Journal of Hypertension: Open Access

Hirohama et al., J Hypertens (Los Angel) 2016, 5:2
DOI: 10.4172/2167-1095.1000225

Research Article Open Access

Renal Denervation Improves Cardiac Diastolic Dysfunction by Restoring Serca2a Transcription in Uninephrectomized Rats

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Abstract

Background: The mortality and morbidity of heart failure with preserved ejection fraction has increased. Sarcoplasmic reticulum Ca^{2+}-ATPase type 2a (SERCA2a) regulates cardiac functions, and a reduction in SERCA2a expression has been documented in left ventricular (LV) diastolic dysfunction. By contrast, SERCA2a overexpression improves LV diastolic dysfunction. Thus, transcriptional regulation of SERCA2a may be a new therapeutic target. The aim of this study was to determine whether renal denervation, a treatment for resistant hypertension, is a regulator of SERCA2a transcription *in vivo*.

Methods: Uninephrectomy and 6-week salt loading in three-week-old male Sprague-Dawley rats were used to devise a cardiac diastolic dysfunction model, and mechanical renal denervation was performed. The expression of SERCA2a and related molecules was evaluated with quantitative polymerase chain reaction and western blot analyses. The maximal positive LV pressure development ($+dP/dt_{max}$) and time constant at the isovolumic relaxation phase (Tau) were determined with cardiac catheters.

Results: Uninephrectomy combined with a high-salt diet significantly reduced the messenger RNA expression and protein abundance of SERCA2a, which were restored by renal denervation. In accordance with changes in SERCA2a transcription, uninephrectomy and the high-salt diet decreased LV diastolic function, which was evaluated by Tau and restored by renal denervation. LV systolic function, measured with $+dp/dt_{max}$, was preserved. Renal denervation did not lower blood pressure, urinary protein levels, cardiac hypertrophy, or fibrosis.

Conclusions: We found that renal denervation is a regulator of SERCA2a transcription *in vivo*. Our data may provide new therapeutic insights into LV diastolic dysfunction and warrant further study.

Keywords: Heart failure; Heart failure with preserved ejection fraction; Renal denervation; SERCA2a

Introduction

The mortality and morbidity of heart failure (HF) has increased due to the increased prevalence of hypertension and the aging of the population. The proportion of patients with HF with preserved ejection fraction (HF-PEF) accounts for more than 50% of the total HF population [1]. Left ventricular (LV) diastolic dysfunction is considered the major underlying pathology in HF-PEF [2]. However, clinical trials to date have failed to show improvements in diastolic dysfunction or cardiovascular outcome [3,4].

Sarcoplasmic reticulum Ca^{2+}-ATPase type 2a (SERCA2a) plays an essential role in Ca^{2+} homeostasis and regulates cardiac functions. Reductions in SERCA2a expression have been widely documented in LV systolic [5] as well as diastolic dysfunction [6,7]. By contrast, SERCA2a overexpression has been shown to improve LV systolic [8] and diastolic [7] dysfunction. Furthermore, clinical trials have demonstrated the beneficial effects of transferring the SERCA2a gene to the heart of systolic HF patients [9], but several obstacles remain to be overcome. The transcriptional regulation of SERCA2a may be a new therapeutic target; however, knowledge of SERCA2a transcription is limited [10-14].

Renal denervation has been used to treat patients with resistant hypertension. Pilot trials have shown that renal denervation improves LV diastolic dysfunction, possibly independent of blood pressure (BP) and heart rate reduction [15,16]. The aim of the present study was to determine whether renal denervation is a regulator of SERCA2a transcription *in vivo*.

Methods

Model of cardiac diastolic dysfunction

All experimental procedures were conducted in accordance with the guidelines for the care and use of laboratory animals approved by The University of Tokyo Graduate School of Medicine. 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-side uninephrectomy. Left-side renal denervation or a sham operation was performed simultaneously as described

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Received May 05, 2016; **Accepted** May 18, 2016; **Published** May 24, 2016

Citation: Hirohama D, Kawakami-Mori F, Ogura S, Mu S, Jimbo R, et al. (2016) Renal Denervation Improves Cardiac Diastolic Dysfunction by Restoring Serca2a Transcription in Uninephrectomized Rats. J Hypertens (Los Angel) 5: 225. doi:10.4172/2167-1095.1000225

below. The procedures were carried out under anesthesia with sodium pentobarbital (20 mg/kg body weight, intraperitoneally). Thereafter, the rats were fed either a normal-salt diet (NS; 0.3% NaCl) or a high-salt diet (HS; 8% NaCl) for 6 weeks. The rats were randomly divided into four groups as follows: 1) NS and sham operation of denervation (NS); 2) NS renal nerve denervated (NS-RDx); 3) HS and sham operation of denervation (HS); and 4) HS and renal nerve denervated (HS-RDx).

Renal denervation

Renal denervation was performed as described previously [17]. 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. These vessels were then stained with a solution of 10% phenol in ethanol. In the sham operation, the renal nerves were isolated but preserved. After the rats were killed, renal tissue norepinephrine (NE) content was measured to confirm total renal denervation [17]. The supernatants were analyzed for endogenous NE by using a high-performance liquid chromatography assay with electrochemical detection. The NE content of the renal tissue in the NS-RDx group was significantly smaller than that in the NS group (2.99 ± 0.52 vs. 100.0 ± 9.11 ng/g tissue; $P < 0.01$) and nearly undetectable, which indicated that renal afferent and efferent denervation was complete [17].

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

RNA was prepared from rat LV tissues with an RNeasy fibrous kit (Qiagen, Venlo, Netherlands). Total RNA was reverse-transcribed with Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, CA). Gene expression was quantitatively analyzed with real-time reverse transcription-PCR as previously described [18]. We used TaqMan Gene Expression Assays with a 7300 Real-Time PCR System (Invitrogen). The ID numbers for the assays were as follows: Rn00667869_m1 for β-actin, RN01499544_m1 for SERCA2a, RN01488777_g1 for β-myosin heavy chain, RN01463848_m1 for collagen 1a, and RN01437681_m1 for collagen 3a.

Western blot analysis

Heart tissue was homogenized on ice with T-PER Tissue Protein Extraction Reagents (Thermo Fisher Scientific, Rockford, IL) for total protein. The reagents contained complete protease inhibitors (Roche Diagnostics, Basel, Switzerland) and phosphatase inhibitors (Roche Diagnostics). Western blots were carried out as described elsewhere [18]. We used primary antibodies to anti-rabbit phospholamban (PLB) Ser16 (A010-12, Badrilla, Leeds, UK; 1:5,000), anti-rabbit phosphorylated PLB Thr17 (sc-17024-R, Santa Cruz Biotechnology, Dallas, TX; 1:400), anti-mouse PLB (ab2865, Abcam, Cambridge, MA; 1:1,000), anti-mouse SERCA2a (MA3-919, Thermo Fisher Scientific 1:2,000), anti-mouse nitro tyrosine (ab7048, Abcam; 1:1,500), anti-mouse 4-hydroxy-2-nonenal (4HNE; Japan Institute for the Control of Aging, Shizuoka, Japan; 15 μg/ml), and anti-rabbit actin (A2066, Sigma-Aldrich, St. Louis, MO; 1:5,000). After the incubation with anti-rabbit (7074P2, Cell Signaling Technology, Danvers, MA; 1:5,000) and anti-mouse (7076, Cell Signaling Technology; 1:5,000) secondary antibodies, signals were visualized with an enhanced chemiluminescence detection system (GE Healthcare UK Ltd., Buckinghamshire, UK) with a LAS 4000 imaging system (FUJIFILM, Tokyo, Japan). Densitometry was performed with Image 1.63 software (National Institutes of Health, Bethesda, MD).

Physiological studies

Systolic BP was measured with the tail-cuff method (P-98A, Softron, Tokyo, Japan) in conscious rats in the NS (n = 9), NS-RDx (n = 8), HS (n = 22), and HS-RDx (n = 13) groups and recorded at 4 and 6 weeks. We measured systolic BP five times at each time point for each rat and calculated the average.

Twenty-four-hour urine samples were collected via metabolic cages at 6 weeks from the NS (n=6), NS-RDx (n=8), HS (n=19), and HS-RDx (n=12) groups, 18 and urinary protein levels were measured. At the completion of invasive LV-pressure measurements, the animals were killed. Blood samples were obtained from the vena cava, and then the heart tissues were harvested and snap-frozen for RNA and protein analyses.

Pathological studies

Left ventricles (four hearts each from the NS, HS, and HS-RDx groups) were fixed with 4% paraformaldehyde, embedded in paraffin, and cut into sections of 3-μm thickness. Azan staining was performed to evaluate perivascular and myocardial interstitial fibrosis.

Hemodynamic measurements

At 6 weeks, rats in the NS (n=9), NS-RDx (n = 8), HS (n=22), and HS-RDx (n=13) groups were anesthetized with sodium pentobarbital (40 mg/kg body weight, intraperitoneally). LV pressures were assessed with a Millar Tip catheter (SPR-320NR, 2 Fr, Millar Instruments, Houston, TX), which was introduced from the right carotid artery and advanced into the LV cavity. After the catheters were inserted, the animals were stabilized hemodynamically for 5 minutes. Thereafter, the heart rate, mean arterial pressure (carotid artery), maximal positive LV pressure development ($+dP/dt_{max}$), and time constant at the isovolumic relaxation phase (Tau) were measured.

Statistical analysis

The data are presented as the means ± standard error of the mean. Comparison among groups was performed with one-way analysis of variance followed by the Tukey-Kramer post hoc test. A P value of less than 0.05 was considered statistically significant.

Results

Changes in SERCA2a messenger RNA (mRNA) and protein expression

SERCA2a mRNA expression in the HS group was significantly lower than that in the NS group (Figure 1a), and the abundance of SERCA2a protein followed the same trend (Figure 1b). The mRNA expression and protein abundance of SERCA2a were restored by renal denervation (Figure 1a and 1b).

Total PLB protein and PLB phosphorylation at serine 16 and threonine 17 were comparable among the groups (Figure 1c). The protein abundance of nitro tyrosine and 4HNE were not altered by the HS (Figure 1d).

Cardiac function and structural remodeling

BP was elevated in the HS group and was not lowered by renal denervation (Figure 1e and Supplementary Figure S1a and 1b).

In accordance with changes in SERCA2a transcription, rats in the HS group showed deteriorated diastolic function, which was evaluated with Tau and restored by renal denervation (Figure 1f).

LV systolic function, measured with $+dp/dt_{max}$, in the HS group was higher than that in the NS group which indicated that LV systolic

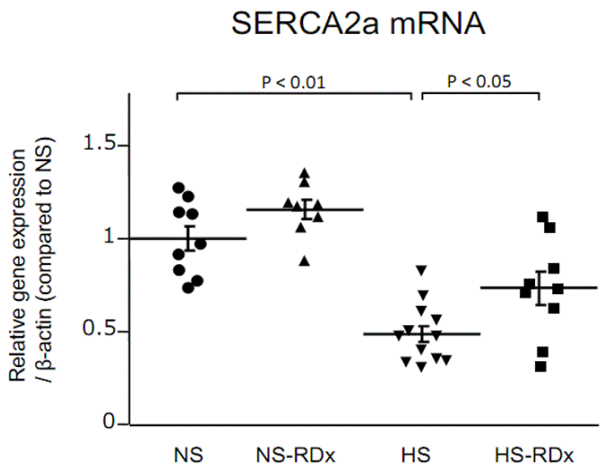

Figure 1a: Renal denervation improved cardiac diastolic dysfunction by restoring sarcoplasmic reticulum Ca2+-ATPase type 2a (SERCA2a) transcription in uninephrectomized rats. (a) Left ventricular gene expression of SERCA2a in the normal-salt diet (NS), NS with renal denervation (NS-RDx), high-salt diet (HS), and HS with renal denervation (HS-RDx) groups was normalized to β-actin expression (n = 8–12). The data are presented as means ± standard error of the mean (SEM). mRNA = messenger RNA.

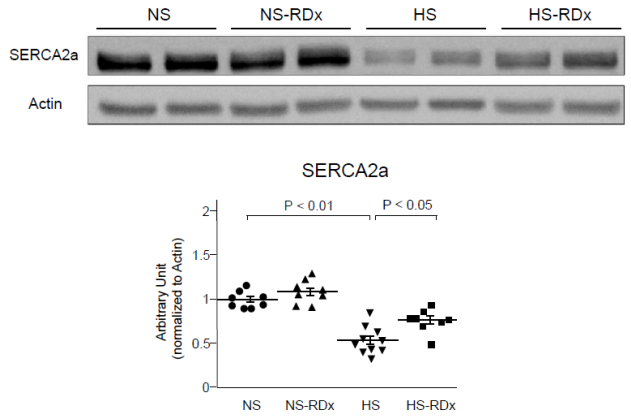

Figure 1b: Representative western blots of the left ventricle and quantification of SERCA2a in the NS, NS-RDx, HS, and HS-RDx groups (n = 7–10). The same amount of protein was loaded, and actin was the loading control. The data are presented as means ± SEM.

function was preserved (Supplementary Table S1). Compared with the LV weight-to-body weight ratio in the NS group, that in the HS group was significantly higher and was not reversed by renal denervation (Figure 1g).

Renal damage

Urinary protein levels in the HS group were significantly higher than those in the NS group. Renal denervation did not affect proteinuria (Supplementary Table S1).

Discussion

SERCA2a transcription is regulated by several signaling pathways [10-14]. Mitochondrial transcriptional factors A and B2 increase SERCA2a transcription [10]. Oxidative stress [11] nuclear factor

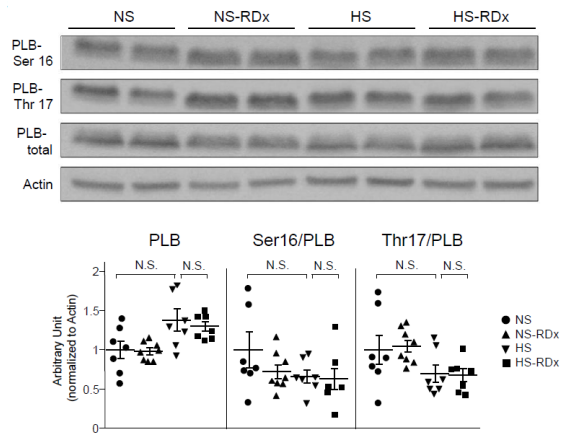

Figure 1c: Representative western blots of the left ventricle and quantification of total phospholamban (PLB) and its phosphorylation at serine 16 (Ser16/PLB) and threonine 17 (Thr17/PLB) in the NS, NS-RDx, HS, and HS-RDx groups (n = 7–10). The same amount of protein was loaded, and actin was the loading control. The data are presented as means ± SEM.

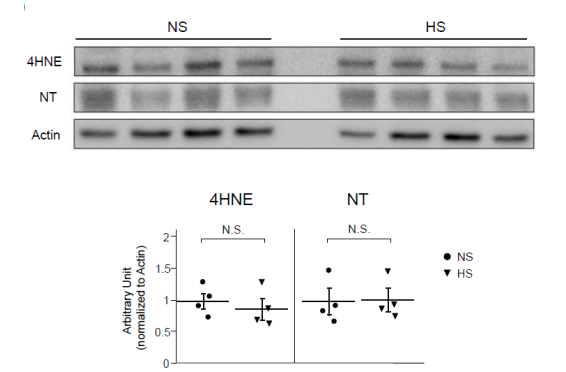

Figure 1d: Representative western blots of the left ventricle and quantification of 4-hydroxy-2-nonenal (4HNE) and nitro tyrosine (NT) in the NS and HS groups (n = 4). The same amount of protein was loaded, and actin was the loading control. The data are presented as means ± SEM.

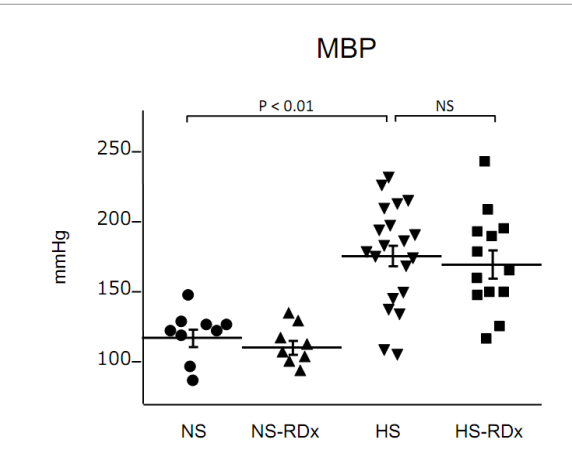

Figure 1e: Mean arterial pressure (MBP) and time constant of left ventricular pressure decay (Tau) in the NS (n = 9), NS-RDx (n = 8), HS (n = 22), and HS-RDx (n = 13) groups. The data are presented as means ± SEM.

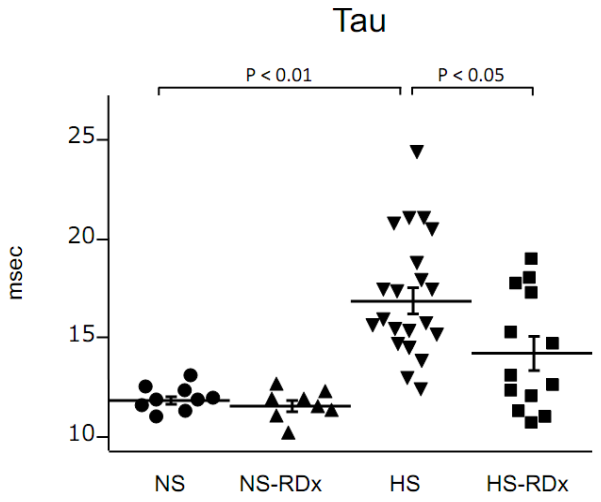

Figure 1f: Mean arterial pressure (MBP) and time constant of left ventricular pressure decay (Tau) in the NS (n = 9), NS-RDx (n = 8), HS (n = 22), and HS-RDx (n = 13) groups. The data are presented as means ± SEM

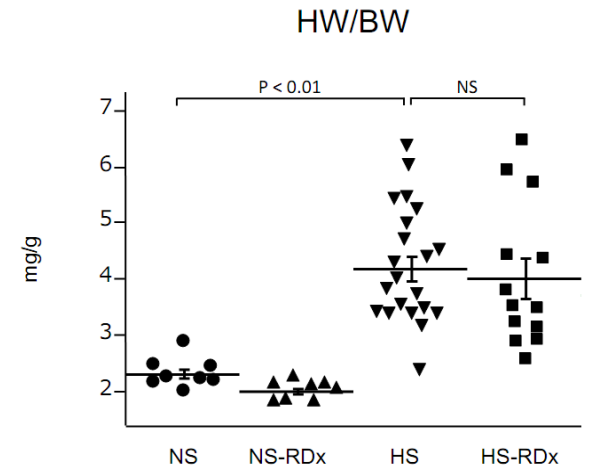

Figure 1g: Heart weight-to-body weight ratio (HW/BW) in the NS (n = 9), NS-RDx (n = 8), HS (n = 22), and HS-RDx (n = 13) groups. The data are presented as means ± SEM.

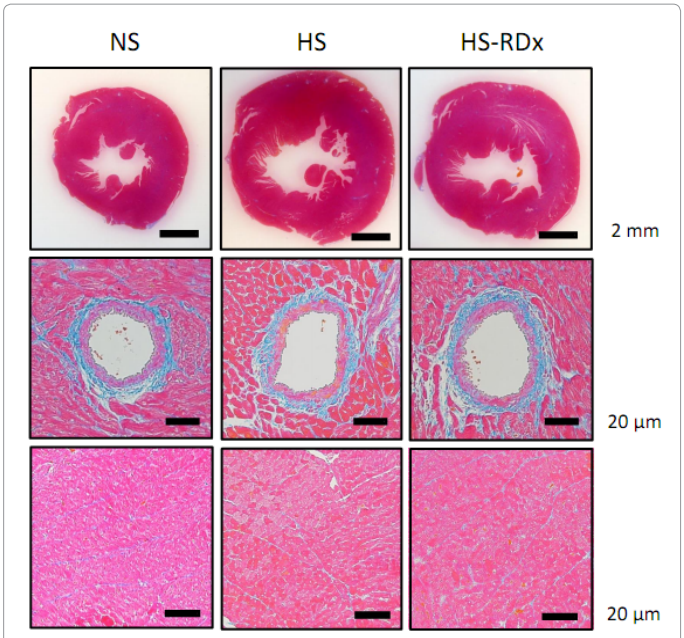

Figure 1h: Azan staining was performed to evaluate cardiac fibrosis in the NS (n = 4), HS (n = 4), and HS-RDx (n = 4) groups. Upper scale bars indicate 2 mm, middle and lower scale bars indicate 20 µm (upper panels, original magnification, 1×; middle and lower panels, original magnification, 100×).

of activated T-lymphocytes, myocyte enhancer factor 2c [12] and mitogen-activated protein kinase-activated protein kinases 2 and 3 suppress SERCA2a transcription [13]. Brain natriuretic peptide also reportedly inhibits the transcription of SERCA2a directly [14]. In the present study, we demonstrated that renal denervation restores SERCA2a transcription and diastolic function in uninephrectomized young rats consuming an HS.

To confirm that low levels of SERCA2a protein impair cardiac function, we evaluated LV diastolic function by measuring Tau. In concordance with SERCA2a level, LV diastolic function was reduced by uninephrectomy and the HS, and this deterioration was reversed by renal denervation. PLB phosphorylation at serine 16 and threonine 17 functionally enhances SERCA2a activity and Ca^{2+} uptake in the sarcoplasmic reticulum [19]. In the present study, however, PLB and its phosphorylation were not significantly different among the experimental groups. This result suggests that the restoration of cardiac diastolic function occurs independently of PLB and its phosphorylation.

As shown in Figure 1f-1h and Supplementary Figures S1a, 1b and 1d neither BP nor cardiac hypertrophy was lowered by renal denervation throughout the experiment. Moreover, a regression curve showing the relationship between cardiac weight and Tau showed significantly different relationships between the renal nerve intact (the NS and HS) group and the denervated group (the NS-RDx and HS-RDx) (Supplementary Figure S1c). These data suggest that renal denervation reversed SERCA2a transcription and diastolic function independently of BP changes or cardiac hypertrophy. Furthermore, we observed no fibrotic changes in the heart (Figure 1h and Supplementary Figures S1e, 1f), which suggests that *in vivo* SERCA2a transcription can be altered independently of profibrotic stimuli.

Owing to our experimental design, we could not discern whether the restoration of SERCA2a transcription was due to the direct or indirect effects of renal denervation on the heart. In the uninephrectomy and HS group, we observed prominent proteinuria that may induce cardio-renal syndrome and affect SERCA2a transcription. However, renal denervation did not restore renal function; therefore, it is doubtful that renal denervation indirectly restored SERCA2a transcription via the preservation of kidney function. A previous study showed that oxidative stress impairs SERCA2a transcription [11]. In the present study, uninephrectomy and the HS did not induce higher oxidative stress, which was evaluated by measuring nitro tyrosine and 4HNE (Figure 1d). Thus, oxidative stress appears unlikely to have played a role in the regulation of SERCA2a transcription in our animal model.

A limitation of our study was the lack of 24-hour continuous BP monitoring. The minor effect of renal denervation on BP reduction is comparable with the result of recent randomized trial [20]. Indeed, 24-hour continuous BP monitoring would provide additional information with which to understand the cardioprotective effect of renal

denervation. Another limitation of the present study was the inability to clarify the differential role of the afferent and efferent nerves, also a consequence of our experimental design. Further *in vivo* studies are required to clarify the factors that alter SERCA2a transcription in this model of cardiac failure and via renal denervation.

Taken together, our data may provide new therapeutic insights into LV diastolic dysfunction, and these findings warrant further study.

Financial Disclosure

This study was supported by Grants-in-Aid for Scientific Research (c) (No. 26461249).

Acknowledgements

We are deeply grateful to Dr. Yoshihide Fujisawa (Life Science Research Center, Faculty of Medicine, Kagawa University) for his instruction and guidance in the field of renal nerve denervation.

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