博士論文

The role of renal sympathetic nerve
in cardio-renal association

（心腎連関における腎交感神経の役割）

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Abstract

Background:

     Chronic kidney disease (CKD) is an independent risk factor for cardiovascular disease (CVD) even from early stage of CKD. Clinically, CKD and heart failure (HF) often worsen each organ concurrently, referred to as the cardiorenal syndrome (CRS). However, the mechanisms and mediators underlying this interaction are poorly understood. Former studies suggests that sympathetic tone is altered in CKD patients and recently renal denervation successfully reduced blood pressure and cardiac function in either hypertensive and HF patients. In this study, I hypothesized that renal denervation has direct cardioprotective effect on cardiac function in CRS.

Methods:

     Three-week-old male Sprague-Dawley rats were subjected to right uninephrectomy with or without left-side renal denervation, and fed normal-salt diet (0.3% NaCl) or high-salt diet (8% NaCl) for 6 weeks. Left ventricle (LV) diastolic function measured as time constant at the isovolumic relaxation phase (Tau) were investigated by cardiac catheterization. Protein level of sarcoplasmic reticulum Ca^{2+}-ATPase type 2a (SERCA2a) and phospholamban (PLB) were determined by Western blotting, and related gene expression was quantified by real time PCR.

Results:

High salt loading induced a significant elevation of blood pressure (BP) and impaired LV relaxation, accompanied by reduced SERCA2a protein and gene expression in the cardiac tissue. Renal denervation improved LV relaxation accompanied by restoring SERCA2a protein and gene expression despite neither BP nor urinary protein levels were altered. BP reduction by hydralazine at the early phase had the tendency to restore LV relaxation, while SERCA2a gene and protein expression remained reduced. PLB-phosphorylation was not altered by renal denervation.

Conclusion:

In this CKD animal model, renal denervation has the cardioprotective effect on cardiac diastolic dysfunction independent from BP reduction as well as renal function possibly through specific restoration of SERCA2a gene and protein expression, providing new insight into the mechanism of CRS.

Key words:

Cardiorenal syndrome, heart failure, heart failure with preserved ejection fraction, renal denervation, renal sympathetic nerve

Abbreviations

AR: adrenergic receptor

BNP: brain natriuretic peptide

BP: blood pressure

Ca: calcium

CKD: chronic kidney disease

CRS: cardiorenal syndrome

CVD: cardiovascular disease

$+dP/dt_{max}$: maximal positive LV-pressure development

eGFR: estimated glomerular filtration rate

ESRD: end-stage renal disease

HF: heart failure

HF-PEF: heart failure with preserved ejection fraction

HE-REF: heart failure with reduced ejection fraction

HR: heart rate

LV: left ventricle

LVEDP : left ventricle end-diastolic pressure

LVPSP: left ventricle peak systolic pressure

MBP: mean arterial pressure

NE: norepinephrine

PLB: phospholamban

RT-PCR: reverse transcription-polymerase chain reaction

SBP: systolic blood pressure

Ser16-PLB: phospholamban phosphorylation at serine 16

SERCA2a: sarcoplasmic reticulum Ca^{2+}-ATPase type 2a

SNS: sympathetic nerve system

SR: sarcoplasmic reticulum

Tau: time constant at the isovolumic relaxation phase

Thr17-PLB: phospholamban phosphorylation at threonine 17

UTR: untranslated region

Introduction

Chronic kidney disease (CKD), defined as either kidney damage represented by proteinuria or a decreased estimated glomerular filtration rate (eGFR) of less than 60 ml/min per 1.73 m^2 for 3 or more months, irrespective of the type of kidney disease [1, 2] **(table 1)**. The prevalence of CKD is dramatically increasing worldwide so does the prevalence of end-stage renal disease (ESRD) [3-5] **(table 2)**. Recent clinical studies have reported that CKD is an independent risk factor for cardiovascular disease (CVD) from its early stage [6-8]. CKD is common and an important independent predictor of death and hospitalization in adults with heart failure (HF) across the spectrum of left ventricular systolic function [9-11]. In addition, CKD-associated mortality is higher in HF with preserved ejection fraction (HF-PEF) than HF with reduced ejection fraction (HF-REF). [10]

Table 1. Definition of chronic kidney disease (CKD).

1. Kidney damage for 3 months, manifest by either: albuminuria (AER 30 mg/d; ACR 30 mg/g), urinary sediment abnormalities, electrolyte and other abnormalities due to tubular disorders, abnormalities detected by histology, structural abnormalities detected by imaging, or history of kidney transplantation 2. GFR < 60 mL/min/1.73m^2 for 3 months

ACR; albumin-creatinine ratio, AER; albumin excretion rate, GFR; glomerular filtration rate

Table 2. Prevalence of chronic kidney disease in Japan.

GFR stage	GFR (mL/min/1.73m^2)	Urinary protein - ±	Urinary protein 1+-
G1	≧90	2,803	61 (0.6%)
G2	60-89	6,187	171 (1.7%)
G3a	45-59	886 (8.6%)	58 (0.6%)
G3b	30-44	106 (1.0%)	24 (0.2%)
G4	15-29	10 (0.1%)	9 (0.1%)
G5	< 15	1 (0.01%)	4 (0.03%)

Unit; ten thousand people.

GFR; glomerular filtration rate.

Cited from Jpn J Nephrol. 2012; 54: 1031-1189.

Clinically, CKD and HF often worsen each organ concurrently. While CKD and HF share many common risk factors, it is clear that CKD itself places patients at higher risk for CVD - most frequently HF [12]. The mechanisms to explain these observations remain unclear, and the complex interdependence of heart and kidney function is referred to as the cardiorenal syndrome (CRS) [13] (**Figure 1**). In cardiorenal syndrome, the impact of CKD on patients with HF likely operates through pathways common to both diseases, including volume and pressure overload, the renin-angiotensin-aldosterone system [14-16], anemia [17, 18], chronic inflammation [19-21], as well as disturbances in phosphate metabolism [22-24].
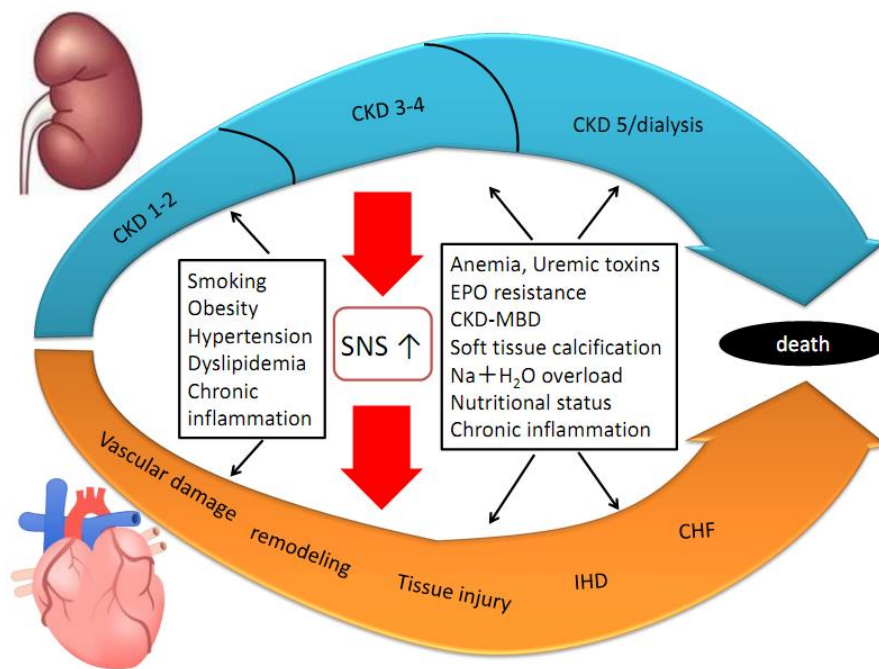
Figure 1. Cardiorenal syndrome in chronic kidney disease.

In cardiorenal syndrome, the impact of chronic kidney disease (CKD) on patients with heart failure (HF) likely operates through pathways common to both diseases.

CHF; chronic heart failure, CKD-MBD; chronic kidney disease-mineral bone disorder, EPO; erythropoietin, IHD; ischemic heart disease, SNS; sympathetic nerve system.

Cited from Am J Coll Cardiol. 2008; 52: 1527-1539, edited

Altered sympathetic cardiovascular regulation is an important mechanism contributing to the association between CKD and increased cardiovascular morbidity and mortality [25]. Clinical studies have demonstrated that plasma catecholamines were elevated [26] and muscle sympathetic nerve activity was enhanced in CKD patients [27, 28]. In addition, the sympathetic overdrive parallels the severity of the CKD from its early stage [29]. Furthermore, plasma NE levels are predictive of both survival and incidents of cardiovascular

events in patients with ESRD [30]. Interestingly, in ESRD patients who had undergone long-term treatment with hemodialysis, elevated muscle sympathetic nerve activity was reduced by bilateral nephrectomy, which appears to be mediated by an afferent signal arising in the failing kidneys [31].

There have been reported that selective catheter-based renal denervation is safe and effective in attaining improved and sustained BP control in patients with resistant hypertension and normal renal function [32-35], as well as reduced renal function [36]. Recently, Brandt et al. showed in a retrospective analysis an improvement in HF-PEF parameters using echocardiography after renal denervation [37].

Furthermore, additional benefits appear to be evident in patients with concomitant metabolic disorders or obstructive sleep apnea [38-40]. These effects are likely to be mediated via alterations in renal afferent signaling, resulting in reductions in both renal and whole-body sympathetic outflow [32, 34].

At present, there are growing concerns whether and how sympathetic nerve activation has the ability to cause cardiac dysfunction. Moreover, the direct effect by renal denervation on heart remains unknown. I therefore hypothesized that renal denervation has direct cardioprotective effect on cardiac dysfunction with young, salt-induced uninephrectomized Sprague-Dawley rats, as a salt induced CKD with sympathetic

overactivation model [41-43] **(Figure 2)**. This study will identify a novel role of renal

denervation on cardiac dysfunction in CKD rats, and find an alternative strategy for the
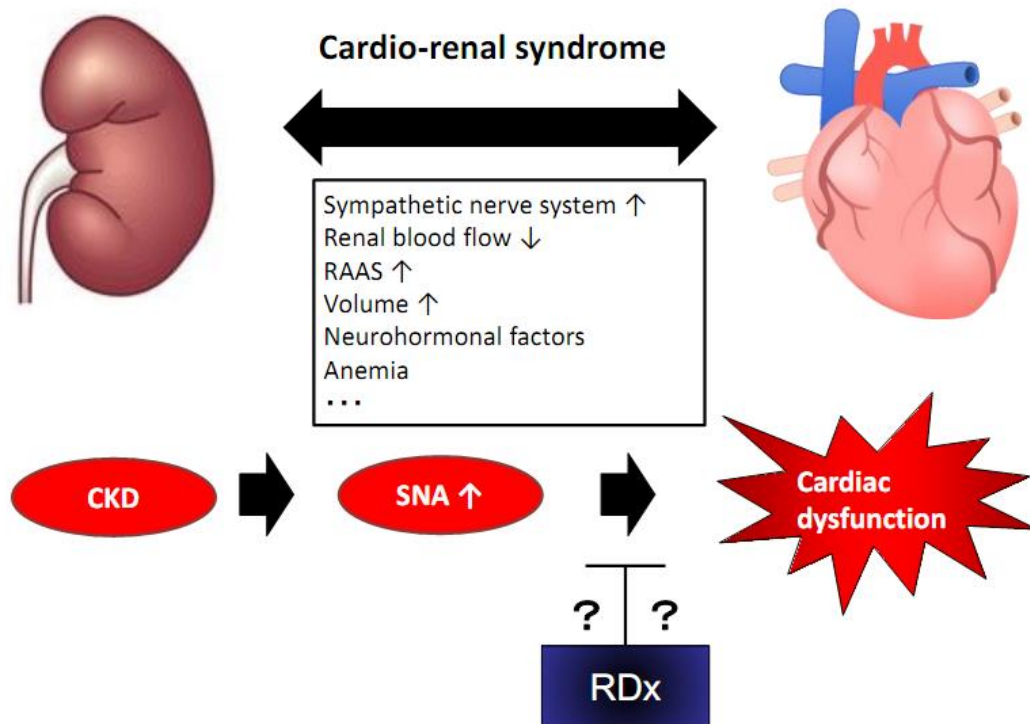
management of CRS.

Figure 2. Hypothesis.
Renal denervation might have cardioprotective effect on cardiac dysfunction.
CKD; chronic kidney disease, RAAS; renin-angiotensin-aldosterone system, RDx; renel
denervation, SNA; sympathetic nerve activity.

Materials and methods

All experimental procedures were conducted in accordance with the guidelines for the care and use of laboratory animals approved by my university. All rats were housed in a room maintained at 23°C to 25°C with a 12-hour light/dark cycle and were given food and water.

Model of chronic kidney disease with salt sensitive hypertension

Three-week-old male Sprague-Dawley rats (45 to 55 g) were purchased from Tokyo Laboratory Animals Science (Tokyo, Japan). All rats were subjected to right uninephrectomy. At the same time, left-side renal denervation as described below or sham operation was performed. The procedures were underwent under anesthesia with sodium pentobarbital (20 mg/kg body weight, intraperitoneally). Thereafter, the rats were fed a normal-salt diet (0.3% NaCl) or high-salt diet (8% NaCl) for 6 weeks. The rats were randomly divided into five groups, as follows: a normal-salt diet group (NS); NS denervated group (NS-RDx); a high-salt diet group (HS); HS denervated group (HS-RDx); HS plus hydralazine (12.5 mg/kg/day in drinking water) treatment group (HS-Hyd). The NS group was used as control **(Figure 3)**.

During the experimental period, body weight was recorded every week. Systolic blood pressure (SBP) measured by the tail-cuff method in conscious rats (P-98A, Softron, Tokyo, Japan) in the NS (n=9), NS-RDx (n=8), HS (n=22), HS-RDx (n=13) and HS-Hyd

(n=17) group were recorded at 4 and 6 weeks. I measured SBP 5 times at each time point for each rat and calculated the average. Twenty-four-hour urine samples were collected using metabolic cages at 6 weeks in the NS (n=6), NS-RDx (n=8), HS (n=19), HS-RDx (n=12) and HS-Hyd (n=15) group [44]. Urinary protein levels, sodium and potassium concentrations were measured. Additionally, urinary norepinephrine (NE) was measured using an HPLC assay with electrochemical detection. At 6 weeks, invasive left ventricle (LV)-pressure measurements were performed and the animals were afterwards sacrificed.
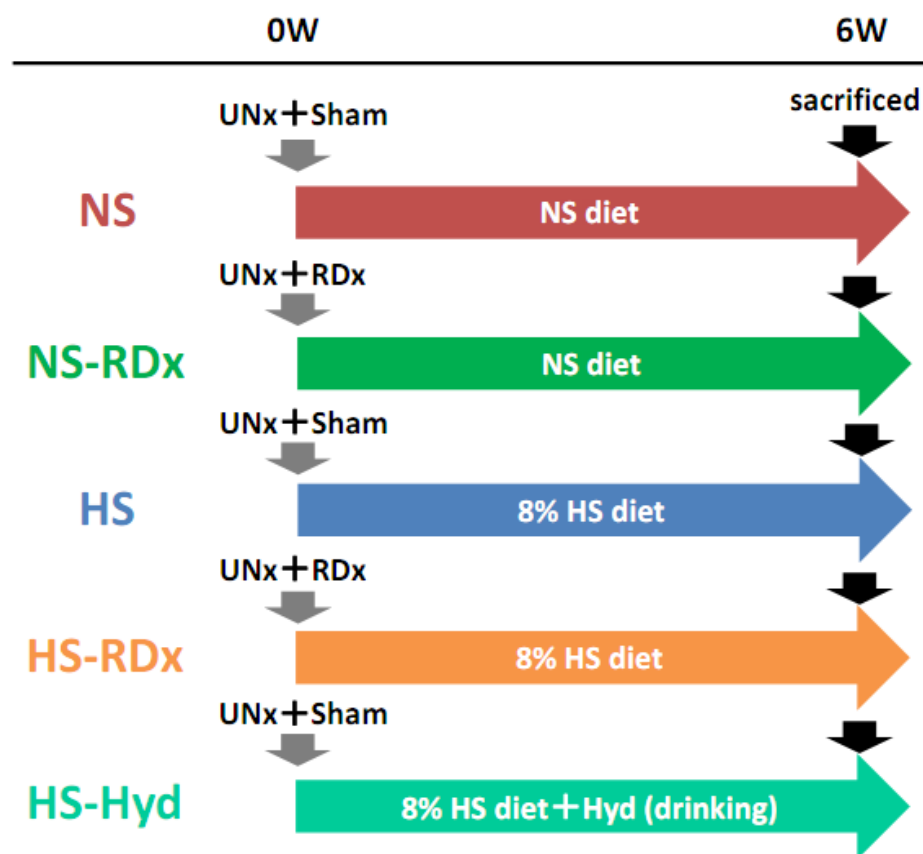
Figure 3. Experimental protocol.
HS; high salt, Hyd; hydralazine, NS; normal salt, RDx; renal denervation, UNx; uninephlectomy

Renal denervation

Renal denervation was performed as described previously [45]. The left renal sympathetic nerve was isolated through a retroperitoneal incision and total renal denervation 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 [45]. This method ablates the afferent and efferent renal nerves [45, 46]. The animals recovered from anesthesia after the end of surgery. In the sham operation, the renal nerves were isolated but preserved. After sacrificing the rats, renal tissue NE content was measured to confirm the total renal denervation [45, 46]. The tissues were stored at -80 °C until analyzed. After the tissues were homogenized in ice-cold 0.4 N perchloric acid, the homogenates were centrifuged at 4,000 g for 20 min. The supernatants were subjected for analysis of endogenous NE using an HPLC assay with electrochemical detection.

Cardiac and renal NE turn over

NE turnover is an in vivo measure of sympathetic nerve system (SNS) activity in sympathetically innervated organs of unanesthetized, unrestrained animals [47]. Cardiac and renal NE turnover was measured as described previously [44, 48]. After the blockade of NE synthesis, tissue NE contents decrease exponentially in accord with the release of NE in

response to incoming efferent nerve impulses. The rate of disappearance of NE thus reflects SNS activity in an individual tissue. For the blockade of NE synthesis, a-methyl-p-tyrosine methyl ester hydrochloride was given intraperitoneally in the three groups (NS, HS and HS-RDx). After the intra-peritoneal injection of 300 mg α-methyl-p-tyrosine methyl ester hydrochloride for blocked of NE synthesis, the NS (n=9), HS (n=11) and HS-RDx (n=7) group were killed at 6 hour. Their hearts and kidneys were removed for analysis of endogenous NE using an HPLC assay with electrochemical detection. The rate constant decline represents the fractional turnover rate of NE or percentage of the pool declining per 6 hours.

Data are plotted as means ± SE for endogenous NE in each group. The line representing the decline in endogenous NE with time was calculated by the method of least squares. The slope, or rate constant of decline, represents the fractional turnover rate of NE or the percentage of the pool declining per hour [47].

Pathological studies

LVs (4 hearts for the NS, HS and HS-RDx group) were fixed with 4% paraformaldehyde, embedded in paraffin, and subsequently cut into sections 3 μm in thickness (3 sections at the level of the papillary muscle for each rat). Azan staining was

performed for evaluation of peri-vascular and myocardial interstitial fibrosis.

Hemodynamic measurement

At 6 weeks, rats in the NS (n=9), NS-RDx (n=8), HS (n=22), HS-RDx (n=13) and

HS-Hyd (n=17) group were anesthetized with sodium pentobarbital (40 mg/kg body weight,

intraperitoneally). LV-pressures were assessed using a Millar Tip catheter (SPR-320NR, 2Fr,

Millar Instruments Inc., Houston, TX), which was introduced from the right carotid artery and

advanced into the LV-cavity. The catheter was connected to a polygraph system (AP-601G;

Nihon Kohden, Tokyo, Japan). The readings were monitored and saved on a computer using

the analog-to-digital PowerLab system (AD Instruments, Colorado Springs, CO). After

inserting catheters, animals were stabilized hemodynamically for 5 minutes. Thereafter, heart

rate (HR), LV-peak systolic pressure (LVPSP), mean arterial pressure (MBP, carotid artery),

maximal positive LV-pressure development ($+dP/dt_{max}$), LV end-diastolic pressure (LVEDP)

and the time constant at the isovolumic relaxation phase (Tau) were determined.

After completion of hemodynamic measurements, the animals were sacrificed. After

rapid excision, blood samples were obtained from vena cava. Thereafter, rats were submitted

to transvenous perfusion with 0.01 M PBS for fresh tissue collection. After careful removal of

atrial and non-cardiac tissue, LV-heart weight and left kidney weight were measured. Then

heart and kidney tissues were harvested snap-frozen for RNA and protein analysis.

Total and micro RNA extraction and quantitative real time reverse transcription-polymerase chain reaction (RT-PCR)

For total RNA, RNA was prepared from rat LV-tissues using an RNeasy fibrous kit (Qiagen, Venlo, Netherlands). 5 μg of total RNA was reverse-transcribed using Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, CA). For micro RNA, RNA was prepared from rat LV-tissues using a mirVana miRNA isolation kit (Invitrogen, Carlsbad, CA). 25 ng of micro RNA was reverse transcribed using MultiScribe Reverse Transcriptase (Invitrogen, Carlsbad, CA).

For real time PCR, we utilized TaqMan Gene Expression Assays with a 7300 Real Time PCR system (Invitrogen, Carlsbad, CA). The ID numbers for the assays are; Rn00667869_m1 for Actb, RN01499544_m1 for Atp2a2, RN00580641_m1 for Nppb, RN01488777_g1 for Myh7, RN01463848_m1 for Col1a1, RN01437681_m1 for Col3a1, RN00567876_m1 for Adra1a, RN00824536_s1 for Adrb1, RN00560650_s1 for Adrb2, RN00562500_m1 for Th, 000602 for hsa-miR-30b, 000419 for has-miR-30c, 000458 for has-miR-133a, 000494 for has-miR-195 and 001973 for U6 SnRNA. Denaturation took place at 95 °C for 15 seconds, and annealing and extension at 60 °C for 1min, for 40 cycles. To

compare gene expression levels, the comparative cycle threshold (Ct) method was used. Beta-actin and U6 were used as an endogenous control to correct for potential variation in RNA loading or inefficiency of amplification.

Western blot analysis

Heart tissue was homogenized on ice with T-PER Tissue Protein Extraction Reagents (Pierce Chemical Co., Rockford, IL) for total protein. The reagents contained complete protease inhibitors (Roche Diagnostics, Basel, Switzerland) and phosphatase inhibitors (Roche Diagnostics, Basel, Switzerland). Each process was performed according to the manufacturer's protocol and protein concentration of the supernatants was measured by using Bradford Protein Assay (Bio-Rad Laboratories, Hercules, CA). Solubilized proteins were mixed with sample buffer, boiled for 5min and the separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The separated proteins were transferred to a PVDF membrane at a constant voltage of 80 V for 120 min, and blocked with Tris-buffered saline solution with Tween-20 containing 4% skimmed milk for 60 min at room temperature. The blocked membranes were then incubated with the following primary antibodies during overnight at 4 °C; rabbit polyclonal anti-phospholamban Ser16 (A010-12, Badrilla, Leeds, UK, 1:5000), rabbit polyclonal anti-phosphorylated-phospholamban Thr17 (sc-17024-R, Santa Cruz

Biotechnology, CA, 1:400), mouse monoclonal anti-phospholamban (ab2865, Abcam, Cambridge, MA, 1:1000), anti-mouse monoclonal Serca2 (MA3-919, Pierce Biotechnology, Rockford, IL, 1:2000), anti-rabbit actin (A2066, Sigma-Aldrich, St. Louis, MO, 1:5000) antibody. Secondary antibodies anti-rabbit (7074P2, Cell Signaling Technology, Danvers, MA, 1:5000), anti-mouse (7076, Cell Signaling Technology, Danvers, MA, 1:5000) were incubated for 60 min at room temperature. Signals were visualized with the enhanced chemiluminescence detection system (GE Healthcare UK Ltd, Buckinghamshire, England) using the LAS 3000 imaging (FUJIFILM, Tokyo, Japan). The protein levels of interest were normalized to rabbit actin. In some experiments, densitometry was performed using NIH Image 1.63 software to evaluate the intensity of the signals. For reuse, a membrane was washed with a stripping buffer at 50 °C for 15 min, and was washed 3 times with 0.1%TBS-Tween 20 buffer.

Statistics

Data are presented as mean ± SEM. Comparison among groups was performed by one-way ANOVA followed by the Tukey-Kramer *post hoc* test. The data were analyzed with a mixed model performed with JMP (SAS Institute, Cary, NC) computer software. A p-value of less than 0.05 was considered statistically significant.

Results

Effect of salt loading and uninephrectomy on blood pressure, renal function, cardiac structure in young Sprague-Dawley rats

SBP determined by tail cuff in conscious rats at both 4 weeks and 6 weeks were significantly elevated in the HS group compared with the NS group **(Figure 4-(A), (B))**. Urinary protein levels were significantly higher in the HS group compared with the NS group **(Figure 5-(A))**, indicating that the HS group had CKD**.** These results are compatible with a previous study [41, 42].

Blood urea and serum creatinine concentration were similar between the NS and the HS group **(Table 3)**. Reflecting high salt diet, urine volume and sodium excretion were significantly higher in the HS group compared with the NS group **(Table 4)**. However, urinary potassium excretion was similar between the NS and the HS group **(Table 4)**.

LV weight to body weight (HW/BW) was significantly increased in the HS group compared with the NS group **(Figure 4-(C))**.

(A)

SBP (4w, tail cuff)

(B)

SBP (6w, tail cuff)
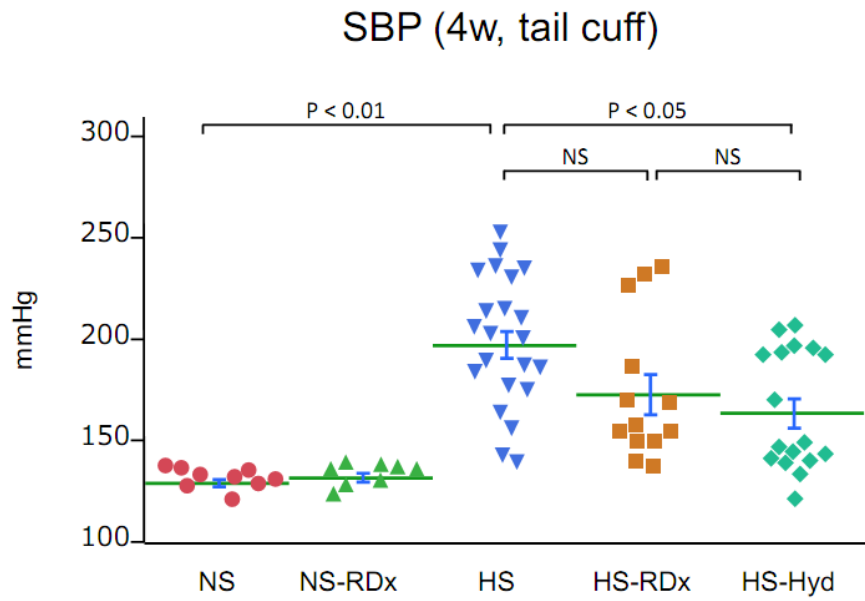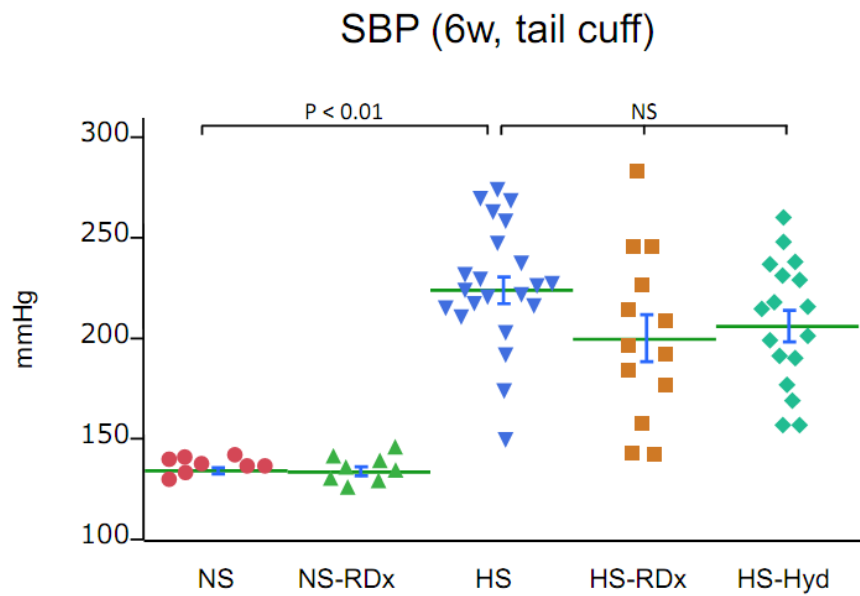
Figure 4. Physiological parameters.

Systolic blood pressure (SBP) at (A) 4 weeks and (B) 6 weeks measured by tail-cuff in NS (n = 9), NS-RDx (n = 8), HS (n = 22), HS-RDx (n = 13) and HS-Hyd (n = 17). Data are presented in means ± SEM.
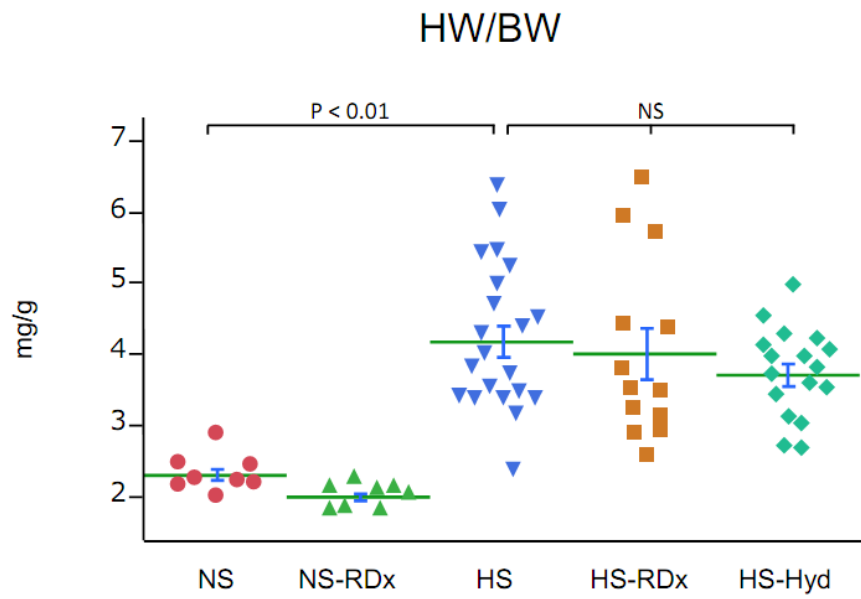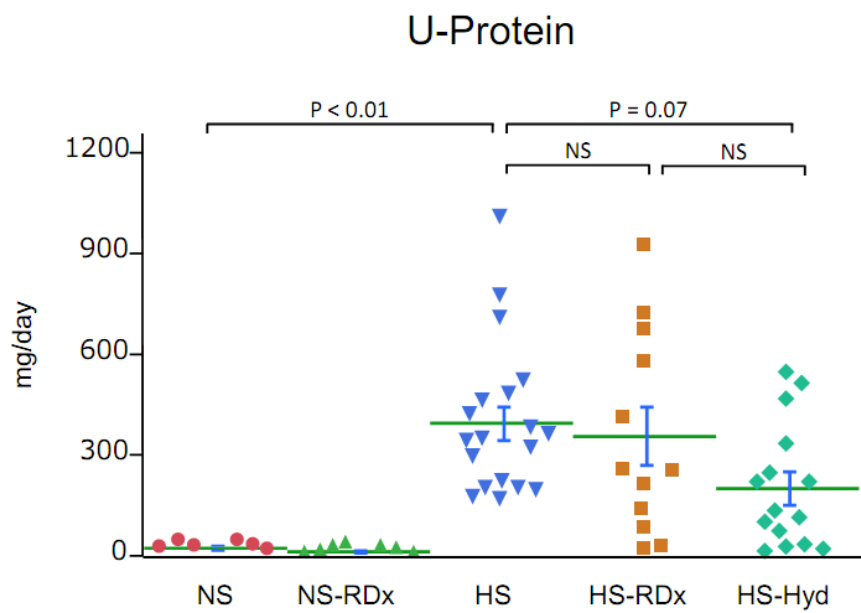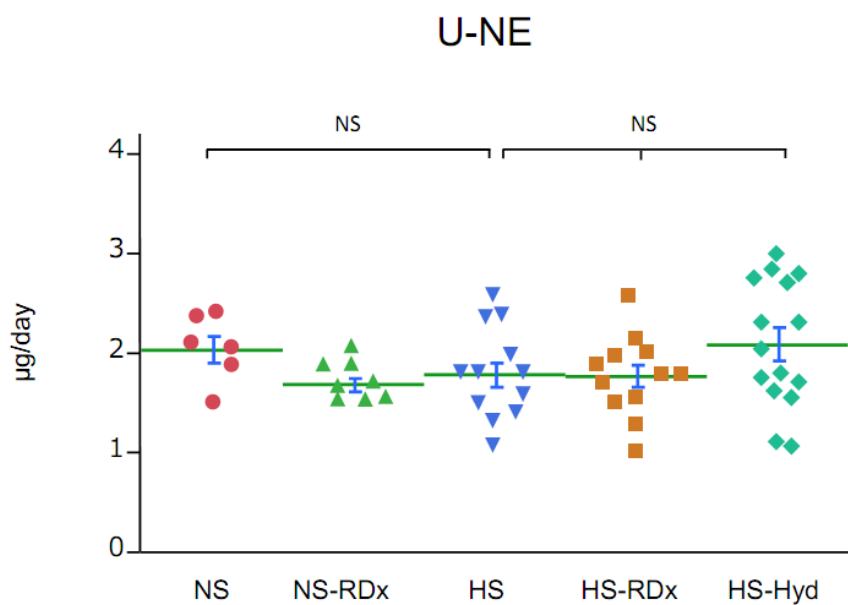
(C)

Figure 4. Physiological parameters

(C) heart weight to body weight (HW/BW) in NS (n = 9), NS-RDx (n = 8), HS (n = 22), HS-RDx (n = 13) and HS-Hyd (n = 17). Data are presented in means ± SEM.

(A)

(B)

Figure 5. Urine parameters.

(A) Urinary protein (U-protein) and (B) urinary norepinephrine (U-NE) in NS (n = 6),
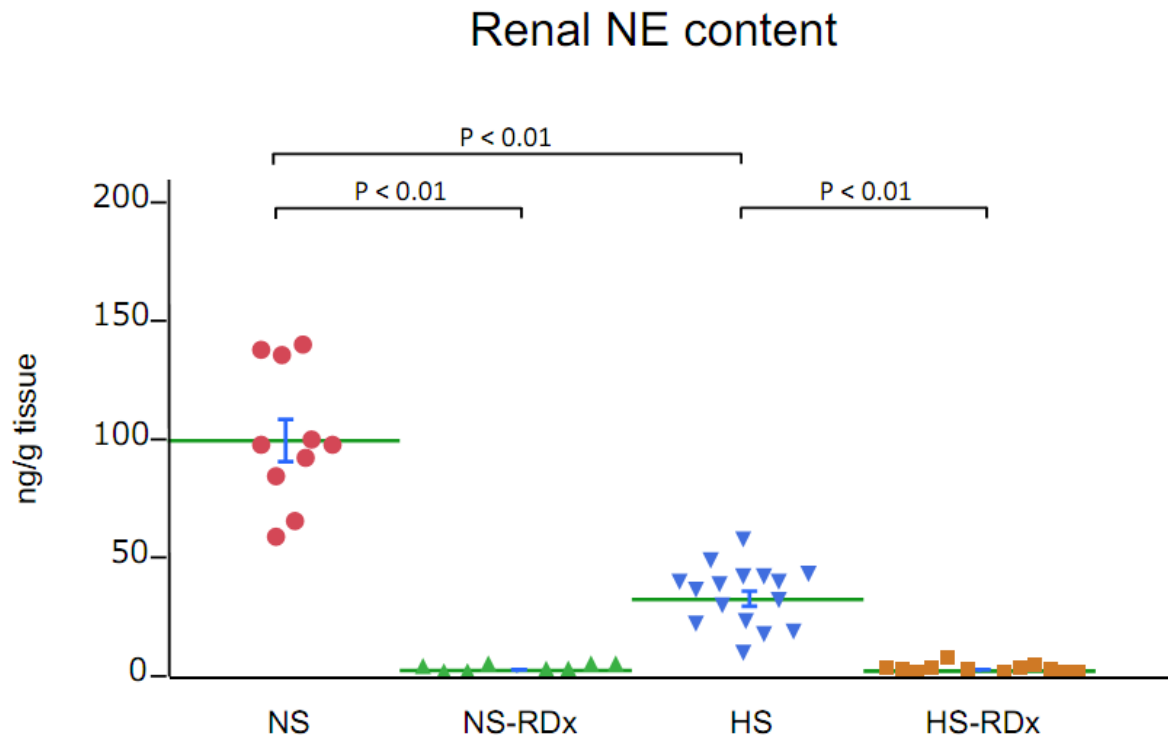NS-RDx (n = 8), HS (n = 19), HS-RDx (n = 12) and HS-Hyd (n = 15). Data are presented in
means ± SEM.

Figure 6. Renal norepinephrine content after renal denervation.

Renal tissue norepinephrine (NE) content was measured in NS (n = 10), NS-RDx (n = 8), HS
(n = 16) and HS-RDx (n = 13). Data are presented in means ± SEM.

Renal denervation was successful in this experiment

The renal tissue NE content in the NS-RDx and HS-RDx group was far smaller than

that of the NS and HS group, which was almost undetectable (< 5 ng/g tissue), indicating that

renal denervation was complete (**Figure 6**). Urinary NE which is an indicator for systemic

sympathetic tone was similar between the groups (**Figure 5-(B)**).

Table 3. Biological and hemodynamic parameters.

	NS (N = 9)	NS-RDx (N = 8)	HS (N = 22)	HS-RDx (N = 13)	HS-Hyd (N = 17)
BW (g)	428.3±13.2	390.0±9.5	310.4±12.4**	296.1±16.0	324.4±12.0
HW (mg)	992.2±40.6	782.4±16.8	1269±39.8**	1182±69.8	1217±47.0
HW/BW (mg/g)	2.32±0.08	2.01±0.06	4.19±0.22**	4.03±0.35	3.73±0.15
KW (mg)	2474±117.3	2048±78.0	3640±165.6**	3300±228.2	3438±195.9
KW/BW (mg/g)	5.79±0.25	5.25±0.15	12.2±0.94**	11.1±0.89	10.5±0.70
SBP (4wks)	129.4±1.69	132.2±1.93	197.5±6.89**	173.2±9.73	164.0±7.09$^{\#}$
SBP (6wks)	134.8±1.35	134.1±2.34	224.7±6.57**	200.2±11.7	206.5±7.61
BUN (mg/dl)	31.5±2.08	24.9±1.28	35.5±4.49	33.0±5.84	40.3±9.52
Cr (mg/dl)	0.22±0.02	0.28±0.01	0.46±0.06	0.33±0.07	0.54±0.06

Data are presented in means ± SEM. BW; body weight, HW; heart weight, KW; right kidney weight, SBP; systolic blood pressure, wks; weeks of age, BUN; serum blood urea nitrogen, Cr; serum creatinine. *P<0.05, **P<0.01 vs. NS. $^{\#}$P<0.05 vs. HS.

Table 4. Urine parameters.

	NS (N = 6)	NS-RDx (N = 8)	HS (N = 19)	HS-RDx (N = 12)	HS-Hyd (N = 15)
UV (ml/day)	25.4±2.63	24.5±2.16	132.2±6.98[**]	122.6±8.30	112.4±6.78
protein (mg/day)	39.4±28.1	15.3±2.66	397.9±51.7[**]	358.9±87.2	203.8±47.5
NE (µg/day)	2.04±0.14	1.70±0.07	1.80±0.12	1.78±0.12	2.09±0.16
Na (mEq/day)	20.7±3.09	19.3±0.84	261.1±18.7[**]	287.1±19.2	290.4±20.4
K (mEq/day)	49.9±5.46	42.0±2.66	41.8±3.19	48.9±3.39	42.9±2.62
Na/K	0.40±0.03	0.47±0.03	6.25±0.13[**]	6.44±0.17	6.68±0.19

Data are presented in means ± SEM. UV; urine volumebody weight, NE; norepinephrine, Na; sodium, K; pottasium. [*]P<0.05, [**]P<0.01 vs. NS.

Pathological studies

In the pathological studies, only mild perivascular fibrosis was observed, but myocardial interstitial fibrosis could not be detected in any group, suggesting cardiac hypertrophy developed in the HS group in the absence of severe fibrosis (**Figure 7**).
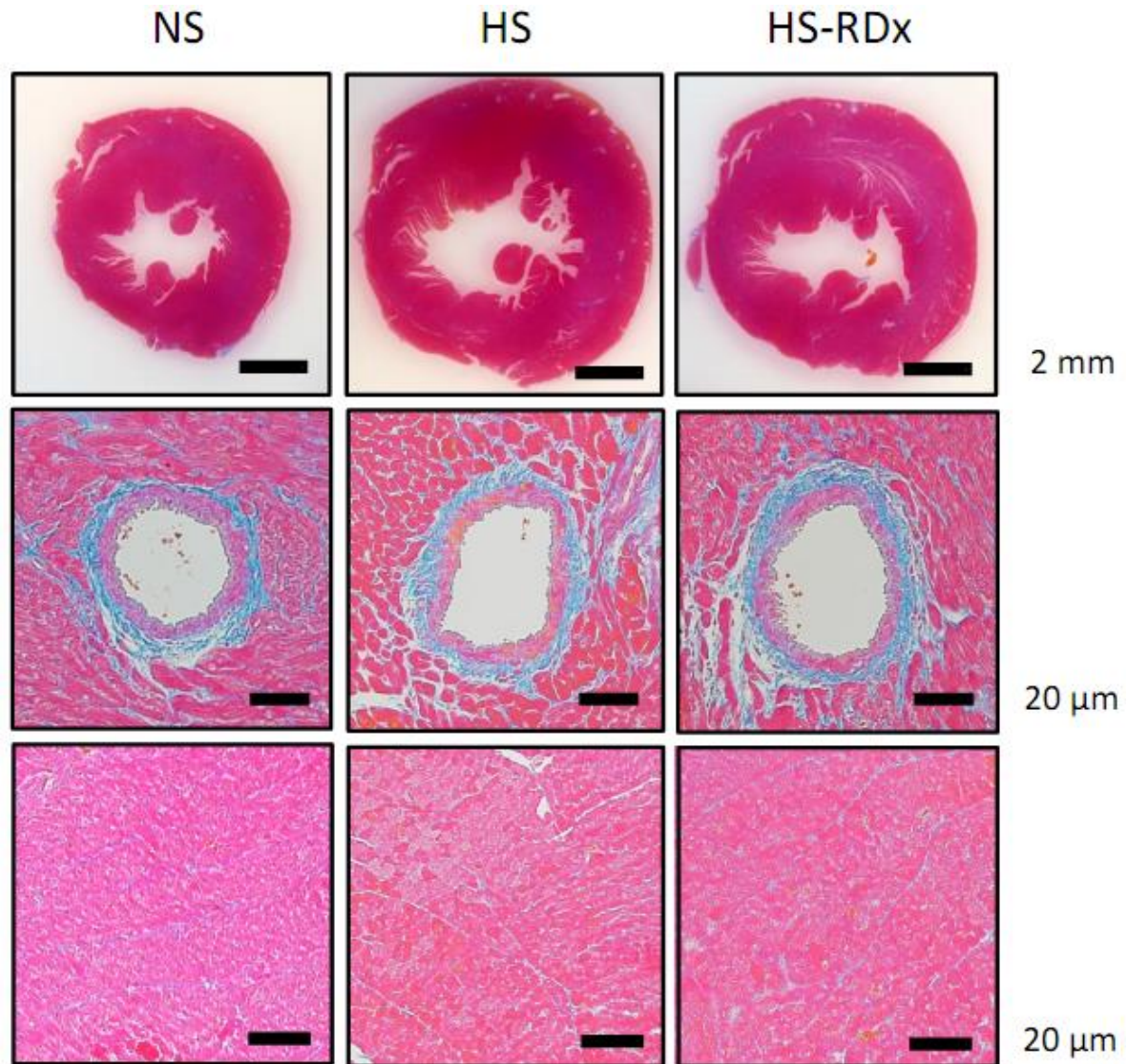

Figure 7. Representative histopathologic micrographs of left ventricle.
Azan staining was performed to evaluate cardiac fibrosis in NS (n = 4), HS (n = 4), HS-RDx (n = 4). Upper bars indicate 2mm, middle and lower bars indicate 20μm (upper panels; original magnification, ×1, middle and lower panels; original magnification, ×100).

Effect of renal denervation or hydralazine on blood pressure, renal function, cardiac structure in young salt-induced CKD rats

Renal denervation tended to lower blood pressure (BP) but did not reach significant effect on blood pressure at both 4 weeks and 6 weeks **(Figure 4-(A), (B))**. On the other hand, hydralazine administration significantly lowered BP at 4 weeks **(Figure 4-(A))**. Urinary protein levels tended to be reduced with the treatment by hydralazine possibly due to antihypertensive effect at the early phase, but not by renal denervation **(Figure 5-(A))**. Neither renal denervation nor hydralazine administration altered urinary sodium and potassium excretion **(Table 4)**. Reflecting the minor effect on blood pressure, the development of cardiac hypertrophy was affected by neither renal denervation nor hydralazine administration **(Figure 4-(C))**.

LV hemodynamics

To evaluate cardiac systolic and diastolic function, I performed cardiac catheterization on anesthetized rats after 6 weeks of high salt loading. These studies demonstrated that BP, measured by determining LVPSP and MBP, were significantly higher in the HS group compared with the NS group **(Figure 8-(C), Table 5)**. There was no difference in HR between the NS and the HS groups **(Table 5)**.

LV systolic function, measured by determining $+\mathrm{dp/dt_{max}}$ was increased in the HS group compared with the NS and HS-Hyd group **(Figure 8-(A))**, indicating that LV systolic function was preserved in these CKD rats and the treatment of hydralazine normalized systolic function. However, LV diastolic function, measured as Tau, was significantly prolonged in the HS group compared with the NS group **(Figure 7-(B))**, indicating that the HS group had cardiac diastolic dysfunction. There was no difference in the LV end-diastolic

pressure (LVEDP) between the groups **(Table 5)**.
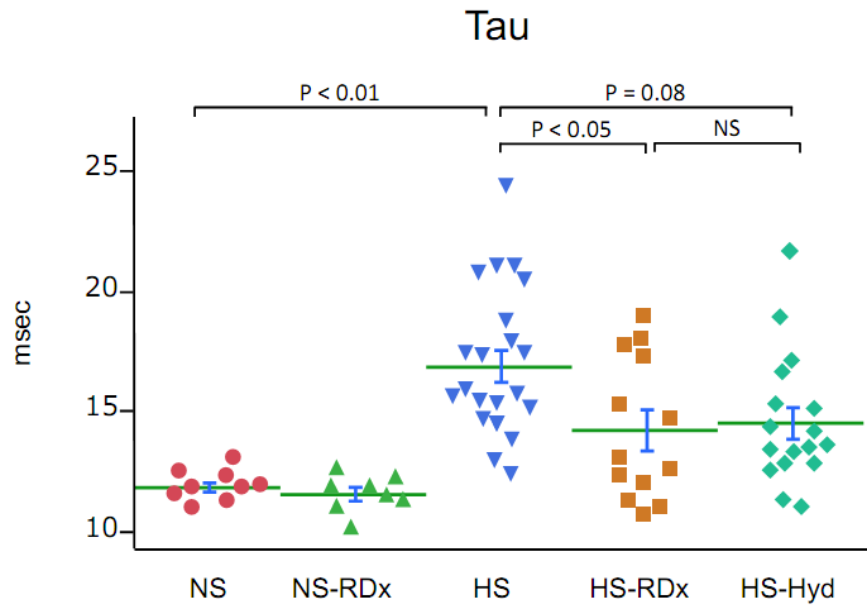
(A)
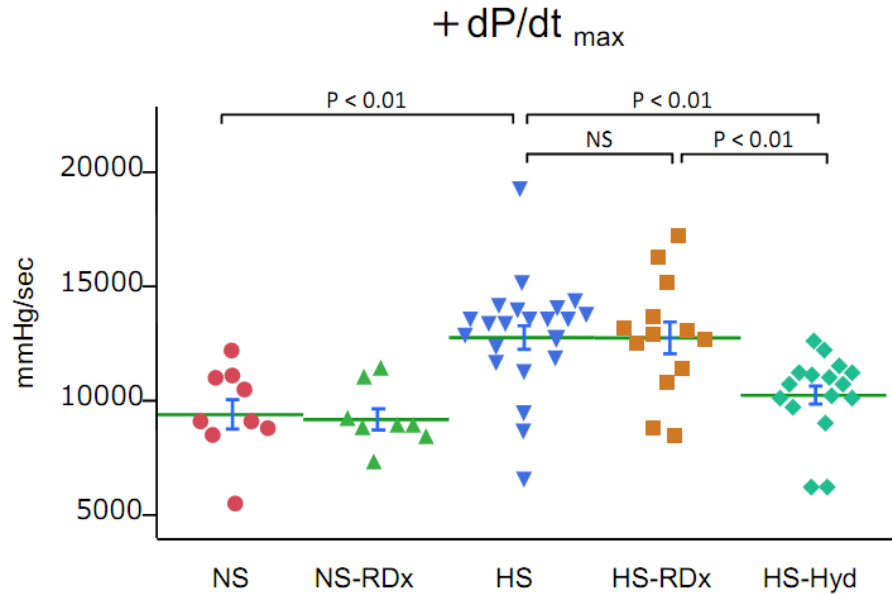
(B)

Figure 8. Cardiac function determined by invasive left ventricle measurement procedures.
(A) maximal positive LV-pressure development ($+dP/dt_{max}$) and (B) time constant of LV-pressure decay (Tau) in NS (n = 9), NS-RDx (n = 8), HS (n = 22), HS-RDx (n = 13) and HS-Hyd (n = 17). Data are presented in means ± SEM.
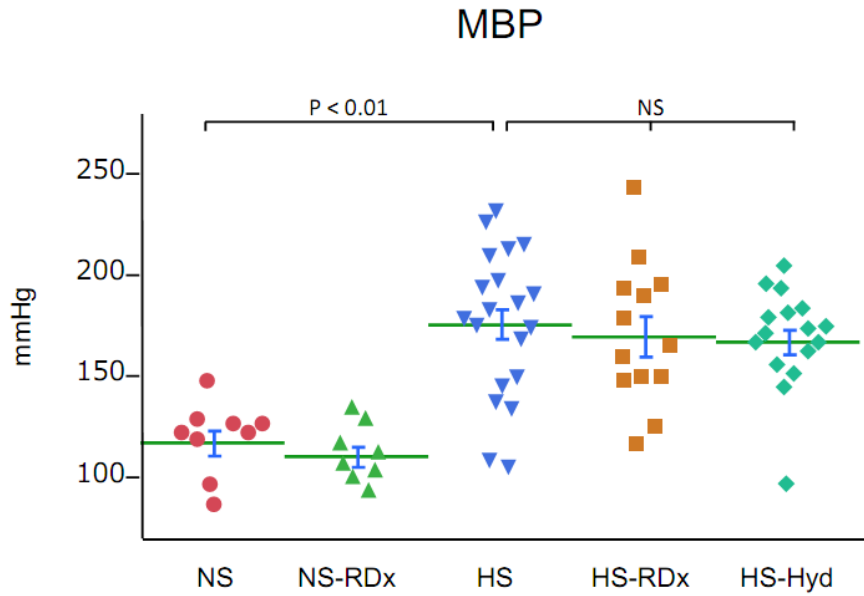
(C)

Figure 8. Cardiac function determined by invasive left ventricle measurement procedures.
(C) mean blood pressure (MBP) in NS (n = 9), NS-RDx (n = 8), HS (n = 22), HS-RDx (n = 13) and HS-Hyd (n = 17). Data are presented in means ± SEM.

This abnormal LV relaxation may be regarded as an early functional change without an increase in LV chamber stiffness, because LV systolic function was preserved, LVEDP was not elevated, and fibrotic change appeared only in the perivascular region as shown above.

Interestingly, renal denervation significantly improved Tau, independent from the BP reduction as well as renal function, suggesting that renal denervation has the direct cardioprotective effect on cardiac diastolic dysfunction.

On the other hand, although systolic and mean BP with the treatment by hydralazine was nearly identical to the untreated HS group, LV diastolic dysfuntion was marginally improved by hydralazine administration **(Figure 8-(B))**. This result may indicate that the BP reduction at the early phase and resultant smaller renal damage would partially prevent LV diastolic dysfunction in the future. HR was altered by neither renal denervation nor the hydralazine administration.

Table 5. Cardiac function determined by invasive LV measurement procedures.

	NS (N = 9)	NS-RDx (N = 8)	HS (N = 22)	HS-RDx (N = 13)	HS-Hyd (N = 17)
HR (bpm)	393.7±6.89	410.5±12.1	397.7±5.59	393.2±9.06	390.2±8.00
MBP (mmHg)	117.7±6.05	110.8±5.00	176.0±7.49[**]	170.0±9.75	167.6±6.31
LVPSP (mmHg)	137.1±5.90	126.4±5.65	209.3±7.49[**]	208.6±12.6	191.5±7.56
+dP/dt max (mmHg/s)	9438±650.5	9233±481.1	12806±530.1[**]	12798±711.4	10289±430.7[##, †]
LVEDP (mmHg)	6.24±0.41	4.46±0.61	6.72±0.33	7.27±1.10	5.97±0.61
Tau (ms)	11.9±0.21	11.6±0.27	16.9±0.66[**]	14.3±0.82[#]	14.6±0.67

Data are presented in means ± SEM. LV; left ventricle, HR; heart rate, MBP; mean blood pressure, LVPSP; LV paek systolic pressure, +dP/dt$_{max}$; maximal positive LV-pressure development, LVEDP; left ventricle end-diastolic pressure, Tau; time constant of LV-pressure decay. [*]$P<0.05$, [**]$P<0.01$ vs. NS. [#]$P<0.05$, [##]$P<0.01$ vs. HS. [†]$P<0.05$ vs. HS-RDx.

Renal denervation recovered cardiac diastolic dysfunction possibly through specific restoration of SERCA2a mRNA and protein expression

Sarcoplasmic reticulum (SR) Ca^{2+}-ATPase type 2a (SERCA2a) plays an essential role in Ca^{2+} homeostasis and regulates cardiac functions. A reduction in the expression of SERCA2a was widely documented in diastolic heart failure [49-52].

As reported previously, the protein abundance of SERCA2a was significantly reduced in the HS group compared with the NS group **(Figure 9-(A), (B))**. Interestingly, renal denervation significantly restored the SERCA2a protein abundance but hydralazine administration did not **(Figure 9-(A), (B))**.

Total phospholamban (PLB) protein was similar in all groups **(Figure 9-(C), (D))**. PLB phosphorylation at serine 16 (Ser16-PLB) and at threonine 17 (Thr17-PLB) functionally enhances SERCA2a activity and Ca^{2+} uptake in the SR. Ser16-PLB to total PLB ratios and Thr17-PLB to total PLB ratios were comparable among the groups **(Figure 9-(C), (D))**.
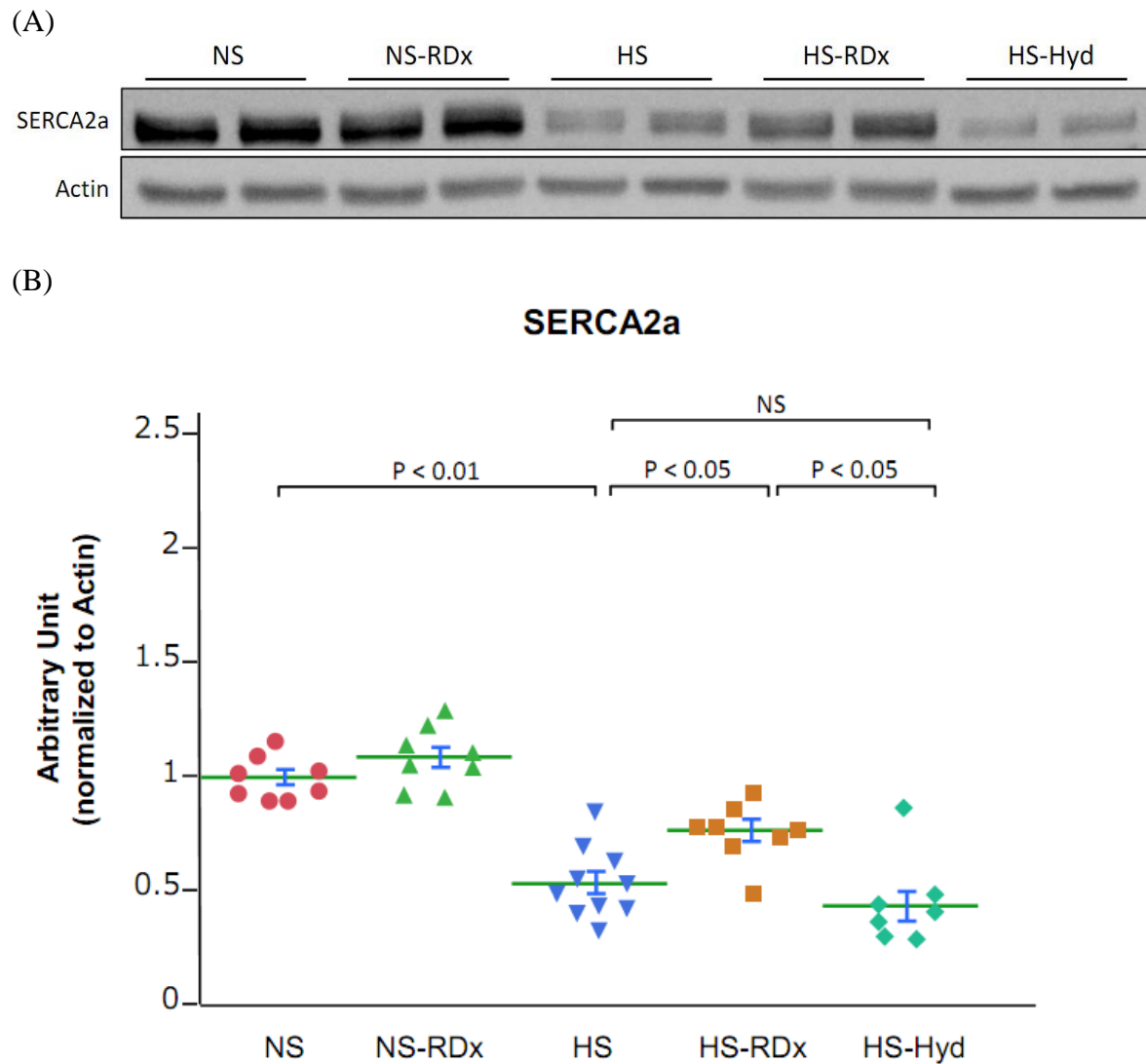
Figure 9. SERCA2a and Phospholamban protein expression of left ventricle.
(A) Representative western blots and (B) quantification of sarcoplasmic reticulum
Ca^{2+}-ATPase type 2a (SERCA2a) in NS, NS-RDx, HS, HS-RDx and HS-Hyd (n = 7-10).
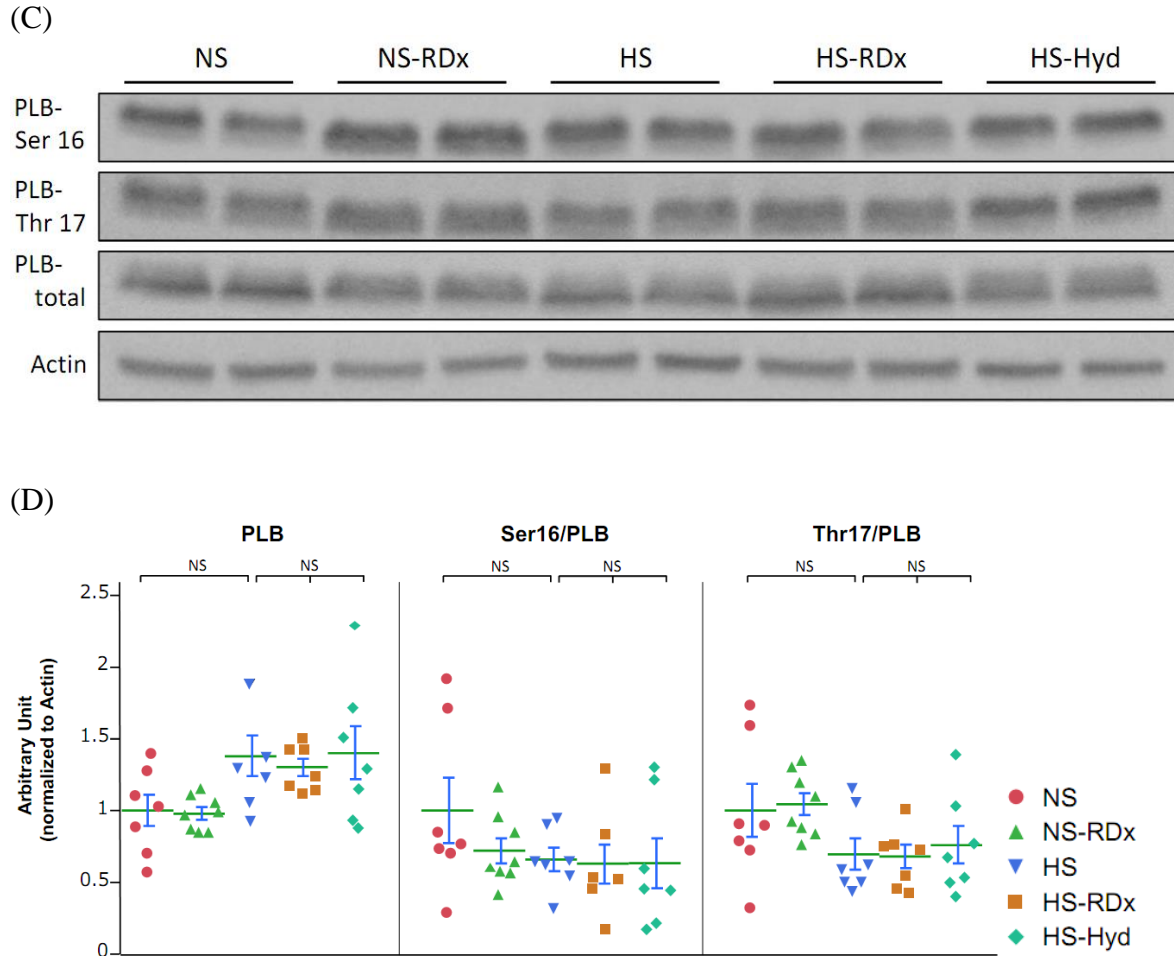Actin was used as loading control. Data are presented in means ± SEM.

Figure 9. SERCA2a and Phospholamban protein expression of left ventricle
(C) Representative western blots and (D) quantification of total phospholamban (PLB) and its phosphorylation at Serine 16 and Threonine 17 in NS, NS-RDx, HS, HS-RDx and HS-Hyd (n = 6-8). Actin was used as loading control. Data are presented in means ± SEM.
PLB-Ser16; PLB phosphorylation at serine 16, PLB-Thr17; PLB phosphorylation at threonine 17, Ser16/PLB; PLB phosphorylation at serine 16 to total PLB ratios, Thr17/PLB; PLB phosphorylation at threonine 17 to total PLB ratios.

Thereafter, in order to assess whether SERCA2a protein expression was regulated by the gene transcription level, I performed quantitative real time reverse transcription-polymerase chain reaction (RT-PCR). The SERCA2a mRNA expression was significantly reduced in the HS group compared with the NS group **(Figure 10-(A))**. Moreover, renal denervation significantly restored the SERCA2a mRNA expression, but hydralazine administration did not **(Figure 10-(A))**. These mRNA expression results were almost parallel to the results of protein expression.

These results indicate that renal denervation and hydralazine have different mechanism on restoring cardiac diastolic function in these CKD rats, and that the transcription of SERCA2a mRNA and protein expression was specifically restored by renal denervation.

Cardiac structural-remodeling related gene expressions

To evaluate whether the LVH observed in the HS group was associated with changes in cardiac gene expression, I performed quantitative real time RT-PCR. The levels of brain natriuretic peptide (BNP) and β-myosin heavy chain (β-MHC) mRNA expression were significantly increased in the HS group compared with the NS group **(Figure 10-(B), (C))**. However, the levels of both BNP and β-MHC were affected by neither renal denervation nor hydralazine administration **(Figure 10-(B), (C))**.
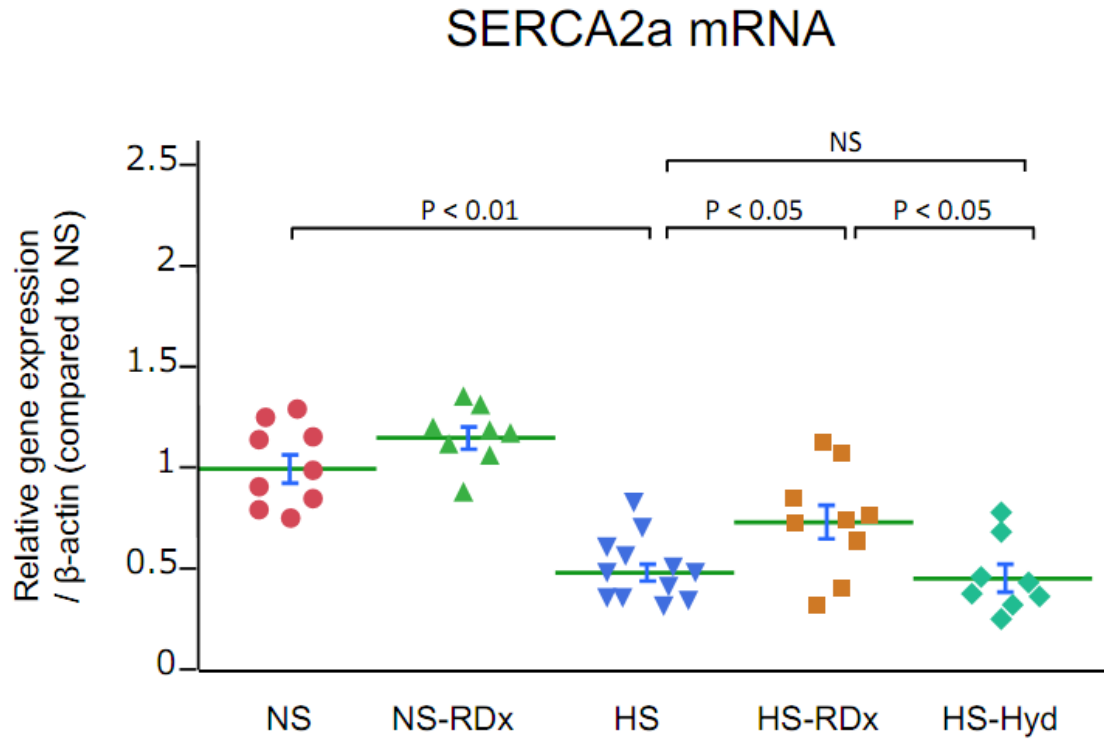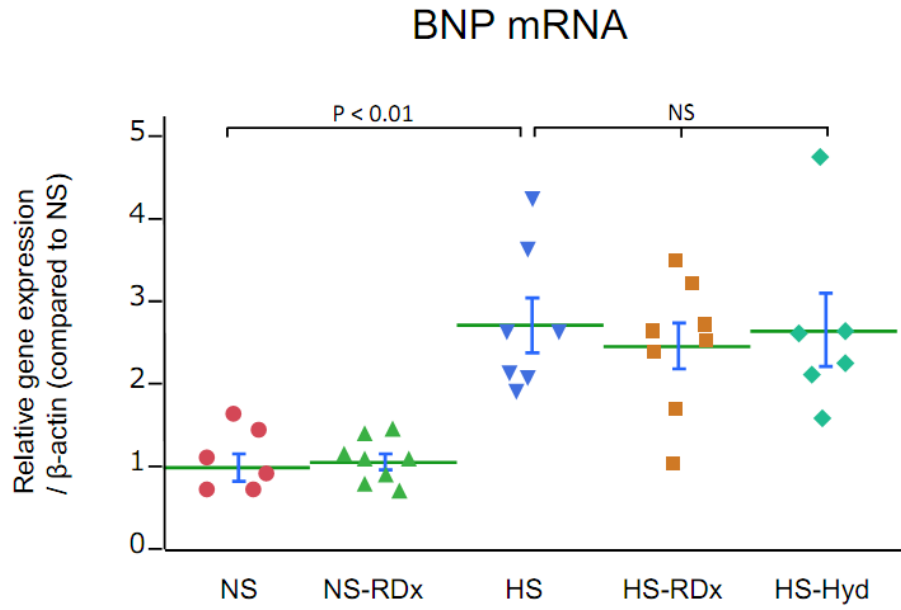
(A)

Figure 10. SERCA2a and related gene expression of left ventricle.
Left ventricular gene expression of (A) sarcoplasmic reticulum Ca^{2+}-ATPase type 2a (SERCA2a) in NS, NS-RDx, HS, HS-RDx and HS-Hyd normalized to β-actin expression. (n = 8 to 12 from each group). Data are presented in means ± SEM.
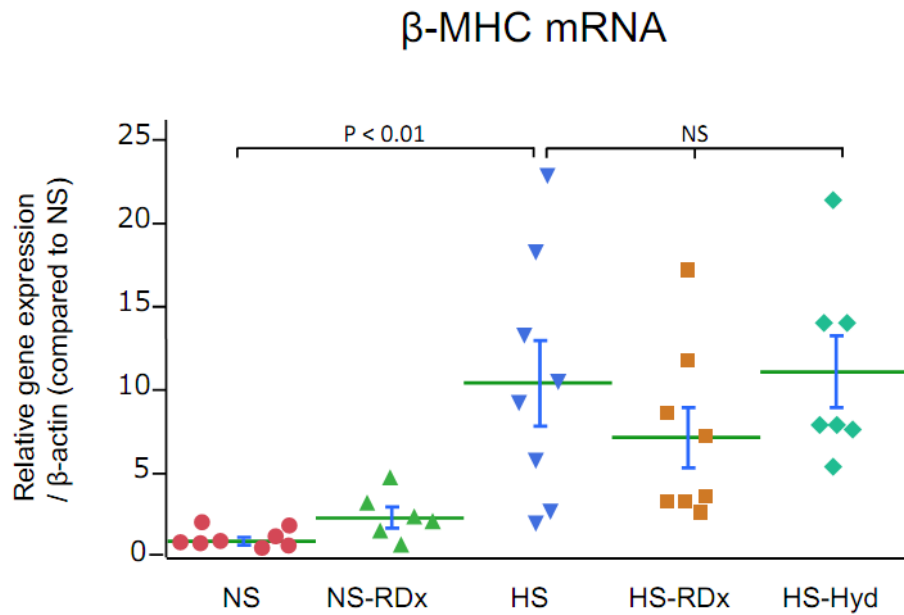
(B)

BNP mRNA

(C)

β-MHC mRNA

Figure 10. SERCA2a and related gene expression of left ventricle.
Left ventricular gene expression of (B) brain natriuretic peptide (BNP) and (C) β-myosin heavy chain (β-MHC) in NS, NS-RDx, HS, HS-RDx and HS-Hyd normalized to β-actin expression. (n = 6 to 10 from each group). Data are presented in means ± SEM.

Additionally, the mRNA levels of Col-1a and Col-3a were identical in both the NS and HS group **(Figure 10-(D), (E))**. These results were consistent with the result of HW/BW as well as pathological studies as shown above.

Cardiac and renal tissue NE content

Cardiac tissue NE content was significantly reduced by high salt loading **(Figure 11)**. It is reported that cardiac NE content is reduced in heart failure with systolic dysfunction models [53-56]. Our results indicate that cardiac NE content is also reduced in diastolic dysfunction. Salt loading also suppress the NE content in the kidney **(Figure 12)**. In contrast neither in the heart nor kidney, NE turnover was affected by salt loading.

Cardiac sympathetic activity was not altered by renal denervation in salt induced CKD rats

In this study, I assessed the mRNA expression of α1a, β1, β2 adrenergic receptors (AR), and tyrosine hydroxylase, which is the late-limiting enzyme in synthesis of catecholamines. The mRNA expressions of these three ARs were not altered by salt loading. The mRNA expression of α1a and β2 ARs were significantly upregulated by renal denervation on normal salt but not on high salt, while the mRNA expression of β1 AR was altered neither on high salt nor on normal salt. **(Figure 13-(A)-(D))**.

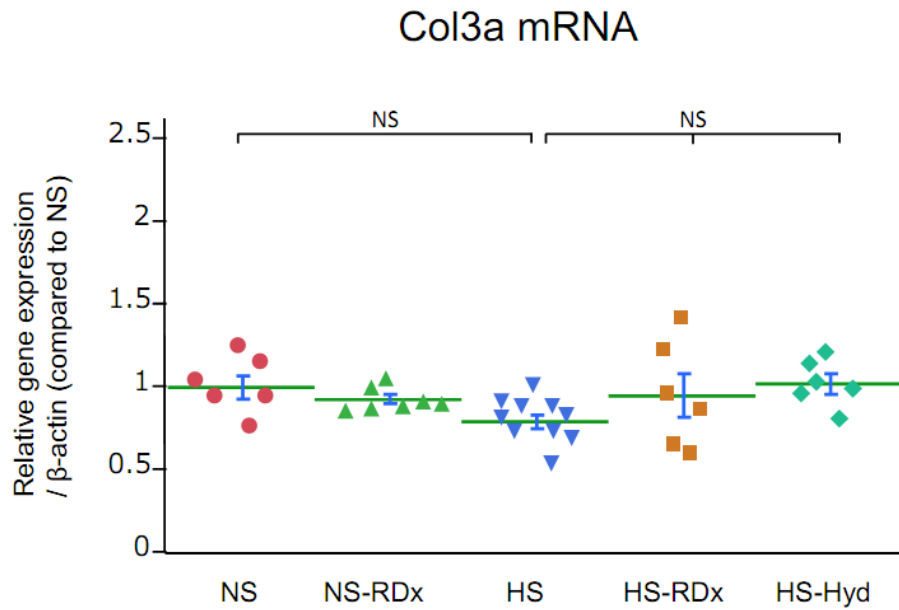(D)
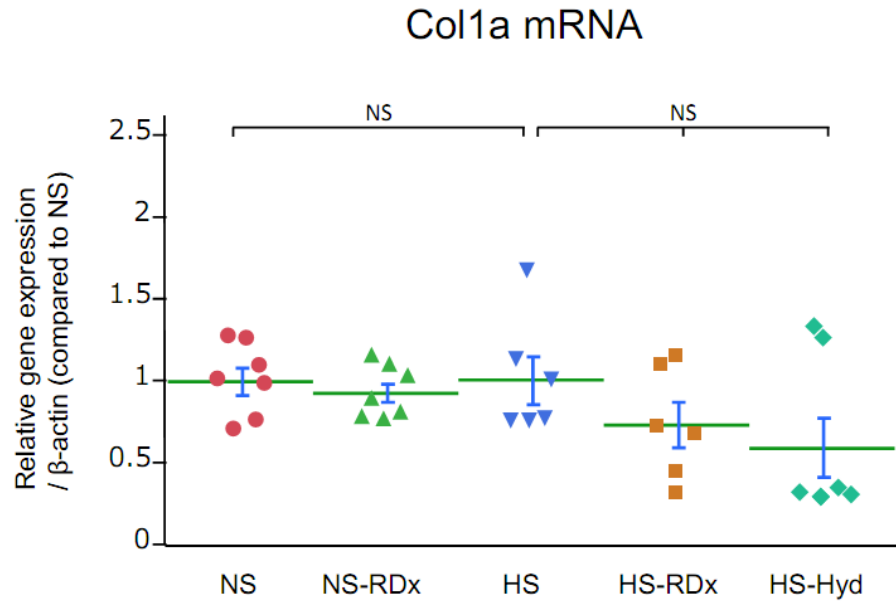
Col1a mRNA

(E)

Col3a mRNA

Figure 10. SERCA2a and related gene expression of left ventricle.
Left ventricular gene expression of (D) collagen 1a (Col1a) and (D) collagen 3a (Col3a) in
NS, NS-RDx, HS, HS-RDx and HS-Hyd normalized to β-actin expression. (n = 6 to 10 from
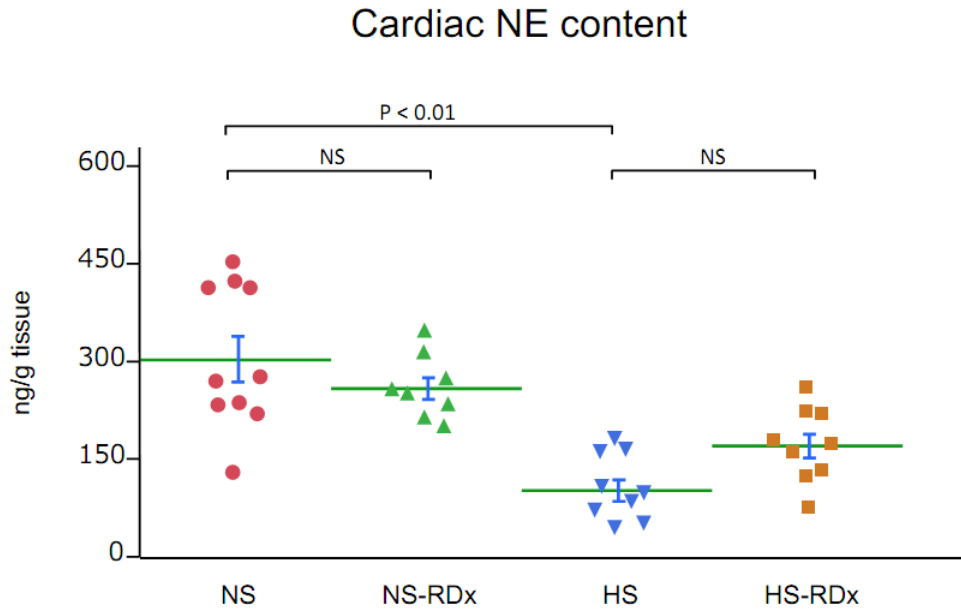each group). Data are presented in means ± SEM.

Figure 11. Cardiac norepinephrine content after renal denervation.
Cardiac tissue norepinephrine (NE) content was measured in NS (n = 10), NS-RDx (n = 8), HS (n = 9) and HS-RDx (n = 9). Data are presented in means ± SEM.
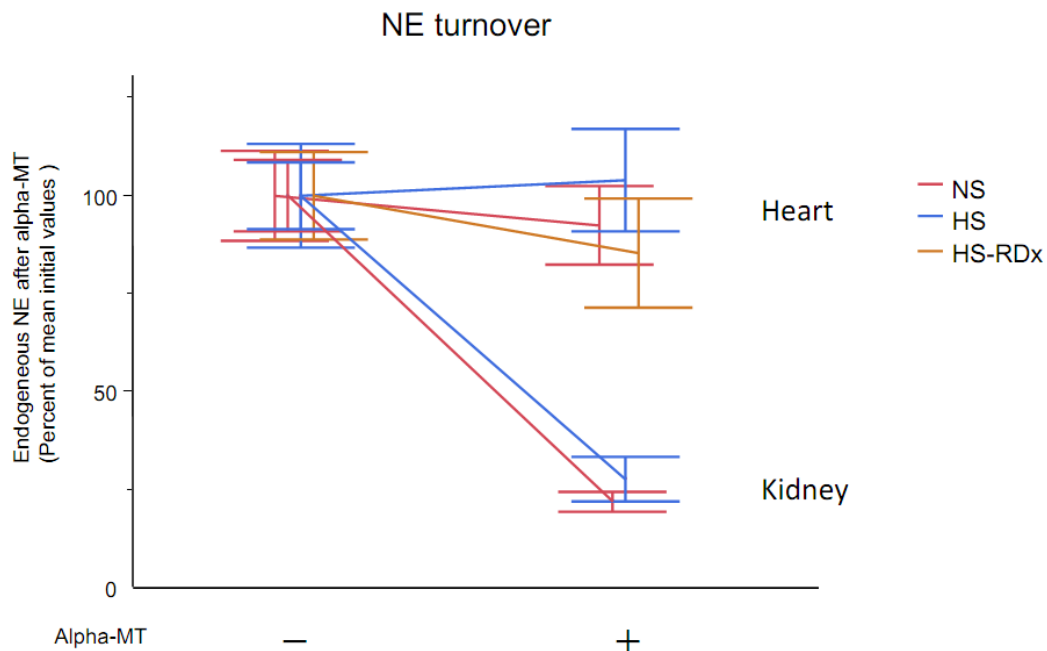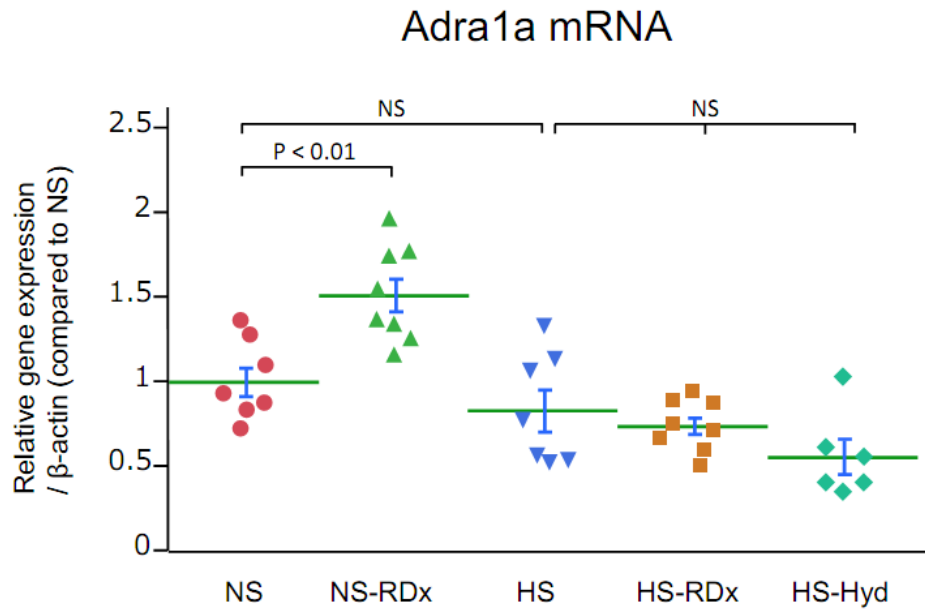

Figure 12. Renal and cardiac norepinephrine turnover rate.
After the intra-peritoneal injection of 300 mg α-methyl-p-tyrosine methyl ester hydrochloride (Alpha-MT) for blocking norepinephrine (NE) synthesis, the NS (n=9), HS (n=11) and HS-RDx (n=7) group were killed at 6 hour. Data are presented in means ± SEM.
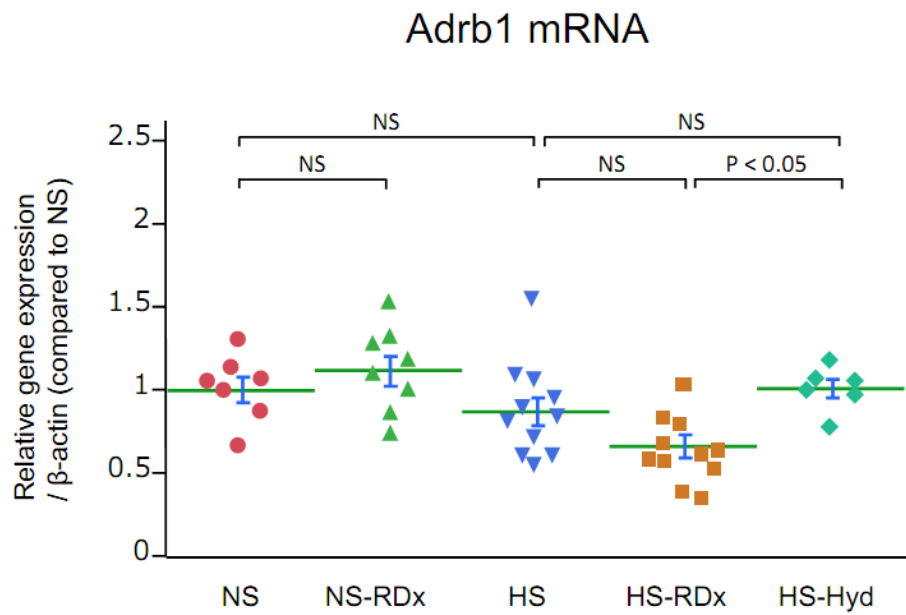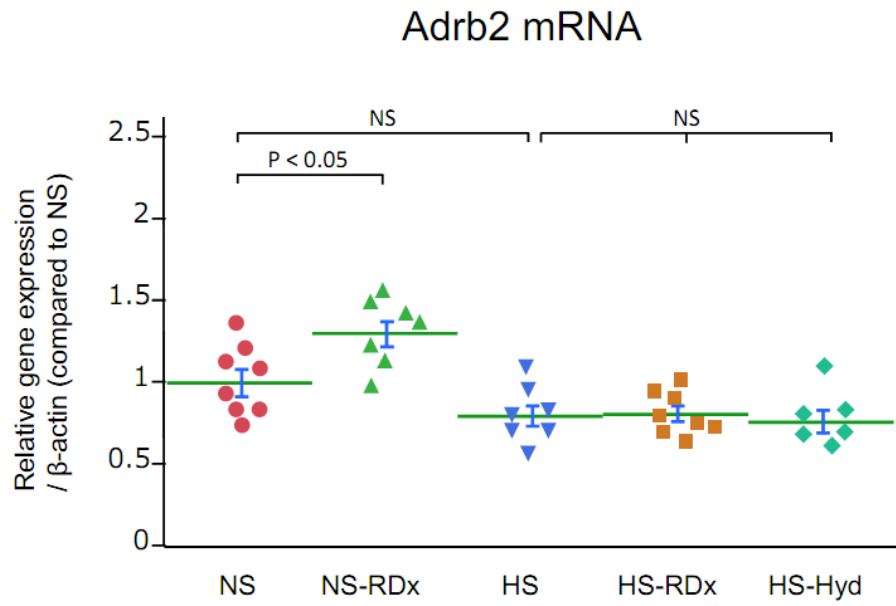
(A)

Adra1a mRNA

(B)

Adrb1 mRNA

Figure 13. Adrenergic receptors and related gene expression of left ventricle.
Left ventricular gene expression of (A) alpha-1a adrenergic receptor (Adra1a) and (B) beta-1 adrenergic receptor (Adrb1) in NS, NS-RDx, HS, HS-RDx and HS-Hyd normalized to β-actin expression. (n = 6 to 11 from each group). Data are presented in means ± SEM.
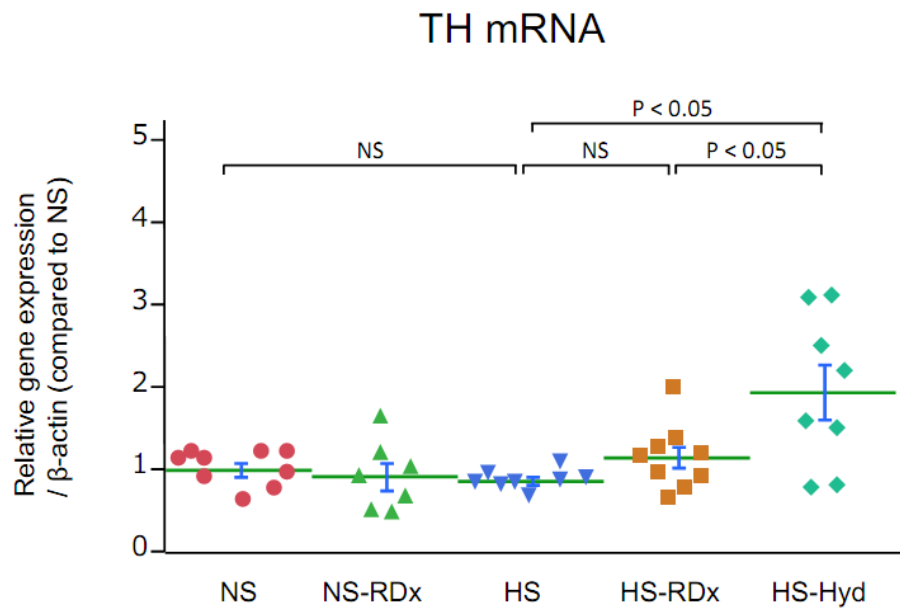
(C)

(D)

Figure 13. Adrenergic receptors and related gene expression of left ventricle.
Left ventricular gene expression of (C) beta-1 adrenergic receptor (Adrb2) and (B) tyrosine hydroxylase (TH) in NS, NS-RDx, HS, HS-RDx and HS-Hyd normalized to β-actin expression. (n = 6 to 9 from each group). Data are presented in means ± SEM

Micro RNA expression

Epigenetics refers to chromatin-based pathways important in the regulation of gene expression, including microRNA (miR)-based mechanisms [57, 58]. To determine whether miRs positively or negatively regulate the SERCA2a mRNA expression, I investigated the miR expression of LV from the NS, HS and HS-RDx group. MiRs target predictions were obtained using TargetScan, microRNA.org databases, and the miRNAMap database. I focused on miRs which are abundant in LV and can bind to complementary sequences on the 3' untranslated region (UTR) of SERCA2a mRNA. As a result, I picked up miR 30b, miR30c, miR133a and miR195. Only miR 30c expression was upregulated in the HS group compared with the NS group **(Figure 14)**. However, none of these miR expressions was altered by renal denervation **(Figure 14)**.

(A)
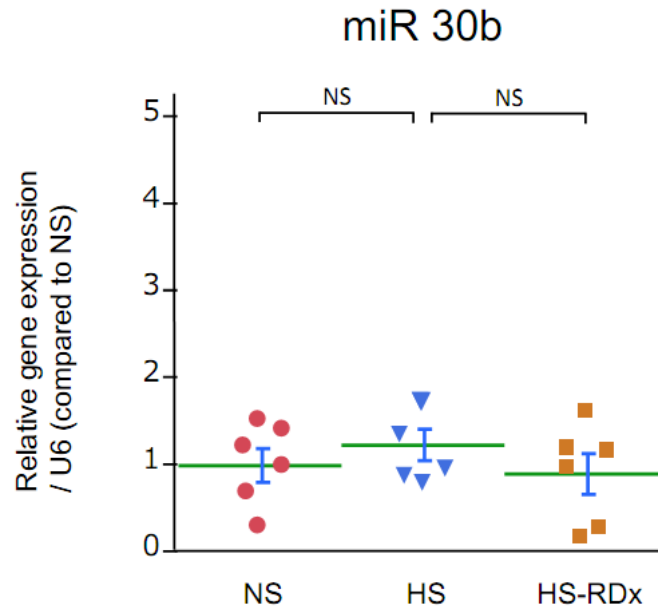
(B)

Figure 14. Micro RNA expression of left ventricle.
Left ventricular gene expression of (A) micro RNA (miR) 30b and (B) miR 30c in NS, HS and HS-RDx normalized to U6 expression. (n = 5 to 6 from each group). Data are presented in means ± SEM.
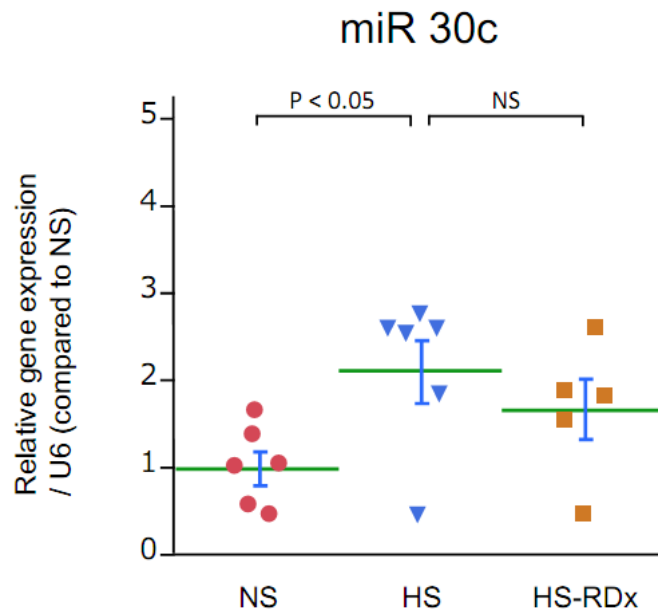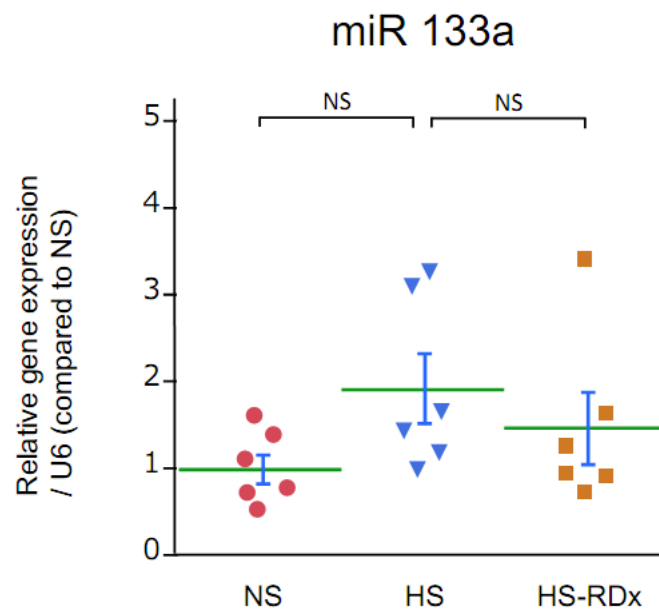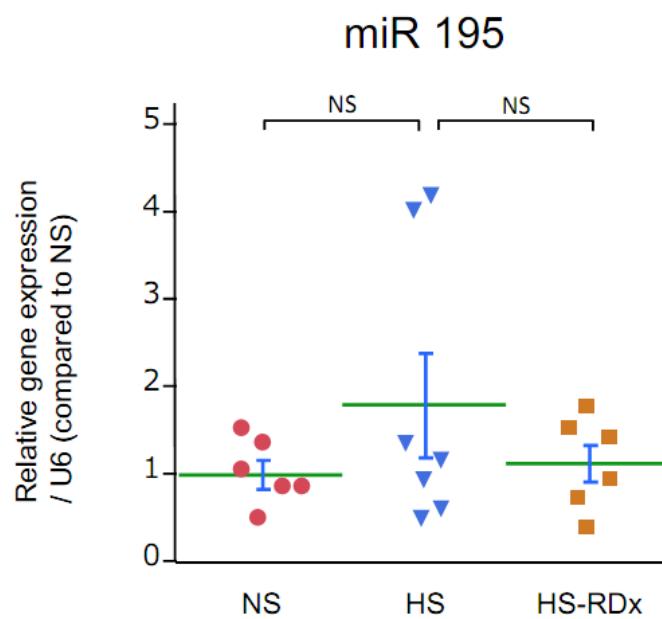
(C)

(D)

Figure 14. Micro RNA expression of left ventricle.
Left ventricular gene expression of (C) micro RNA (miR) 133a and (B) miR 195 in NS, HS and HS-RDx normalized to U6 expression. (n = 5 to 6 from each group). Data are presented in means ± SEM.

Discussion

In this study, I established a model of early stage diastolic dysfunction in salt induced CKD rats. Moreover, for the first time, I demonstrated that renal denervation improved cardiac diastolic dysfunction independent of BP reduction as well as renal function possibly through the specific restoration of SERCA2a gene and protein expression in LV.

The population prevalence of HF has increased, so has the proportion of patients with HF-PEF, accounting for more than 50% of the total HF population [59]. LV diastolic dysfunction and adverse cardiac remodeling are considered major underlying pathologies in HF-PEF [60]. However, there are many issues remain to be fully understood, and pharmacotherapies tested to date have not shown improvements in diastolic dysfunction, cardiac remodeling, or cardiovascular outcome [61-64].

CKD is a common and an important independent predictor of death and hospitalization in adults with HF across the spectrum of LV systolic function [9-11]. In addition, CKD-associated mortality is higher in HF-PEF than HF-REF [10]. Therefore the treatment strategy for HF-PEF in CRS is truly required.

SERCA2a plays an essential role in Ca^{2+} homeostasis and regulates cardiac functions through maintaining SR Ca^{2+} contents and excitation-contraction coupling. A reduction in the expression of SERCA2a was widely documented in cardiac systolic dysfunction [65-68] as well as diastolic dysfunction [49-52]. In contrast, overexpression of SERCA2a was shown to improve cardiac function [69, 70]. These beneficial results observed in preclinical testing have led to clinical trials in patients with HF to enhance Ca^{2+} uptake, and these trials demonstrated safety and suggested benefit for advanced HF patients [71, 72].

In the present study, a reduction in SERCA2a protein level observed in salt induced CKD rats was accompanied by the development of cardiac diastolic dysfunction. This

indicates that reduced SR Ca^{2+} stores and slow Ca^{2+} transient decay cause cardiac diastolic dysfunction. While interestingly, renal denervation restored the expression of SERCA2a protein accompanied by the improvement of cardiac diastolic dysfunction.

The regulations of SERCA2a expression and function are not fully known, and may likely be complex. In fact, the activity of SERCA2a is regulated by a number of other molecules including PLB [73-77], protein phosphatase 1 (PP1) [78, 79], inhibitor-1 (I-1) [79-81], and protein kinase Cα (PKCα) [82, 83], which together constitute a signaling pathway. Among them, PLB and its phosphorylation at either serine 16 (Ser-16-PLB) [73, 74], threonine 17 (Thr-17-PLB) [75, 76], or both [77], have been thought to be the most essential and important factors to regulate the SERCA2a function in HF. In the present study, however, Ser16-PLB to total PLB and Thr17-PLB to total PLB ratios were not significantly altered among the groups. Taken together, these suggest that renal denervation restores the SERCA2a mRNA transcription, and then leads to the improvement of protein expression and activity in cardiac diastolic dysfunction.

The sympathetic nerve system produces NE and increases the heart rate, conduction velocity, and myocardial contraction and relaxation. In CKD, sympathetic neural tone is upregulated [26-29], and sympathetic overactivity independently predicts cardiovascular events and mortality in ESRD [30]. Many studies have indicated that salt loading suppresses sympathetic tone. [84-87]. In concordance with the former studies, in the present study, salt loading suppressed cardiac and renal NE contents, however, the urinary NE was not suppressed. It suggests that whole body sympathetic tone and organ specific sympathetic tone are regulated differently by salt loading and whole body sympathetic tone is activated in our CKD model.

Organ specific sympathetic tone in the kidney and heart are regulated by both

directly and indirectly; direct stimulation by central nervous system and indirect regulation via reflexes by multiple pathway. Pressure on pelvis increases afferent renal nerve activity and leads to a decrease in efferent renal sympathetic nerve activity and a natriuresis, i.e., a renorenal reflex response [88]. When the afferent limb of the renorenal reflex is interrupted by dorsal rhizotomy, an operation that severs the afferent renal nerve fibers at the entrance to the ganglionic dorsal root, salt loading suppressed NE turnover at posterior hypothalamic nuclei [89-91] and fails to suppress renal sympathetic tone through impaired arterial baroreflex function [92]. These observations suggest that afferent signals from diseased kidneys to integrative structures in the brain may result in activation of sympathetic outflow in whole body.

NE content is the sum of synthesized, released and reuptaken NE. Therefore, the content of NE in the organ does not necessarily suggest physiological active NE. And, we measured NE turnover by halting de novo synthesis of NE by alpha-methyl tyrosine in the kidney and the heart. In those targeted organs, NE turnover were comparable between normal salt and high salt loading. Additionally, renal denervation changed neither NE contents nor NE turnover in the heart. It suggests that salt loading may affect NE synthesis in the heart and its effect is also impaired in the kidney. And renal denervation does not affect salt-effect on NE synthesis. To confirm this hypothesis, I evaluated tyrosine hydroxylase, the rate-limiting enzyme for NE synthesis. So far, enzymatic activity of tyrosine hydroxylase cannot be measured, thus we measured mRNA, however, I could not detect the changes of its transcription. In future, novel technique is developed, this hypothesis may be clarified.

Although renal denervation did not alter NE status, it successfully restored diastolic dysfunction. To clarify the possibility that adrenergic receptor status may change after renal denervation, I investigated those receptor expressions. However, I could not observe

significant changes in adrenergic receptors. I assume that renal denervation does not modify cardiac sympathetic tone in our model but other unknown factors play key roles in reserving SERCA2a transcription and subsequent restoration of cardiac function. It is supported by the observations that renal denervation did not change the heart rate in either salt-loaded or normal salt diet groups.

To further clarify the molecular basis of regulation on SERCA2a transcription, I focused on the epigenetical modulation. Epigenetics refers to chromatin-based pathways important in the regulation of gene expression, including miR-based mechanisms. MiRNAs are short, endogenous, single-stranded RNA molecules that play a role as posttranscriptional regulators. MiRNAs are on average only 22 nucleotides long and are found in all eukaryotic cells. MiRNAs bind to complementary sequences on the 3' UTR of target mRNA, usually resulting in translational silencing, either by cleaving and degrading the target mRNA or blocking ribosomal translation of the targeted mRNA [57, 58]. In the present study, miRs may not regulate SERCA2a expression in LV, however, other epigenetics mechanisms such as DNA methylation, histone density and posttranslational modifications are future perspectives.

Among factors that links renal denervation and SERCA2a transcription, oxidative stress is a candidate. In CKD model rat, oxidative stress in the brain is increased and leads to hypertension and organ damages [43]. In that study, antioxidant therapy successfully reversed renal damages, however, in the present study, renal denervation did not protect proteinuria. It requires further investigations to clarify the cardioprotective effect of renal denervation in CKD model.

While the vast majority of animal studies did demonstrate favorable effects of renal denervation on BP, in this study, renal denervation neither prevented or delayed the onset of BP nor ameliorated the magnitude of hypertension. This is the first report of renal denervation

on BP using young, uninephrectomized rats on high salt diet. There are isolated contrary reports in the DOCA-NaCl and one-kidney, one-clip (1K-1C) Goldblatt models suggesting that renal denervation can not prevent development of hypertension [93, 94]. Those data and my study indicate that uninephrectomized rodent models are less responsible to renal denervation. In clinical study, some investigators have indicated that the treatment effect to catheter-based renal denervation is of lesser magnitude and the population of nonresponders may be larger than previously believed [95, 96].

In conclusion, I for the first time demonstrated that renal denervation improved cardiac diastolic dysfunction independent of BP reduction as well as renal function possibly through the specific restoration of SERCA2a gene and protein expression in LV **(Figure 15)**.
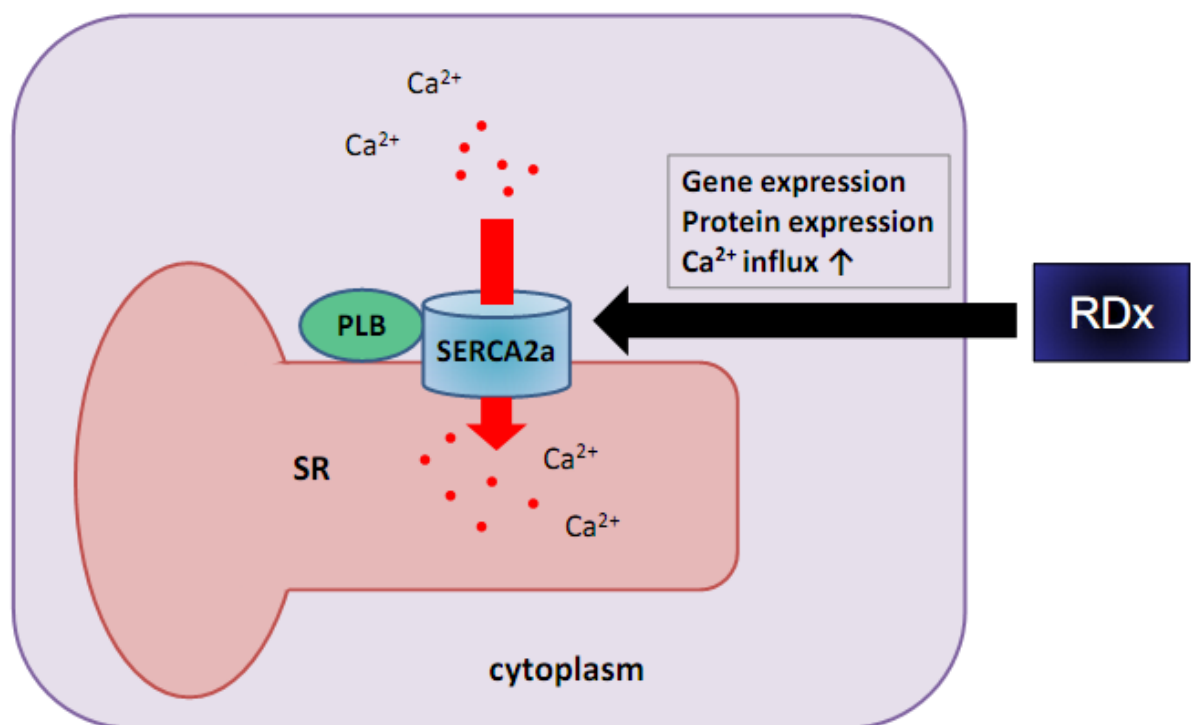

Figure 15. Proposed mechanisms of renal denervation on cardiac diastolic dysfunction.
PLB; phospholamban, RDx; renal denervation, SERCA2a ; sarcoplasmic reticulum Ca^{2+}-ATPase type 2a, SR; sarcoplasmic reticulum.

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