</td>
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<tr>
<td>6 mo</td>
<td>1.80±0.31</td>
<td>1.99±0.28</td>
<td>2.10±0.26</td>
<td>2.17±0.29</td>
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<td>PW, mm</td>
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<td>6 mo</td>
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<td>FS, %</td>
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<td>6 mo</td>
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<td>33.51±2.89</td>
<td>38.50±1.75</td>
<td>35.22±1.59‡</td>
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<td>mvcf, circ/s</td>
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<td>2.31±0.21</td>
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<td>2.27±0.09</td>
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<tr>
<td>6 mo</td>
<td>2.11±0.05</td>
<td>1.88±0.25</td>
<td>1.95±0.22</td>
<td>1.96±0.14</td>
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</tbody>
</table>

AR indicates aortic regurgitation; LVEDD, left ventricular end-diastolic dimension; LVESD, left ventricular end-systolic dimension; LV, left ventricular; SW, septal wall thickness; PW, posterior wall thickness; RWT, relative wall thickness (SW+PW)/LVEDD; FS, fractional shortening; and mvcf, mean velocity of circumferential fiber shortening. Values are means±SEM. *P<0.05, †P<0.01, ‡P<0.005 versus sham. §P<0.01 versus AR rats.

**Cell Culture**

Studies were performed in immortalized human proximal tubular cells (HPTCs).\textsuperscript{33} HPTCs were incubated with vehicle or NE (0.01, 1, and 100 nmol/L) for 24 hours. After incubation with NE, mRNA was extracted and the mRNA expression levels of AGT were analyzed by real-time polymerase chain reaction using previously described...
conditions.23 The oligonucleotide primer sequences were (sense and antisense) as follows: human GAPDH, 5′-TGCAACCACCAACT-GCTTAGC-3′ and 5′-GGCATGGACTGGTCTG-3′; and human-AGT, 5′-AATCTGTTGTCAAGAGTCT-3′ and 5′-TCTCTCTACCCGCT-3′. All data were normalized for the expression of GAPDH.

Statistical Analyses
All values are presented as mean ± SEM. Statistical comparisons of differences among groups were performed with 1-way repeated-measures ANOVA followed by the Newman-Keuls post hoc test. Systolic blood pressure (SBP), diastolic blood pressure (DBP), urinary albumin-to-creatinine ratio (UACR), urinary albumin excretion rate (UalbV), urinary AGT excretion rate (UAGTV), and echocardiography were compared by use of 2-way repeated-measures ANOVA followed by the Bonferroni post hoc test. Values of P < 0.05 were considered statistically significant. Data and statistical analyses were performed with GraphPad Prism version 5 for Windows (GraphPad Software, San Diego, CA).

Results
ARB Inhibits AR-Induced LV Dilatation and De Novo Albuminuria in Rats
During the 6-month treatment period, there were no differences in SBP between AR and sham rats (Figure 1A). In contrast, DBP decreased significantly in AR rats compared with sham rats (Figure 1B). Treatment with olmesartan and hydralazine significantly lowered SBP, but not DBP, compared with untreated AR rats. There were no significant differences in SBP and DBP between AR rats treated with olmesartan and hydralazine. Plasma BNP levels were higher in AR rats than in sham rats (Figure IA in the online-only Data Supplement). Treatment with olmesartan, but not with hydralazine, suppressed the increase in plasma BNP levels in AR rats. AR rats had marked LV enlargement and hypertrophy at 6 months as shown in Table 1 and Table I in the online-only Data Supplement, respectively. Compared with sham rats, AR rats exhibited LV end-diastolic dimension and LV end-systolic dimension dilatation and lowered fractional shortening. LV mass estimated by echocardiography was significantly increased in AR rats. Wall thickness was similar in all groups. However, relative wall thickness was lower in AR rats, as expected from the eccentric pattern of LV remodeling. AR increased mRNA expression of βMHC and BNP and decreased mRNA expression of αMHC in LV tissues, markers of cardiac hypertrophy34,35 and heart failure36 (Figure IIA–IC in the online-only Data Supplement). Treatment with olmesartan, but not hydralazine, significantly attenuated LV hypertrophy in AR rats. LV interstitial fibrosis is a late feature in our model.37,38 AR rats had significantly greater LV tissue collagen content and collagen I and III mRNA expression than sham rats (Figure IIIA–IIIC in the online-only Data Supplement). All of these changes were attenuated by olmesartan treatment but not by hydralazine.

UalbV and UACR were significantly higher in AR rats than in sham rats at 3 months after the AR operation (Figure 1C). Furthermore, UalbV progressively increased over time in AR rats. At 6 months, UalbV was 0.70 ± 0.06 and 3.59 ± 0.15 mg/d in the sham and AR rats, respectively (P < 0.001). At 6 months, the plasma creatinine levels tended to be increased and CCR tended to be decreased in AR rats compared with sham rats, although these differences were not statistically significant (Table I in the online-only Data Supplement). Treatment with olmesartan suppressed the increases in UalbV (0.60 ± 0.08 mg/d; P < 0.001), UACR, and other parameters in AR rats (Figure 1C). In contrast, hydralazine did not affect these parameters in AR rats. These data indicate that chronic cardiac volume overload caused by AR induces albuminuria independently of changes in blood pressure.

ARB Suppresses AR-Induced Increases in Kidney AngII, RAS Components, and NE Levels
We next investigated the mechanism responsible for the intrarenal SNS and RAS activations in AR rats. In protocol 1, AR rats had significantly greater plasma and kidney NE levels at 6 months after the induction of AR compared with sham rats (Figure 2A and 2B). These increases in plasma and kidney tissue NE levels were significantly decreased by
Olmesartan but not by hydralazine. Interestingly, AR rats had significantly higher kidney AngII levels, but not plasma AngII levels, compared with sham rats (Figure 2C and Table I in the online-only Data Supplement). The renal cortical tissue mRNA levels of AT1a receptor and AGT were also increased in AR rats compared with sham rats (Figure 2D and 2E). On the other hand, renin mRNA levels remained unchanged (Figure IVB in the online-only Data Supplement). Renal renin activity tended to be increased in AR rats, but these changes were not statistically significant (Figure IVA). These results suggest that chronic cardiac volume overload caused by AR induces sympathetic hyperactivity and activates the intrarenal SNS and RAS. Treatment with olmesartan, but not with hydralazine, suppressed the increases in kidney AngII and mRNA levels of AT1a receptor and AGT in AR rats. We also measured the U_{AGT}V rate in AR rats. As shown in Figure 2F, AR rats had markedly increased U_{AGT}V levels at 6 months after the AR operation compared with sham rats. Treatment with olmesartan, but not with hydralazine, suppressed the AR-induced increase in U_{AGT}V levels.

**ARB Prevents Glomerular Podocyte Injury and Suppresses the Production of Glomerular Reactive Oxygen Species and the Activation of NADPH Oxidase in AR Rats**

We further examined glomerular podocyte injury by immunostaining for desmin.26,40 The glomerular desmin-positive area was significantly increased in AR rats compared with sham rats (Figure 3A). To confirm the presence of podocyte injury, we determined the gene expression of glomerular nephrin and podocin, components of the slit diaphragm between 2 adjacent podocytes, using real-time polymerase chain reaction with laser capture microdissection. As shown in Figure 3B and 3C, glomerular nephrin and podocin mRNA levels were significantly lower in AR rats than in sham rats. The AR-induced increase in glomerular desmin staining and decreases in nephrin and podocin mRNA levels were prevented by treatment with olmesartan but not with hydralazine.
However, glomerular sclerosis, as evaluated by the periodic acid–Schiff–positive area, was not prominent and did not differ among the groups (2.15 ± 0.15%, 2.49 ± 0.08%, 1.88 ± 0.20%, and 2.25 ± 0.17% in the sham, AR, AR plus olmesartan, and AR plus hydralazine groups, respectively).

AR rats had significant increases in glomerular and tubulointerstitial DHE fluorescence compared with sham rats (Figure 3D). Treatment with olmesartan, but not with hydralazine, prevented the AR-induced increase in DHE fluorescence. The renal cortical TBARS content, but not plasma TBARS content, was significantly higher in AR rats than in sham rats (Figure 3E and Table I in the online-only Data Supplement). Treatment with olmesartan, but not with hydralazine, prevented the AR-induced increase in TBARS content in renal cortical tissue. AR rats also had increases in mRNA levels of renal cortical p22phox and gp91phox, which were prevented by olmesartan but not by hydralazine (Figure 3F and 3G).

**Chronic Renal Denervation Inhibits AR-Induced Albuminuria Independently of Changes in Blood Pressure and Cardiac Function**

To examine the effects of sympathetic nerve activation on renal injury, we next carried out complete inhibition of kidney SNS by renal denervation in AR rats. In protocol 2, rats underwent UNX and RDX before AR operation. RDX did not significantly affect SBP, DBP, or cardiac structural and functional parameters (Figure 4A and 4B and Table 2). However, RDX significantly decreased the gene expression of myocardial markers associated with fetal gene programming, including βMHC and BNP. RDX also decreased cardiac fibrotic markers such as collagen content and mRNA expression of collagen I and III in the LV (Figure II and III in the online-only Data Supplement). On the other hand, cardiac output was maintained in our AR rat model subjected to RDX (Table 2). The absence of significant differences in SBP and cardiac output between the sham and AR rats suggests that there are no major differences in systemic vascular resistance between these 2 groups. In UNX-RDX-AR rats, treatment with olmesartan and hydralazine similarly lowered SBP but not DBP. RDX plus olmesartan significantly decreased LV mass, LV tissue collagen content, and mRNA levels of βMHC, BNP, and collagen I and III; increased αMHC in LV tissues; improved fractional shortening, mean velocity of circumferential fiber shortening, and relative wall thickness; and prevented the accumulation of plasma BNP levels compared with UNX-RDX-AR rats (Table 2 and Figures IB, II, and III and Table II in the online-only Data Supplement).

$U_{\text{alb}}$ and UACR (Figure 4C) were increased in UNX-AR rats 2 months after AR operation, and they increased progressively during the 6-month treatment period, with $U_{\text{alb}}$ reaching 1.36 ± 0.15 and 4.80 ± 0.20 mg/d at 6 months in UNX-sham and UNX-AR rats, respectively ($P<0.005$). It is noteworthy that RDX alone significantly reduced the $U_{\text{alb}}$ (1.73 ± 0.21 mg/d; $P<0.005$) and UACR in UNX-AR rats (Figure 4C). Moreover, RDX plus olmesartan almost completely suppressed albuminuria (0.20 ± 0.03 mg/d; $P<0.001$) and normalized UACR in UNX-AR rats. At 6 months, the plasma creatinine levels tended to be increased and CCr tended to be decreased in UNX-AR rats compared with UNX-sham rats. RDX alone or in combination with olmesartan tended to decrease plasma creatinine levels and tended to increase CCr in UNX-AR rats compared with UNX-sham rats, although these differences were not statistically significant (Table II in the online-only Data Supplement).

**Chronic Renal Denervation Prevents AR-Induced Increases in Kidney AngII and NE Levels**

RDX plus olmesartan significantly reduced the AR-induced increase in plasma NE levels in protocol 2 (Figure 5A).
UNX-AR rats had significantly greater kidney NE and AngII levels compared with UNX-sham rats (Figure 5B and 5C). As shown in Figure 5B, the kidney NE content was almost undetectable (<3 ng/g tissues) in all RDX rats, confirming complete renal denervation.13,20 RDX suppressed the AR-induced increases in kidney AngII levels (Figure 5C) and AGT and AT1a receptor mRNA levels in renal cortical tissues (Figure 5E and 5D), glomerular AGT mRNA level (1.67±0.08, 1.10±0.05, and 0.9±0.08-fold in UNX-AR, UNX-RDX-AR, and UNX-RDX-AR+olmesartan, respectively; P<0.05), and UAGTV (Figure 5F). These levels were further decreased by RDX combined with olmesartan.

Chronic Renal Denervation Suppresses Glomerular Podocyte Injury in AR Rats

In protocol 2, RDX alone and RDX plus olmesartan markedly suppressed the AR-induced increase in the glomerular desmin-positive area and the decreases in glomerular nephrin and podocin mRNA levels in UNX-AR rats (Figure 6A–6C). RDX significantly suppressed the AR-induced increases in kidney TBARS content and DHE fluorescence and the increases in p22phox and gp91phox mRNA levels compared with those in UNX-RDX-AR rats. UNX-RDX-AR plus hydralazine, respectively).

### Table 2. Echocardiographic Data at Baseline and 6 Months After Aortic Regurgitation or Sham Operation in Protocol 2

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<tr>
<th></th>
<th>UNX (n=6)</th>
<th>UNX + AR (n=10)</th>
<th>UNX + RDX (n=6)</th>
<th>UNX + RDX + AR (n=12)</th>
<th>UNX + RDX + AR + Olmesartan (n=8)</th>
<th>UNX + RDX + AR + Hydralazine (n=8)</th>
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<tbody>
<tr>
<td>LVEDD, mm</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>0 mo</td>
<td>7.32±0.30</td>
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<tr>
<td>6 mo</td>
<td>8.96±0.27</td>
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<td>9.05±0.19</td>
<td>10.68±0.38*</td>
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<td>6 mo</td>
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<td>6.78±0.22</td>
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<td>1517±119</td>
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<td>1475±107¶#</td>
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<tr>
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<td>1.63±0.08</td>
<td>1.60±0.15</td>
<td>1.59±0.03</td>
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<td>2.21±0.24</td>
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</tr>
<tr>
<td>6 mo</td>
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<td>6 mo</td>
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<td>97.25±7.00</td>
<td>105.90±7.21</td>
<td>102.56±4.45</td>
<td>108.80±8.21</td>
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</table>

AR, aortic regurgitation; UNX, uninephrectomy; RDX, left renal denervation; LV, left ventricular; LVEDD, left ventricular end-diastolic dimension; LVEDS, left ventricular end-systolic dimension; SW, septal wall thickness; PW, posterior wall thickness; RWT, relative wall thickness ([SW + PW]/LVEDD); FS, fractional shortening; mvcf, mean velocity of circumferential fiber shortening; and CO2, cardiac output. Values are mean±SEM.

*P<0.05, †P<0.005 versus UNX.
‡P<0.05.
§P<0.005 versus UNX + AR.
¶P<0.05.
##P<0.01 versus UNX + RDX + AR.
NE Directly Increases AGT Gene Expression in HPTCs

To confirm the possible contribution of sympathetic nervous activation to local AGT gene expression in the kidney, we performed an in vitro study using immortalized HPTCs. In this experiment, exposure to NE for 24 hours significantly and dose-dependently increased AGT mRNA levels in HPTCs (Figure V in the online-only Data Supplement).

Discussion

In the present study, we first found that chronic cardiac volume overload induced by AR initiates the onset of albuminuria via glomerular podocyte injury. Second, AR-induced SNS activation plays an important role in the pathogenesis of glomerular podocyte injury by activating the RAS in the kidney. Third, intrarenal AGT expression, but not renin, is directly stimulated by intrarenal NE, enhancing kidney AngII production. Fourth, renal denervation suppresses pathological activation of intrarenal RAS, which prevents the onset and progression of albuminuria in chronic AR rats.

Sympathetic hyperactivity is a hallmark of progressive heart failure. The cardiac sympathetic afferent reflex is a sympathoexcitatory cardiovascular reflex that contributes to the enhanced sympathetic outflow in CHF. It is well recognized that sympathetic hyperactivity activates the RAS.

In the present study, the AR-induced increases in plasma and kidney NE levels were associated with increases in kidney AngII levels. These data indicate that chronic cardiac volume overload caused by AR enhances sympathetic outflow from the heart and that systemic sympathetic hyperactivity leads to intrarenal NE production. In turn, NE stimulates intrarenal AngII production, suggesting pathological activation of intrarenal RAS activity in AR rats. Renal denervation prevented AR-induced increases in kidney NE and AngII levels. Renal denervation combined with an ARB further suppressed glomerular podocyte injury and reactive oxygen species (ROS) production and prevented albuminuria. These data support the concept that AR-induced activation of the SNS is essentially involved in the onset and progression of albuminuria.

Acute hyperactivity of the SNS stimulates renin secretion via the /adrenergic receptor–dependent pathway at the juxtaglomerular apparatus. However, we found that the increases in kidney AngII levels in AR rats were not accompanied by increases in renal renin activity or its mRNA expression, suggesting the existence of alternative pathway(s) for intrarenal RAS activation. In this regard, we have provided substantial evidence that kidney AGT is an essential regulator of kidney AngII levels. Furthermore, early studies by Nakamura and Johns reported that mild stimulation of the renal nerve increased AGT but not renin mRNA levels in rat kidney, suggesting that a certain level of sympathetic nerve activation needs to be achieved to stimulate renal renin in some pathophysiological condition. Similarly, an in vitro study reported that isoproterenol stimulated AGT gene expression in proximal tubular cells. Furthermore, sympathetic hyperactivity-induced heart failure increased renal...
renin mRNA expression in an early, but not a late, stage of heart failure.\textsuperscript{46} In the present study, we found that the chronic AR-induced augmentation of kidney AngII was associated with upregulation of kidney AGT levels. In vitro studies confirmed that NE significantly increased AGT gene expression in HPTCs in a dose-dependent manner. These data support the concept that, during conditions of chronic volume overload on the heart, chronic elevation of kidney NE content stimulates local AGT expression, leading to AngII production in the kidney. AGT is abundantly expressed in proximal tubular cells in the kidney.\textsuperscript{39} However, in the present study, we also detected AGT mRNA in glomeruli, and AGT mRNA levels were significantly increased in both renal cortical tissues and glomeruli. These data agree with those of recent studies\textsuperscript{47,48} indicating that glomerular injury is associated with an increase in glomerular AGT expression. Collectively, it is possible that augmentation of AGT expression in glomeruli mediates local AngII production, leading to injuries of glomerular podocytes and other cells, although the present study did not clarify the precise mechanisms responsible for intraglomerular AngII regulation because of technical difficulties.

Our preclinical\textsuperscript{24,47} and clinical\textsuperscript{49} studies revealed that treatment with ARBs decreased, rather than increased, AngII levels in the kidney by blocking AT1 receptor–mediated stimulation of kidney AGT production. Consistent with previous studies,\textsuperscript{24,47,49} we found that treatment with an ARB suppressed the increases in kidney AGT levels in AR rats. Inappropriate activation of RAS results in the formation of ROS via the NADPH oxidase–dependent pathways.\textsuperscript{50} In mice overexpressing AGT in the kidney, renal injury was associated with NADPH oxidase–dependent ROS production.\textsuperscript{51} It has also been reported that AngII directly increases ROS formation through an NADPH oxidase–dependent pathways in podocytes, thereby accelerating albuminuria.\textsuperscript{52} In the present study, podocyte injury and albuminuria were associated with increased intrarenal production of AngII, ROS, and NADPH oxidase components in AR rats, changes that were suppressed by treatment with olmesartan but not hydralazine. These data suggest that intrarenal AngII-induced increases in ROS contribute to the pathogenesis of AR-induced glomerular podocyte injury and albuminuria. Renal denervation also attenuated the AR-induced ROS production in the kidney, suggesting that the intrarenal SNS is also involved in this process.

Renal denervation did not affect SBP or echocardiographic parameters in AR rats. We also found that renal denervation significantly decreased collagen content and the mRNA expression of collagen I and III, in addition to altering the expression of fetal gene programming in AR rats, although these levels were not fully normalized. However, the results...
of echocardiography showed that renal denervation did not significantly affect the AR-induced changes in myocardial structure or function. We are currently unable to explain this discrepancy between the results of echocardiography and the changes observed at the molecular level. Nevertheless, we think that the molecular changes induced by renal denervation were not sufficient to cause myocardial structural or functional changes detectable on echocardiography. In fact, the combination of renal denervation and olmesartan almost completely prevented the AR-induced changes in LV molecular parameters, as well as its structure and function. A recent study showed that sympathectomy did not affect renal blood flow in a 5/6 nephrectomy animal model.53 In addition, previous studies in animals and humans showed that renal denervation did not affect renal blood flow or vascular resistance.20,54,55 Consistent with these earlier findings, our preliminary data suggest that chronic renal denervation does not significantly affect renal blood flow in anesthetized AR rats (data not shown). Taken together, our results suggest that renal denervation elicits renoprotective effects via mechanisms that are not simply explained by changes in blood pressure, renal blood flow, or myocardial function.

Conclusions
The possible mechanisms responsible for the onset of albuminuria under the condition of cardiac volume overload are depicted in Figure 7. Here, we have proposed a concept that ties our results together with those of previous studies.56-57 Chronic cardiac volume overload leads to SNS activation, resulting in augmentation of intrarenal RAS and NADPH oxidase–dependent ROS production. In turn, these events may contribute to the onset of renal injury, including glomerular podocyte injury and albuminuria. Thus, our data strongly support the hypothesis that coactivation of the SNS and RAS mediates de novo renal injury and confirm the presence of an interactive network underlying the cardiorenal syndrome during the progression of heart failure.

Acknowledgments
We are grateful to Daiichi-Sankyo Co. Ltd. for supplying olmesartan.

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Disclosures
None.

References


**CLINICAL PERSPECTIVE**

There is increasing awareness of the cardiorenal syndrome in patients with myocardial disease. The presence of chronic kidney disease is a significant and independent risk factor for poor prognosis in patients with chronic heart failure. In the present study, we demonstrated that inappropriate activation of the sympathetic nervous system and intrarenal renin-angiotensin system mediates de novo albuminuria in rats subjected to aortic regurgitation. Renal denervation reduced intrarenal angiotensinogen and angiotensin II levels and decreased podocyte injury and albuminuria in aortic regurgitation rats. Furthermore, treatment with olmesartan, to block renin-angiotensin system activity, enhanced the suppressive effects of renal denervation on albuminuria. Renal denervation also significantly reduced the induction of genes involved in fetal gene programming such as β-myosin heavy chain and brain natriuretic peptide and reduced markers of cardiac fibrosis such as collagen I and III mRNA levels and collagen content in the left ventricle. Our results imply that patients with cardiac dysfunction may be at increased risk for de novo renal dysfunction or worsening of preexisting asymptomatic renal dysfunction through sympathetic nerve hyperactivity. Recent clinical trials have demonstrated that catheter-based renal denervation can safely reduce blood pressure in treatment-resistant hypertensive patients without significant adverse events. The molecular mechanism underlying this pathophysiological condition may provide a new therapeutic target for patients with cardiovascular disease to prevent further renal injury while providing stronger protection of both organs.
Renal Sympathetic Denervation Suppresses De Novo Podocyte Injury and Albuminuria in Rats With Aortic Regurgitation

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Kazi Rafiq, PhD, Takahisa Noma, MD, PhD, Yoshihide Fujisawa, PhD, Yasuhiro Ishihara, MD, PhD, Yoshie Arai, BSc, A.H.M. Nurun Nabi, PhD, Fumiaki Suzuki, PhD, Yukiko Nagai, PhD, Daisuke Nakano, PhD, Hirofumi Hitomi, MD, PhD, Kento Kitada, MS, Maki Urushihara, MD, PhD, Hiroyuki Kobori, MD, PhD, Masakazu Kohno, MD, PhD and Akira Nishiyama, MD, PhD

SUPPLEMENTAL MATERIAL
### Table I. Biological and hemodynamic parameters in protocol 1

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<th>sham (n = 8)</th>
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<td>bwt (g)</td>
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<td>Plasma BUN (mg/dL)</td>
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<td>Creatinine Clearance (mL/min/kg)</td>
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<td>Plasma AngII (fmol/mL)</td>
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*P<0.05, **P<0.01 vs. sham. #P<0.05, ##P<0.01, ###P<0.005 vs. AR rats. Values are mean±SEM. LKwt; left kidney weight, bwt; body weight, Hwt; heart weight, LVwt; left ventricular weight, BUN; blood urea nitrogen, TBARS, thiobarbituric acid reactive substances.
Table II. Biological and hemodynamic parameters in protocol 2

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<th>UNX (n = 6)</th>
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<th>UNX + RDX (n = 6)</th>
<th>UNX + RDX + AR (n = 12)</th>
<th>UNX + RDX + AR + olmesartan (n = 8)</th>
<th>UNX + RDX + AR + hydralazine (n = 8)</th>
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<td>6 month</td>
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<td>748±15</td>
<td>729±26</td>
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<td><strong>Hwt/bwt (mg/g)</strong></td>
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<td>1.87±0.02</td>
<td>2.84±0.07†</td>
<td>1.81±0.11‡</td>
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<td><strong>LVwt/bwt (mg/g)</strong></td>
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<td>2.05±0.08†</td>
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<td>1.36±0.08†</td>
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<td><strong>Plasma BUN (mg/dL)</strong></td>
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<td><strong>Plasma creatinine</strong></td>
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<td><strong>Creatinine Clearance</strong></td>
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<td>1.90±0.18</td>
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<td><strong>Plasma AngII (fmol/mL)</strong></td>
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<td>59.84±9.02†</td>
<td>30.48±3.10</td>
<td>26.75±2.25‡</td>
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<td><strong>Plasma TBARS (µmol/L)</strong></td>
<td>9.25±0.71</td>
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<td>10.39±0.91</td>
<td>8.81±1.20</td>
<td>9.75±0.90</td>
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†P < 0.05 vs. UNX. ‡P < 0.05, ‡‡P < 0.01, ‡‡‡P < 0.005 vs. UNX + AR. §§P < 0.01, §§§P < 0.005 vs. UNX + RDX + AR. Values are mean±SEM. LKwt; left kidney weight, bwt; body weight, Hwt; heart weight, LVwt; left ventricular weight, BUN; blood urea nitrogen, TBARS, thiobarbituric acid reactive substances.
Plasma brain natriuretic peptide (BNP) levels. (A) Plasma BNP levels at 6 months after AR or sham operation in protocol 1. Plasma BNP levels are higher in AR rats than in sham rats. Treatment with olmesartan, but not with hydralazine, prevents the increases in plasma BNP levels in AR rats. ***P<0.005 vs. sham; ##P<0.01 vs. AR rats. (B) Plasma BNP levels at 6 months after AR or sham operation in protocol 2. UNX-AR rats show increased plasma BNP levels, which is tends to be decreased by RDX. In contrast, RDX plus olmesartan, but not RDX plus hydralazine, prevents the increases in plasma BNP levels. ††P<0.01 vs. UNX; †††P<0.005 vs. UNX + AR; §§P<0.01 vs. UNX + RDX + AR.
Figure II

Gene expressions in left ventricle. (A) αMHC (B) βMHC and (C) BNP gene expressions in LV tissues from protocol 1. αMHC gene expression was markedly decreased, and βMHC as well as BNP genes expression were markedly increased in AR rats than in sham rats. Treatment with olmesartan, but not with hydralazine, prevents the AR induced changes in genes expression in LV tissues. *P<0.05, **P<0.01, ***P<0.005 vs. sham; #P<0.05, ##P<0.01 vs. AR rats. (D) αMHC (E) βMHC and (F) BNP gene expression in LV tissues from protocol 2. UNX-AR rats showed decreased αMHC gene expression, and increased βMHC as well as BNP genes expression in LV tissues, which are attenuated by RDX. In contrast, RDX plus olmesartan, but not RDX plus hydralazine, prevented the AR induced changes. †P<0.05, ††P<0.01, †††P<0.005 vs. UNX; ‡P<0.05, ‡‡P<0.01 vs. UNX + AR; § P<0.05 vs. UNX + RDX + AR. MHC, myosin heavy chain.
Figure III.

**Collagen content and Collagen gene expressions in LV tissues.** (A) Collagen content (B) Collagen I and (C) Collagen III gene expressions in LV tissues from protocol 1. Collagen content as well as Collagen III gene expression were markedly increased in AR rats than in sham rats. Treatment with olmesartan, but not with hydralazine, significantly suppressed the AR induced Collagen content and gene expression in LV tissues in AR rats. *P*<0.05, **P*<0.01, ***P*<0.005 vs. sham; #*P*<0.05, ##*P*<0.01 vs. AR rats. (D) Collagen content (E) Collagen I and (F) Collagen gene III expression in LV tissues from protocol 2. UNX-AR rats showed increased Collagen content as well as Collagen I, Collagen III genes expression in LV tissues. Collagen content as well as Collagen genes expression were suppressed by RDX. In contrast, RDX plus olmesartan, but not RDX plus hydralazine, prevented the AR induced changes in LV tissues. †*P*<0.05, ††*P*<0.01, †††*P*<0.005 vs. UNX; ‡*P*<0.05, ‡‡*P*<0.01 vs. UNX + AR; §*P*<0.05 vs. UNX + RDX + AR.
Figure IV.

Renal cortical tissues renin activity and renin mRNA levels at 6 months after AR or sham operation. Renin activity in renal cortical tissues in protocols 1 (A) and 2 (C). Renin mRNA levels in renal cortical tissues in protocols 1 (B) and 2 (D). Neither AR nor UNX-AR affects renin activity or renin mRNA levels in renal cortical tissues. Olmesartan treatment increases renal renin activity in protocol 1 and 2. There are no differences in renin mRNA levels between any of the groups in protocol 1 and 2. RT-PCR data are expressed as fold-changes compared with sham or UNX after normalization for the expression of GAPDH. 

### P < 0.001 vs. AR; ‡‡‡‡ P < 0.001 vs. UNX + AR.
**Figure V.**

**Effects of norepinephrine (NE) on AGT gene expression in human proximal tubular cells (HPTCs).** Application of NE for 24 hours significantly increases AGT gene expression in a dose-dependent manner. RT-PCR data are expressed as fold-changes compared with the control group after normalization for the expression of GAPDH. *n*=4 for group. *P*<0.05, **P*<0.01 vs. control.