Renal Sympathetic Denervation Suppresses de novo Podocyte Injury and Albuminuria in Rats with Aortic Regurgitation

Running title: Rafiq et al.; SNS and RAS as mediators of cardiorenal connection

Kazi Rafiq, PhD¹; Takahisa Noma, MD, PhD²; Yoshihide Fujisawa, PhD³; Yasuhiro Ishihara, MD, PhD²; Yoshie Arai, BSc⁴; A.H.M. Nurun Nabi, PhD⁴; Fumiaki Suzuki, PhD⁴; Yukiko Nagai, PhD³; Daisuke Nakano, PhD¹; Hirofumi Hitomi, MD, PhD¹; Kento Kitada, MS¹; Maki Urushihara, MD, PhD⁵; Hiroyuki Kobori, MD, PhD¹; Masakazu Kohno, MD, PhD²; Akira Nishiyama, MD, PhD¹

¹Dept of Pharmacology, ²Dept of Cardiorenal & Cerebrovascular Medicine, ³Life Science Research Center, Faculty of Medicine, Kagawa University, Kagawa, Japan; ⁴Laboratory of Animal Biochemistry, Faculty of Applied Biological Sciences, Gifu University, Gifu, Japan; ⁵Depts of Medicine & Physiology & Hypertension & Renal Center of Excellence, Tulane University Health Sciences Center, New Orleans, LA

Correspondence:
Takahisa Noma, MD, PhD
Department of Cardiorenal and Cerebrovascular Medicine Faculty of Medicine
Kagawa University
1750-1 Ikenobe, Miki-cho, Kita-gun
Kagawa 761-0793, Japan
Phone: +81(87) 891-2151
Fax: +81(87)891-2151
Email: no@kms.ac.jp

Journal Subject Codes: [10] Cardio-renal physiology/ pathophysiology; [128] ACE/Angiotension receptors; [85] Autonomic, reflex, and neurohumoral control of circulation; [148] Heart failure - basic studies
Abstract:

**Background** - The presence of chronic kidney disease is a significant independent risk factor for poor prognosis in patients with chronic heart failure (CHF). 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 co-activating the sympathetic nervous system (SNS) and the renin-angiotensin system (RAS) in the kidney. We also examined the therapeutic potential of renal denervation and RAS inhibition to suppress renal injury in CHF.

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

**Conclusions** - In this chronic cardiac volume overload animal model, activation of the SNS augments kidney RAS and oxidative stress, which act as crucial cardio-renal mediators. Renal denervation and olmesartan prevent the onset and progression of renal injury, providing new insight into the treatment of cardio-renal syndrome.

**Key words:** aortic regurgitation, albuminuria, sympathetic nervous system (SNS), renin-angiotensin system (RAS), cardio-renal interaction
Introduction

Chronic heart failure (CHF) and chronic kidney disease (CKD) often co-exist, and exacerbate each other, resulting in poorer clinical outcomes, including faster progression to end-stage renal disease and further progression of CHF. This co-existence of CHF and CKD is commonly referred to the cardio-renal syndrome. It is now clear that the presence of CKD in patients with CHF results in an incurable state of CHF with very poor prognosis.

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. 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.

The sympathetic nervous system (SNS) and/or renin-angiotensin system (RAS) have been suggested as possible cardio-renal mediators. Sympathetic nerve activity is increased in patients with CHF, and may influence cardiovascular and renal prognosis. Similarly, CKD is often accompanied by increased sympathetic nerve activity and is improved by renal denervation. It has also been documented that RAS intervention with angiotensin converting enzyme inhibitors and angiotensin II (AngII) receptor blockers (ARBs) protect the heart and kidney independently of their effects on blood pressure lowering.

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 co-activating 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 at 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 as 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 three groups and treated with vehicle (AR, n=12), olmesartan (0.03% in chow, approximately 15 mg/kg body weight/day; Daiichi-Sankyo Co., Ltd., Tokyo, Japan; n=12), or hydralazine (0.075% in chow, approximately 50 mg/kg body weight/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 were measured in conscious rats by tail-cuff plethysmography (BP-98A; Softron Co., Tokyo, Japan). Twenty four-hour urine samples were collected at baseline and every month during treatment to determine urinary albumin, creatinine and angiotensinogen (AGT) levels.
Protocol 2

The rats were subjected to right uninephrectomy (UNX). Then, left-side renal denervation (RDX) was performed. Thereafter, AR or a sham operation was performed at 9 weeks of age. At 10 weeks of age, the rats were divided into six groups for a 6-month treatment period, as follows: vehicle-treated rats (UNX, n=6), vehicle-treated AR rats (UNX-AR, n=10), vehicle-treated denervated rats (UNX-RDX, n=6), vehicle-treated denervated-AR rats (UNX-RDX-AR, n=12), and UNX-RDX-AR plus olmesartan (0.03% in chow; n=8) or hydralazine (0.075% in chow; n=8) treatment. Blood pressure measurements and urine collection were performed as described above. In this protocol, right UNX was performed to prevent reno-renal reflexes from the right kidney, as previously described 17, 18.

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, i.p.). Complete RDX was achieved by cutting all of the visible renal nerves from the renal artery and vein, and painting these vessels with a solution of 10% phenol in ethanol 13. This method ablates the afferent and efferent renal nerves 13, 20. After sacrificing the rats, 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, i.p.) and xylazine (10 mg/kg, i.p.) with a SONOS5500 (Philips Medical Systems, Andover, MA, USA) 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. Immediately after collecting the blood, half of the right kidney was homogenized in cold methanol and processed to measure the AngII content, while the other half of the kidney tissue was cut and fixed in 10% buffered paraformaldehyde or embedded in OCT 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 (PAS) reagent. Immunohistochemistry for desmin, was performed as previously described. Frozen, OCT-embedded kidney tissue was cryosectioned into 10-μm-thick sections, which were stained with 10 μmol/L dihydroethidium (DHE) solution (Invitrogen, Carlsbad, CA, USA). DHE fluorescence intensity was measured as previously described. Images were obtained by confocal laser-scanning fluorescence microscopy (Radiance2100; Bio-Rad Laboratories, Hercules, CA, USA).

**Laser Capture Microdissected (LCM) Techniques**

To measure glomerular AGT, nephrin and podocin mRNA levels, the glomeruli were microdissected using a LCM (LM-200, Arcturus Bioscience, Mountain View, CA, USA). Glomerular mRNA was extracted using RNeAqueous-Micro kits (Ambion, Austin, TX, USA), as previously described.

**RT-PCR**

The mRNA expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), AGT, renin, nephrin,
podocin, p22phox and gp91phox was analyzed by real-time PCR using a LightCycler FastStart DNA Master SYBR Green I kit. Angiotensin type 1a (AT1a) receptor mRNA expression was measured using TaqMan Gene Expression Assay (Assay ID: Rn00578456_m1, Applied Biosystems, Foster City, CA, USA), and an ABI Prism 7000 Sequence Detection System (Applied Biosystems). PCR was performed using the previously described conditions with the following oligonucleotide primer sequences (sense and antisense): GAPDH, 5'-TGAACGGAAGCTCAGTTGTA-3' and 5'-TGGTGAGGAGGCCTGTAT-3'; AGT, 5'-TTGTGTGAGGAGGCCTGTAT-3' and 5'-TGCTGAGGATGTCCTG-3'; renin, 5'-TTGGGTGCTGAGCAATCT-3' and 5'-CCACATTTTGAGTTATCC-3'; nephrin, 5'-CAGAGTGACGACTATTAGGA-3' and 5'-GACCAGTAAACTGCCGTTATCC-3'; podocin, 5'-CTTCATGAGGTGAACCA-3' and 5'-GATGGCTTGGGACACATGAG-3'; p22phox, 5'-TCCACTTACTGCTGTCCGT-3' and 5'-TCAATGGGAGTCCACTGCT-3'; gp91phox, 5'-TGGTGATGTTAGTGGGAGC-3' and 5'-CTTTCTTGCATCTGGGTCT-3'; myosin heavy chain (MHC), 5'-GGCAGAGGAGAGGGCGGAGA-3' and 5'-GCGAGGCTCTTTCTGCTGGACA-3'; α MHC, 5'-GCCGACATTGCCGGAGTCCAGGCCTTCCACA-3' and 5'-AGGCTCCAGGTCTCAGGGCTTC-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.

**Urine Parameters**

Urinary albumin and creatinine concentrations were measured using assay kits for albumin (Code No. AKRAL-120, Shibayagi Co., Shibukawa, Japan) and creatinine (micro CRE-test; Wako Co., Ltd.), respectively. Urinary concentrations of AGT were measured using a Rat Total Angiotensinogen Assay Kit (Code No. 27414, IBL Co., Ltd., Fujioka, Japan), as previously described. Creatinine (Cr) clearance (CCr) was calculated using the equation: CCr
(mL/min/kg) = [urinary Cr (mg/dL) × urinary volume (mL) / plasma Cr (mg/dL)] × [1000 / body weight (g)] × [1 / 1440 (min)], as previously described \(^\text{30}\).

**Other Analytical Procedures**

Renal cortical tissue renin activity \(^\text{31}\) and plasma and renal cortical tissues NE levels \(^\text{13}\) were measured, as previously described. Plasma and kidney AngII concentrations were measured by a radioimmunoassay, as previously described \(^\text{24, 25}\). The degree of lipid peroxidation in plasma and renal cortical tissue was evaluated using biochemical assays for thiobarbituric acid reaction substances (TBARS), as previously described \(^\text{22}\). Collagen content in the LV tissues was determined based on hydroxyproline concentrations, as previously described \(^\text{32}\). Plasma brain natriuretic peptide (BNP) was measured using an AssayMaz Rat BNP-45 (rBNP-45) assay kit (ASSAYPRO, St. Charles, MO, USA). Plasma blood urea nitrogen (BUN) was measured using an automatic analyzer (Model 7020, HITACHI, Tokyo, Japan).

**Cell Culture**

Studies were performed in immortalized human proximal tubular cells (HPTCs) \(^\text{33}\). 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 PCR using previously described conditions \(^\text{23}\). The oligonucleotide primer sequences were (sense and antisense): human GAPDH, 5'-TGCACCACCAACTGCTTAGC-3' and 5'-GGCATGGACTGTGGTCATGAG-3'; human-AGT, 5'-AACTGGTGCTGCAAGGATCT-3' and 5'-TCTCTCTCATCCGCTTCAAG-3'. All data were normalized for the expression of GAPDH.

**Statistical Analyses**

All values are presented as means ± SEM. Statistical comparisons of differences among groups were performed using one-way repeated-measures analysis of variance (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 (U_{alb}V), urinary AGT excretion rate (U_{AGT}V) and echocardiography were compared using two-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 using GraphPad Prism version 5 for Windows (Graph Pad Software, San Diego, CA, USA).

**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*). By contrast, DBP decreased significantly in AR rats, as compared with that in 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 (*Supplemental Figure 1A*). 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 *Supplemental Table 1*, respectively. Compared with sham rats, AR rats exhibited LV end-diastolic dimension and LV end-systolic dimension dilatation, and lowered fractional shortening (FS). 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 $\beta$MHC and BNP, and decreased mRNA expression of $\alpha$MHC in LV tissues,
markers of cardiac hypertrophy \textsuperscript{34,35} and heart failure \textsuperscript{36} (Supplemental Figure 2A-C). Treatment with olmesartan, but not hydralazine, significantly attenuated LV hypertrophy in AR rats. LV interstitial fibrosis is a late feature in our model \textsuperscript{37,38}. AR rats had significantly greater LV tissue collagen content as well as collagen I and III mRNA expression than sham rats (Supplemental Figure 3A-C). All of these changes were attenuated by olmesartan treatment but not by hydralazine.

\( U_{\text{alb}} \) and UACR were significantly higher in AR rats than in sham rats at 3 months after AR operation (Figure 1C). Furthermore, \( U_{\text{alb}} \) progressively increased over time in AR rats. At 6 months, \( U_{\text{alb}} \) was 0.70±0.06 and 3.59±0.15 mg/day 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 (Supplemental Table 1). Treatment with olmesartan suppressed the increases in \( U_{\text{alb}} \) (0.60±0.08 mg/day, \( P<0.001 \)), UACR and other parameters in AR rats (Figure 1C). By 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 inducing AR, as compared with sham rats (Figures 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 as compared with sham rats (Figure 2C and Supplemental Table 1). The renal
cortical tissue mRNA levels of AT1a receptor and AGT were also increased in AR rats, as
compared with sham rats (Figures 2D and 2E). On the other hand, renin mRNA levels remained
unchanged (Supplemental Figure 4B). Renal renin activity tended to be increased in AR rats,
but these changes were not statistically significant (Supplemental Figure 4A). 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 hydralazine,
suppressed the increases in kidney AngII and mRNA levels of AT1a receptor and AGT in AR rats.
We also evaluated AGT gene expression in the glomeruli by LCM techniques. Glomerular AGT
gene expression was upregulated in AR rats, an effect of AR that was prevented by olmesartan,
but not by hydralazine (by 1.00±0.09-, 1.83±0.14-, 1.12±0.08- and 1.90±0.09-fold in the sham,
AR, AR plus olmesartan and AR plus hydralazine groups, respectively, P<0.05). Since urinary
AGT levels provide a specific index of kidney AGT expression 39, we also measured the U\textsubscript{AGT}V rate
in AR rats. As shown in Figure 2F, AR rats had markedly increased U\textsubscript{AGT}V levels at 6 months
after the AR operation as compared with sham rats. Treatment with olmesartan, but not with
hydralazine, suppressed the AR-induced increase in U\textsubscript{AGT}V levels.

**ARB Prevents Glomerular Podocyte Injury, and Suppresses the Production of Glomerular
Reactive Oxygen Species (ROS) 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 two adjacent
podocytes, using RT-PCR with LCM. As shown in Figures 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 PAS-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, as 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 Supplemental Table 1). 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 (Figures 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, 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 (Supplemental Figure 2 and 3). 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 two 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, collagen I and III, and increased αMHC in LV tissues, improved FS, mean velocity of circumferential fiber shortening (mvCF) and relative wall thickness, and prevented the augmentation of plasma BNP levels, as compared with UNX-RDX-AR rats (Table 2, Supplemental Figures 1B, 2 and 3, and Supplemental Table 2).

U_{abV} and UACR (Figure 4C) were increased in UNX-AR rats at 2 months after AR operation, and these increased progressively during the 6-month treatment period, with U_{abV} reaching 1.36±0.15 and 4.80±0.20 mg/day at 6 months in UNX-sham and UNX-AR rats, respectively (P<0.005). It is noteworthy that RDX alone significantly reduced the U_{abV} (1.73±0.21 mg/day, P<0.005) and UACR in UNX-AR rats (Figure 4C). Moreover, RDX plus olmesartan almost completely suppressed albuminuria (0.20±0.03 mg/day, 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 be decreased plasma creatinine levels and tended to be increased CCr in UNX-AR rats compared with UNX-sham rats, although these differences were not statistically significant (Supplemental Table 2).

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 as compared with UNX-sham rats (Figure 5B, 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. RDX suppressed the AR-induced increases in kidney AngII levels (Figure 5C), AGT and AT1a receptor mRNA levels in renal cortical tissues (Figures 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 plus olmesartan, respectively, *P*<0.05), and U_{AGT}V (Figure 5F). These levels were further decreased by RDX in combination 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 (Figures 6A-C). RDX significantly suppressed the AR-induced increases in kidney TBARS content and DHE fluorescence, as well as the increases in p22^{phox} and gp91^{phox} mRNA levels, as compared with those in UNX-AR rats (Figures 6D-G). RDX plus olmesartan further attenuated the increases in kidney TBARS content and DHE fluorescence, as well as the increases in p22^{phox} and gp91^{phox} mRNA levels, as compared with those in UNX-RDX-AR rats. The glomerular PAS positive area was increased in UNX-AR rats and UNX-sham rats, and was attenuated by RDX alone or in combination with olmesartan (3.60±0.09, 3.55±0.16, 2.57±0.12, 2.85±0.19, 1.96±0.10 and 2.81±0.25% in UNX, UNX-AR, UNX-RDX, UNX-RDX-AR, UNX-RDX-AR plus olmesartan, and UNX-RDX-AR plus hydralazine, respectively).

**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 (Supplemental Figure 5).

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 chronic heart failure. 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 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 in combination with an ARB further suppressed glomerular podocyte injury and 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 β-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 in order to stimulate renal renin in some pathophysiological condition. Similarly, 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 stage, but not in a chronic stage of heart failure. 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. 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 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 intra-glomerular AngII regulation because of technical difficulties.

Our preclinical \(^{24,47}\) and clinical \(^{49}\) 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 \(^{24,47,49}\), 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 \(^{50}\). In mice overexpressing AGT in the kidney, renal injury was associated with NADPH oxidase-dependent ROS production \(^{51}\). It has also been reported that AngII directly increases ROS formation through a NADPH oxidase-dependent pathways in podocytes, thereby accelerating albuminuria \(^{52}\). 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 play 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, and also altered 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. Recent study showed that sympathectomy did not affect renal blood flow (RBF) in a 5/6 nephrectomy animal model. In addition, previous studies in animals and humans showed that renal denervation did not affect RBF or vascular resistance. Consistent with these earlier findings, our preliminary data suggest that chronic renal denervation does not significantly affect RBF 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, RBF 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. 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 co-activation of the SNS and RAS mediates de novo renal injury, and confirm the presence of an
interactive network underlying cardio-renal syndrome during the progression of heart failure.

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

Funding Sources: This work was supported in part by grant-in-aid for scientific research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (20590253 to Akira Nishiyama, and 22790792 to Hirofumi Hitomi) and the National Institute of Diabetes and Digestive and Kidney Diseases (R01DK072408 to Hiroyuki Kobori).

Conflict of Interest Disclosures: None

References:


44. Nakamura A, Johns EJ. Effect of renal nerves on expression of renin and angiotensinogen


55. Salman IM, Sattar MA, Abdullah NA, Ameer OZ, Hussain FB, Hye Khan MA, Yam MF,


### Table 1. Echocardiographic data at baseline and 6 month after AR or sham operation in protocol 1

<table>
<thead>
<tr>
<th></th>
<th>sham (n = 8)</th>
<th>AR (n = 12)</th>
<th>AR + olmesartan (n = 12)</th>
<th>AR + hydralazine (n = 12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LVEDD, mm</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 month</td>
<td>6.55±0.21</td>
<td>6.49±0.23</td>
<td>6.51±0.17</td>
<td>6.57±0.22</td>
</tr>
<tr>
<td>6 month</td>
<td>8.10±0.40</td>
<td>10.59±0.71*</td>
<td>10.05±0.35*</td>
<td>10.25±0.29*</td>
</tr>
<tr>
<td>LVESD, mm</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 month</td>
<td>3.59±0.16</td>
<td>3.43±0.18</td>
<td>3.67±0.15</td>
<td>3.68±0.21</td>
</tr>
<tr>
<td>6 month</td>
<td>4.80±0.35</td>
<td>6.70±0.67*</td>
<td>6.50±0.34*</td>
<td>6.60±0.26*</td>
</tr>
<tr>
<td>LV mass</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 month</td>
<td>803±67</td>
<td>731±38</td>
<td>869±47</td>
<td>857±50</td>
</tr>
<tr>
<td>6 month</td>
<td>1452±23</td>
<td>2393±152***</td>
<td>1710±122##</td>
<td>2224±122***</td>
</tr>
<tr>
<td>SW, mm</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 month</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>
</tr>
<tr>
<td>6 month</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>
</tr>
<tr>
<td>PW, mm</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 month</td>
<td>1.50±0.05</td>
<td>1.57±0.15</td>
<td>1.58±0.05</td>
<td>1.61±0.09</td>
</tr>
<tr>
<td>6 month</td>
<td>1.67±0.29</td>
<td>1.88±0.22</td>
<td>1.90±0.27</td>
<td>2.00±0.35</td>
</tr>
<tr>
<td>RWT</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 month</td>
<td>0.47±0.09</td>
<td>0.49±0.08</td>
<td>0.50±0.05</td>
<td>0.47±0.09</td>
</tr>
<tr>
<td>6 month</td>
<td>0.43±0.01</td>
<td>0.37±0.01*</td>
<td>0.41±0.12</td>
<td>0.41±0.07</td>
</tr>
<tr>
<td>FS, %</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 month</td>
<td>42.89±2.38</td>
<td>44.00±2.34</td>
<td>44.76±1.56</td>
<td>45.08±2.33</td>
</tr>
<tr>
<td>6 month</td>
<td>40.78±2.11</td>
<td>33.51±2.89**</td>
<td>38.50±1.75</td>
<td>35.22±1.59**</td>
</tr>
<tr>
<td>mvcf, circ/sec</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 month</td>
<td>2.37±0.13</td>
<td>2.31±0.21</td>
<td>2.34±0.10</td>
<td>2.27±0.09</td>
</tr>
<tr>
<td>6 month</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>
</tr>
</tbody>
</table>

Values are mean±SEM. *P<0.05, **P<0.01, ***P<0.005 vs. sham. *P<0.05, **P<0.01 vs. AR rats. AR; 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, mvcf; mean velocity of circumferential fiber shortening.
Table 2. Echocardiographic data at baseline and 6 month after AR or sham operation in protocol 2

<table>
<thead>
<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>
</tr>
</thead>
<tbody>
<tr>
<td><strong>LVEDD, mm</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 month</td>
<td>7.32±0.30</td>
<td>7.39±0.27</td>
<td>7.22±0.12</td>
<td>7.32±0.16</td>
<td>7.39±0.12</td>
<td>7.44±0.29</td>
</tr>
<tr>
<td>6 month</td>
<td>8.96±0.27</td>
<td>10.54±0.21†</td>
<td>9.05±0.19</td>
<td>10.68±0.38†</td>
<td>9.80±0.47†</td>
<td>9.93±0.73†</td>
</tr>
<tr>
<td><strong>LVESD, mm</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 month</td>
<td>4.01±0.31</td>
<td>4.28±0.27</td>
<td>4.05±0.18</td>
<td>4.13±0.17</td>
<td>4.16±0.18</td>
<td>4.26±0.26</td>
</tr>
<tr>
<td>6 month</td>
<td>6.88±0.27</td>
<td>7.88±0.29†</td>
<td>6.78±0.22</td>
<td>7.67±0.36†</td>
<td>6.88±0.45</td>
<td>6.98±0.74</td>
</tr>
<tr>
<td><strong>LV mass</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 month</td>
<td>827±37</td>
<td>896±47</td>
<td>913±36</td>
<td>915±56</td>
<td>827±48</td>
<td>870±75</td>
</tr>
<tr>
<td>6 month</td>
<td>1562±60</td>
<td>1783±158†</td>
<td>1517±119</td>
<td>1920±120††</td>
<td>1475±107†††</td>
<td>1768±177†††</td>
</tr>
<tr>
<td><strong>SW, mm</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 month</td>
<td>1.60±0.07</td>
<td>1.63±0.08</td>
<td>1.60±0.15</td>
<td>1.59±0.03</td>
<td>1.68±0.16</td>
<td>1.55±0.06</td>
</tr>
<tr>
<td>6 month</td>
<td>1.77±0.09</td>
<td>2.02±0.30</td>
<td>1.82±0.07</td>
<td>1.80±0.19</td>
<td>2.00±0.28</td>
<td>1.95±0.37</td>
</tr>
<tr>
<td><strong>PW, mm</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 month</td>
<td>1.50±0.05</td>
<td>1.55±0.06</td>
<td>1.53±0.08</td>
<td>1.54±0.17</td>
<td>1.52±0.06</td>
<td>1.57±0.20</td>
</tr>
<tr>
<td>6 month</td>
<td>1.67±0.29</td>
<td>1.67±0.23</td>
<td>1.71±0.12</td>
<td>1.85±0.09</td>
<td>1.87±0.30</td>
<td>1.77±0.08</td>
</tr>
<tr>
<td><strong>RWT</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 month</td>
<td>0.42±0.09</td>
<td>0.43±0.05</td>
<td>0.43±0.07</td>
<td>0.42±0.12</td>
<td>0.43±0.06</td>
<td>0.41±0.05</td>
</tr>
<tr>
<td>6 month</td>
<td>0.38±0.01†</td>
<td>0.35±0.01‡</td>
<td>0.39±0.08</td>
<td>0.34±0.04†</td>
<td>0.39±0.01‡, §</td>
<td>0.37±0.09</td>
</tr>
<tr>
<td><strong>FS, %</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 month</td>
<td>45.04±3.10</td>
<td>43.20±2.11</td>
<td>44.52±2.41</td>
<td>44.30±2.50</td>
<td>44.00±2.64</td>
<td>43.75±2.55</td>
</tr>
<tr>
<td>6 month</td>
<td>38.75±1.00</td>
<td>33.08±2.05†</td>
<td>40.15±1.56</td>
<td>31.76±1.25†</td>
<td>37.00±3.03†</td>
<td>33.72±3.33</td>
</tr>
<tr>
<td><strong>mvcf, circ/sec</strong></td>
<td>2.45±0.13</td>
<td>2.21±0.24</td>
<td>2.28±0.17</td>
<td>2.43±0.19</td>
<td>2.35±0.25</td>
<td>2.33±0.08</td>
</tr>
<tr>
<td>6 month</td>
<td>2.05±0.10</td>
<td>1.78±0.15</td>
<td>1.76±0.30</td>
<td>1.85±0.22</td>
<td>1.72±0.18</td>
<td>1.92±0.15</td>
</tr>
<tr>
<td><strong>CO, mL/min</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 month</td>
<td>88.08±4.56</td>
<td>88.48±6.80</td>
<td>83.41±2.96</td>
<td>87.46±6.76</td>
<td>81.48±4.74</td>
<td>82.82±4.18</td>
</tr>
<tr>
<td>6 month</td>
<td>91.57±8.00</td>
<td>108.95±7.07</td>
<td>97.25±7.00</td>
<td>105.90±7.21</td>
<td>102.56±4.45</td>
<td>108.80±8.21</td>
</tr>
</tbody>
</table>

Values are mean±SEM. †P<0.05, ††P<0.01, †††P<0.005 vs. UNX. ‡P<0.05, ‡‡P<0.01, ‡‡‡P<0.005 vs. UNX + AR. ‡P<0.05, §§P<0.01 vs. UNX + RDX + AR. AR; aortic regurgitation, UNX; uninephrectomy, RDX; left renal denervation, CO; cardiac output, LV; left ventricular, LVEDD; left ventricular end diastolic dimension, LVESD; 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.
Figure Legends:

Figure 1. Systolic blood pressure (SBP), diastolic blood pressure (DBP) and urinary albumin to creatinine ratio (UACR) profiles in protocol 1. (A) SBP (B) DBP. AR- and sham-operated rats have similar SBP, and treatment with olmesartan or hydralazine lowered SBP to equivalent levels. DBP is lower in AR rats than in sham rats. By 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.

Figure 2. Plasma and kidney norepinephrine (NE) levels, kidney angiotensin II (AngII) content, mRNA levels of angiotensinogen (AGT) and angiotensin type 1a (AT1a) receptor in renal cortical tissues, and urinary angiotensinogen excretion rate (U_{AGT}V) in protocol 1. (A) Plasma NE levels and (B) kidney tissue NE content at 6 months after the sham or AR operation. AR rats have increased plasma and kidney NE levels. Treatment with olmesartan suppresses the increases in plasma and kidney NE levels in AR rats. (C) Kidney AngII content, (D) AT1a receptor mRNA levels and (E) AGT mRNA levels in renal cortical tissues. AR rats have increased kidney AngII content and mRNA levels of AT1a receptor and AGT. Olmesartan suppresses the increases in AngII levels and the upregulation of AT1a receptor and AGT mRNA levels in AR rats. RT-PCR data are expressed as fold-changes compared with the sham group after normalization for the expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). (F) U_{AGT}V is augmented in AR rats, as compared with sham rats. The increase in U_{AGT}V is suppressed by olmesartan in AR rats. *P<0.05, **P<0.01, ***P<0.005, ****P<0.001 vs. sham; #P<0.05, ##P<0.01, ###P<0.005, ####P<0.001 vs. AR.
Figure 3. Podocyte injury, and reactive oxygen species and NADPH oxidase levels in the kidney in protocol 1. (A) Representative immunohistochemical images with staining for desmin, a marker of podocyte injury (original magnification, ×200), and the relative desmin-stained area in glomeruli as a percentage of total glomerular area. (B) Nephrin and (C) podocin mRNA levels in glomeruli. AR rats have a greater desmin-positive area (brown) in the glomeruli, with decreases in glomerular nephrin and podocin mRNA levels. Treatment with olmesartan suppresses the AR-induced increases in the desmin-positive area, and prevents the decreases in glomerular nephrin and podocin mRNA levels. (D) Representative images of dihydroethidium (DHE) staining (original magnification, ×100). (E) Renal cortical tissue thiobarbituric acid reactive substances (TBARS). (F) p22phox and (G) gp91phox mRNA levels in renal cortical tissue. All of these parameters are increased in AR rats compared with sham rats. The increases in renal cortical TBARS, DHE staining, and p22phox and gp91phox mRNA levels in AR rats are attenuated by olmesartan. *P<0.05, **P<0.01, ***P<0.005, ****P<0.001 vs. sham.

Figure 4. Systolic blood pressure (SBP), diastolic blood pressure (DBP) and urinary albumin to creatinine ratio (UACR) profiles in protocol 2. (A) UNX and UNX-AR rats have similar SBP. In UNX-AR rats, RDX does not significantly lower SBP. On the other hand, both olmesartan and hydralazine decrease SBP in UNX-RDX-AR rats to equivalent levels. (B) DBP is lower in UNX-AR rats than in UNX rats. RDX alone does not affect DBP in UNX-AR rats, but RDX in combination with olmesartan or hydralazine decreases DBP to equivalent levels in UNX-RDX-AR rats. (C) UNX-AR rats develop marked albuminuria, which is attenuated by RDX. Furthermore, RDX in combination with olmesartan blocks the onset and progression of albuminuria. †P<0.05, ††P<0.01, †††P<0.005 vs. UNX; ‡‡P<0.01, ‡‡‡P<0.005, ‡‡‡‡P<0.001 vs. UNX-AR; §§P<0.01 vs. UNX-RDX-AR. UNX: uninephrectomy;
RDX: left-side renal denervation.

**Figure 5.** Plasma and kidney norepinephrine (NE) levels, Kidney angiotensin II (AngII) content and mRNA levels of angiotensinogen (AGT) and AT1α receptor in renal cortical tissues, Urinary angiotensinogen excretion rate ($U_{AGT}V$) in protocol 2. (A) UNX-AR rats show increased plasma NE levels, which are tends to be decrease by RDX. However, RDX plus olmesartan suppresses the increases in plasma NE levels. (B) UNX-AR rats show markedly increased kidney NE content. By contrast, the kidney NE content in all renal denervated rats is at or below the undetectable range (below 3 ng/g tissue). (C) Kidney AngII content, (D) AT1α receptor mRNA levels and (E) AGT mRNA levels in renal cortical tissues. UNX-AR rats show increased kidney AngII content and AT1α receptor and AGT mRNA levels, which are suppressed by RDX. RDX plus Olmesartan further decreases these renal RAS components. RT-PCR data are expressed as fold-changes compared with UNX after normalization for the expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). (F) $U_{AGT}V$ is markedly increased in UNX-AR rats, which is suppressed by RDX. In contrast, RDX plus olmesartan further attenuates $U_{AGT}V$. *P<0.05, **P<0.01, ***P<0.005, ****P<0.001 vs. UNX; †P<0.05, ††P<0.01, †††P<0.001 vs. UNX-AR; §P<0.05, §§P<0.01 vs. UNX-RDX-AR.

UNX: uninephrectomy; RDX: left-side renal denervation.

**Figure 6.** Podocyte injury, and reactive oxygen species and NADPH oxidase levels in the kidney in protocol 2. (A) Representative desmin-stained images (original magnification, ×200) and relative desmin-positive area, and (B) nephrin and (C) podocin mRNA levels in glomeruli. UNX-AR rats have a greater desmin-positive area in the glomeruli with reductions in glomerular nephrin and podocin mRNA levels. In these rats, RDX reduces the desmin-positive area and attenuates the reductions in glomerular nephrin and podocin mRNA levels.
RDX in combination with olmesartan further enhances these changes. (D) Representative images of dihydroethidium (DHE) staining (original magnification, ×100). (E) Renal cortical tissue thiobarbituric acid reactive substances (TBARS). (F) \( p22^{\text{phox}} \) and (G) \( \text{gp91}^{\text{phox}} \) mRNA levels in renal cortical tissue. These parameters are increased in UNX-AR rats, while RDX attenuates the AR-induced increases in renal cortical TBARS, DHE staining, and \( p22^{\text{phox}} \) and \( \text{gp91}^{\text{phox}} \) mRNA levels. These suppressive effects of RDX are further enhanced by olmesartan in UNX-RDX-AR rats. \( \dagger P<0.05, \dagger\dagger P<0.01, \dagger\dagger\dagger P<0.001 \) vs. UNX; \( \ddagger P<0.05, \ddagger\ddagger P<0.01, \ddagger\ddagger\ddagger P<0.001 \) vs. UNX-AR; \( \S P<0.05 \) vs. UNX-RDX-AR. UNX: uninephrectomy; RDX: left-side renal denervation.

**Figure 7.** Schematic diagram summarizing the cardio-renal syndrome during conditions of impaired cardiac function. Chronic cardiac volume overload activates the sympathetic nervous system and thereby increases plasma and kidney norepinephrine (NE) levels. In turn, the increased kidney NE stimulates angiotensinogen (AGT) expression and subsequently angiotensin II (AngII) production in the cortical tissues. Activation of the sympathetic nervous system and local AngII stimulates NADPH oxidase-dependent reactive oxygen species (ROS) generation in the kidney. Increases in kidney AngII, NE and oxidative stress lead to podocyte injury and albuminuria. Renal denervation (RDX) plus AngII blockade by an AngII receptor blocker (ARB) suppresses sympathetic nervous activation and the increases in kidney NE, AGT, AngII and oxidative stress, thereby preventing the onset and progression of podocyte injury and albuminuria.
Renal Sympathetic Denervation Suppresses de novo Podocyte Injury and Albuminuria in Rats with Aortic Regurgitation


_Circulation_. published online February 10, 2012;
_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2012 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/early/2012/02/10/CIRCULATIONAHA.111.064097

Data Supplement (unedited) at:
http://circ.ahajournals.org/content/suppl/2012/02/10/CIRCULATIONAHA.111.064097.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation_ is online at:
http://circ.ahajournals.org/subscriptions/
Renal Sympathetic Denervation Suppresses de novo Podocyte Injury and Albuminuria in Rats with Aortic Regurgitation

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

<table>
<thead>
<tr>
<th></th>
<th>sham</th>
<th>AR</th>
<th>AR + olmesartan</th>
<th>AR + hydralazine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n = 8)</td>
<td>(n = 12)</td>
<td>(n = 12)</td>
<td>(n = 12)</td>
</tr>
<tr>
<td>bwt (g)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 month</td>
<td>250±2.90</td>
<td>246±2.89</td>
<td>248±2.10</td>
<td>249±1.82</td>
</tr>
<tr>
<td>6 month</td>
<td>652±9.21</td>
<td>699±20.19</td>
<td>645±18.12</td>
<td>703±19.20</td>
</tr>
<tr>
<td>LKwt/bwt (mg/g)</td>
<td>3.26±0.03</td>
<td>3.61±0.12*</td>
<td>3.01±0.08#</td>
<td>3.54±0.06*</td>
</tr>
<tr>
<td>Hwt/bwt (mg/g)</td>
<td>2.02±0.03</td>
<td>3.01±0.17**</td>
<td>2.17±0.10#</td>
<td>2.93±0.11*</td>
</tr>
<tr>
<td>LVwt/bwt (mg/g)</td>
<td>1.49±0.02</td>
<td>2.34±0.10**</td>
<td>1.61±0.08#</td>
<td>2.27±0.10*</td>
</tr>
<tr>
<td>Plasma BUN (mg/dL)</td>
<td>23.75±3.75</td>
<td>27.91±5.30</td>
<td>22.50±1.25</td>
<td>28.15±3.12</td>
</tr>
<tr>
<td>Plasma creatinine</td>
<td>0.60±0.05</td>
<td>0.81±0.06</td>
<td>0.70±0.03</td>
<td>0.80±0.02</td>
</tr>
<tr>
<td>Creatinine Clearance (mL/min/kg)</td>
<td>2.25±0.22</td>
<td>1.76±0.12</td>
<td>2.00±0.19</td>
<td>1.78±0.16</td>
</tr>
<tr>
<td>Plasma AngII (fmol/mL)</td>
<td>20.04±2.27</td>
<td>28.24±2.80</td>
<td>175.00±19.17###</td>
<td>20.21±3.44</td>
</tr>
<tr>
<td>Plasma TBARS (µmol/L)</td>
<td>8.59±0.66</td>
<td>11.50±0.54</td>
<td>8.83±0.64</td>
<td>10.89±1.19</td>
</tr>
</tbody>
</table>

*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

<table>
<thead>
<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>
</tr>
</thead>
<tbody>
<tr>
<td>bwt (g)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 month</td>
<td>272±6.4</td>
<td>282±2.3</td>
<td>278±2.4</td>
<td>279±2.5</td>
<td>278±2.7</td>
<td>281±3.3</td>
</tr>
<tr>
<td>6 month</td>
<td>723±21</td>
<td>741±22</td>
<td>726±24</td>
<td>748±15</td>
<td>729±26</td>
<td>722±25</td>
</tr>
<tr>
<td>Hwt/bwt (mg/g)</td>
<td>1.82±0.02</td>
<td>2.82±0.10†</td>
<td>1.87±0.02</td>
<td>2.84±0.07†</td>
<td>1.81±0.11‡</td>
<td>2.72±0.16†</td>
</tr>
<tr>
<td>LVwt/bwt (mg/g)</td>
<td>1.35±0.02</td>
<td>2.05±0.08†</td>
<td>1.43±0.02</td>
<td>1.91±0.06†</td>
<td>1.36±0.08‡</td>
<td>1.80±0.10</td>
</tr>
<tr>
<td>Plasma BUN (mg/dL)</td>
<td>25.68±4.27</td>
<td>28.90±6.15</td>
<td>21.31±2.83</td>
<td>17.55±0.74</td>
<td>20.86±1.07</td>
<td>22.15±2.81</td>
</tr>
<tr>
<td>Plasma creatinine (mg/dL)</td>
<td>0.62±0.13</td>
<td>0.69±0.15</td>
<td>0.50±0.04</td>
<td>0.45±0.01</td>
<td>0.42±0.01</td>
<td>0.48±0.02</td>
</tr>
<tr>
<td>Creatinine Clearance (mL/min/kg)</td>
<td>1.99±0.11</td>
<td>1.70±0.23</td>
<td>2.30±0.20</td>
<td>1.90±0.18</td>
<td>2.00±0.21</td>
<td>1.95±0.15</td>
</tr>
<tr>
<td>Plasma AngII (fmol/mL)</td>
<td>28.15±2.87</td>
<td>59.84±9.02†</td>
<td>30.48±3.10</td>
<td>26.75±2.25‡</td>
<td>193.23±20.07†††§§§</td>
<td>28.95±2.98</td>
</tr>
<tr>
<td>Plasma TBARS (µmol/L)</td>
<td>9.25±0.71</td>
<td>13.00±1.01</td>
<td>8.65±0.55</td>
<td>10.39±0.91</td>
<td>8.81±1.20</td>
<td>9.75±0.90</td>
</tr>
</tbody>
</table>

†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.
Figure 1

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.
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 UNIX after normalization for the expression of GAPDH. ###P<0.001 vs. AR; ‡‡‡‡P<0.001 vs. UNIX + 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.