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Salt Loading Exacerbates Diastolic Dysfunction and Cardiac Remodeling in Young Female Ren2 Rats

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Abstract

Objective—Recent data would suggest pre-menopausal insulin resistant women are more prone to diastolic dysfunction than men, yet it is unclear why. We and others have reported that transgenic (mRen2)27 (Ren2) rats overexpressing the murine renin transgene are insulin resistant due to oxidative stress in insulin sensitive tissues. As increased salt intake promotes inflammation and oxidative stress, we hypothesized that excess dietary salt would promote diastolic dysfunction in transgenic females under conditions of excess tissue Ang II and circulating aldosterone levels.

Materials/methods—For this purpose we evaluated cardiac function in young female Ren2 rats or age-matched Sprague-Dawley (SD) littermates exposed to a high (4%) salt or normal rat chow intake for three weeks.

Results—Compared to SD littermates, at 10 weeks of age, female Ren2 rats fed normal chow showed elevations in left ventricular (LV) systolic pressures, LV and cardiomyocyte hypertrophy, and displayed reductions in LV initial filling rate accompanied by increases in 3-nitrotyrosine content as a marker of oxidant stress. Following 3 weeks of a salt diet, female Ren2 rats exhibited no further changes in LV systolic pressure, insulin resistance, or markers of hypertrophy but exaggerated increases in type 1 collagen, 3-nitrotyrosine content, and diastolic dysfunction. These

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findings occurred in parallel with ultrastructural findings of pericapillary fibrosis, increased LV remodeling, and mitochondrial biogenesis.

Conclusion—These data suggest that a diet high in salt in hypertensive female Ren2 rats promotes greater oxidative stress, maladaptive LV remodeling, fibrosis, and associated diastolic dysfunction without further changes in LV systolic pressure or hypertrophy.

Keywords

angiotensin II; TG (mRen2) 27 rat; Diastolic Dysfunction; Cardiac Remodeling; Oxidative Stress; Perivascular Fibrosis

Introduction

It is increasingly evident there is sexual dimorphism in cardiac structure and function. Population based studies support the notion that cardiovascular risk is attenuated in pre-menopausal women compared to men and that this protection is lost with estrogen withdrawal associated with menopause [1,2]. Further, there are notable discrepancies in the response of the heart to alterations in load from chronic elevations in systolic blood pressure in women compared to men [3,4]. In this regard, women have a propensity to develop more concentric hypertrophy relative to the eccentric pattern most commonly observed in men in response to load alterations [3–5]. Concentric hypertrophy and increased cardiac fibrosis can lead to reductions in left ventricular (LV) compliance and relaxation, characteristics that predispose women to diastolic dysfunction compared to men who are more prone to systolic abnormalities.

Women who exhibit systemic insulin resistance are at greater risk for development of diastolic heart failure under conditions of insulin resistance [7,8]; these data suggest a fundamental difference between males and females in cardiac adaptation to nutritional factors and other stressors. To this point, we recently demonstrated earlier progression to diastolic dysfunction in young female C57Bl6 mice fed a western diet high in fat and fructose compared to diet and age-matched males [9,10]. Moreover, the early progression to diastolic dysfunction was associated in females with enhanced whole-body insulin resistance. While there is evidence from human and rodent models that females are more prone than men to diet-induced diastolic dysfunction [9–11], factors promoting this cardiac sexual dimorphic response to dietary factors are poorly understood.

In addition to excess fat and carbohydrates, the high salt intake characteristic of western diets may be another factor that promotes cardiac functional abnormalities, including diastolic dysfunction in females [12]. There is little extant data as to whether high salt negates the protective effects of estrogen and other sex-related factors on diastolic function. To address the effect of increased dietary salt intake on diastolic dysfunction in female rats, we utilized the TG(mRen2)27 (Ren2) rat that over-expresses the mouse renin transgene and has elevated plasma aldosterone levels as well as increased cardiac tissue angiotensin II (Ang II) levels. The female Ren2 rat exhibits normal body mass yet develops insulin resistance, hypertension and diastolic dysfunction [13]. The insulin resistance in the Ren2 rat has been shown to be directly related to increased skeletal muscle oxidative stress [13]. Therefore, to investigate the impact of increased dietary salt on altered cardiac structure and function and potential underlying mechanisms, we fed female Ren2 rats a high salt diet over three weeks and followed indices of cardiac oxidative stress, fibrosis and ultrastructural abnormalities and associated cardiac dysfunction.

Materials and Methods

Animals and Treatments

All animal procedures were approved by the animal care and use committees at the University of Missouri and the Harry S. Truman Veterans Memorial Hospital and housed in accordance with NIH guidelines. Female Ren2 (6–7 weeks of age) and age-matched female Sprague-Dawley (SD) rats were randomly assigned to feed on normal chow (Ren2-C and SD-C, respectively) or chow containing 4% salt (Ren2-HS and SD-HS, respectively) for 21 days.

Baseline parameters - Systolic Blood Pressure (SBP), quantitative insulin sensitivity check index (QUICKI), and Body Weight (BW)

Hemodynamic measurements were performed on anesthetized and ventilated Ren2 and SD rats with thiobutabarbital, as previously described [15,16]. Briefly, a fluid filled plastic catheter was placed in the right carotid artery to monitor LV SBP and heart rate (HR). After an acclimation period of at least 10 minutes, SBP and HR were recorded every 10 minutes with a Biopac recorder and data acquisition software for a period of 90 minutes to calculate an average for each variable. The QUICKI was derived using the inverse of the sum of the logarithms of the fasting insulin and fasting glucose. Total BWs were obtained at time of sacrifice.

In Vivo Cine-Magnetic Resonance Imaging (MRI)

High resolution MRI imaging was performed at the end of the treatment periods using a Varian 7T horizontal bore MRI (Varian Inc., Palo Alto, CA) with EKG gating (SA Instruments, Stony Brook, NY) [17]. Animals were anesthetized using 2–3.5% isoflurane on a nose-cone non-rebreathing system supplying continuous oxygen. A series of cine images of the left ventricle in both long and short axis views were acquired at 12 equally spaced time points throughout the entire cardiac cycle with a frame rate of 9–13 minutes. The total acquisition time of each cine sequence was approximately six minutes with in plane spatial resolution of $156 \times 137 \mu\text{m}^2$ (long-axis view) or $156 \times 156 \mu\text{m}^2$ (short-axis view) and two signal averages. Heart wall thickness and LV volume measurements were determined using VnmrJ (Varian, Inc.) and ImageJTM.

LV Diastolic function extracted from cine-MRI

As previously described, the first derivatives of LV volume (LVV) against time were calculated to extract filling rates and relaxation times during diastole [17]. Diastolic initial filing rate (IFR) was defined as the slope of the first four time points on the early diastolic curve. Diastolic relaxation time (DRT) was defined as the time duration from the end of systolic phase to the peak filling phase. Normalized DRT, which is the ratio of DRT to the R-R interval, was used to compare LV diastolic relaxation among groups, where normalized DRT = $[\text{DRT} \times (\text{HR}/6,000)]$.

Measurement of Heart Tissue 3-nitrotyrosine (3-NT) Content

Generation of 3-NT was measured by immunohistochemistry. Briefly, 4 μm paraffin LV sections were de-paraffinized, rehydrated, and epitopes were retrieved in citrate buffer [18,19]. Endogenous peroxidases were then quenched with 3% H_2O_2 and non-specific binding sites were blocked with avidin, biotin, and protein block (Dako, Carpinteria, CA). The sections were then incubated with 1:150 primary rabbit polyclonal anti-nitrotyrosine antibody (Chemicon, Temecula, CA). Sections were then washed and incubated with secondary antibodies, biotinylated link and streptavidin-HRP, (Dako, Carpinteria, CA). After several rinses with distilled water, diaminobenzidine was applied for 10 minutes. The

sections were again rinsed with distilled water, stained with hematoxylin for 1 minute, dehydrated, and mounted with a Permount (Fisher) media. The slides were evaluated under a bright field (Nikon 50i) microscope and the 40X images were captured with a cool snap^{cf} camera. Images were analyzed and the signal intensities measured with MetaVue (Boyce Scientific Inc. Gary Summit, MO).

Immunofluorescent Studies

LV tissue sections were fixed in 3% paraformaldehyde [18,19], dehydrated with ethanol, infiltrated with low-melting (50° C) paraplast, and embedded in high-melting (56° C) paraplast. Blocks were sectioned by 4 µm, deparaffinized in CitriSolv, and rehydrated in ethanol and HEPES wash buffer. Non-specific binding sites on the sections were blocked with 5% BSA and 5% of the serum that secondary antibodies were generated. Then, the section were incubated with 1:200 mouse cytochrome oxidase IV (Cox IV) (Mitoscience), 1:500 mouse Serca2a (Novus), 1:100 rabbit SERCA2a (294-5 (Bethyl Lab), 1:100 rabbit SERCA2a c674 (Bethyl Lab), 1:400 phospholamban (Pierce), 1:100 mouse Collagen I and 1:100 rabbit collagen III (Abcam) and 1:50 rabbit goat Nox4 (Santa Cruz Biotechnology) antibodies, in 10 fold diluted blocker. After 24 hours, the slides were washed (3 × 15 minutes) and were incubated with 1:300 of appropriate secondary antibodies. After 4 hours, the slides were washed, mounted with Mowiol, and examined using a biphoton laser confocal scanning microscope, images captured by using the LSM Imaging system and signal intensities measured by MetaVue analysis.

Quantification of Peri-arterial Cardiac Fibrosis

Five micron sections of left ventricle were stained with picro-sirius red according to manufacturing procedure. Slides were analyzed with a Nikon50i microscope and 10 images were captured with a cool snap^{cf} camera and auto leveled with Photoshop. Morphometric analysis was performed using MetaVue software. In each image, the areas of hot pink color and their intensities which is representative of periarterial fibrosis were quantified and normalized to the size of the arteries.

Transmission Electron Microscopy (TEM) Methods

Heart tissue was placed in primary EM fixative as previously described [9]. Briefly, following secondary fixation, specimens were then placed on a rocker overnight, embedded, and polymerized at 60° C for 24 hours. 85 nm thin sections were then stained with 5% uranyl acetate and Sato's Triple lead stain for viewing by TEM. A Joel 1400-EX transmission electron microscope (Joel Ltd, Tokyo, Japan) was utilized to view generated images.

Statistical Analysis

Statistical analyses were performed using Sigma Plot 12.0 (Systat Software, San Jose, CA) software. Two-way ANOVA and Bonferroni's post-hoc test were used as appropriate. All values are expressed as mean ± standard error.

Results

Hemodynamic Parameters

We previously reported in ten week old female Ren2 rats, less severe elevations in SBP compared to males at this same age [19]. In the current study, at 10 weeks of age systolic pressures were elevated in the Ren2 compared to SD females. Two-way ANOVA indicate that systolic pressures were significantly elevated in Ren2-C and Ren2-HS compared to SD-C and SD-HS, respectively (Table 1). However there were no further differences between

salt treatment and controls in the Ren2 for systolic pressure or insulin sensitivity by QUICKI (0.33 ± 0.006 vs 0.32 ± 0.009 , respectively). Also, there were no differences detected in HR or BW among the groups.

Four Percent Salt Diet Promotes Diastolic Dysfunction in Female Ren2 Rats

Relaxation abnormalities and diastolic dysfunction are characterized by the inability of the left ventricle to fill during the early phases of diastole in women. Using high-resolution cine-MRI, we measured IFR and relaxation time. At a similar age, male Ren2 rats develop diastolic dysfunction in parallel with the increases in SBP [15]; in our current work, female Ren2 rats developed delays in IFR compared to SD controls (Fig. 1A) that was further augmented in the presence of a 4% salt diet ($p < 0.05$). There was also a significant strain effect for female Ren2 rats treated with salt, compared to their salt-treated SD counterparts (Fig. 1B).

Four Percent Salt Diet Induces Myocardial Collagen Alterations and Fibrosis in Female Ren2 Rats

The relationship between cardiac extracellular matrix degradation and remodeling of the collagen network are closely related to the progressive development of diastolic dysfunction and progressive heart failure [20]. Alterations in collagen content determine the extent of extracellular matrix remodeling and fibrosis whereby type 1 collagen provides more tensile strength (stiffness) and type 3 promotes increased elasticity. There was a significant treatment effect with salt-induced increases in type 1 collagen content (Fig. 1A) that temporally correlated with ultrastructural observations utilizing TEM of pericapillary fibrosis with salt in the female Ren2 (Fig. 1B). This salt-induced remodeling of the collagen network characterized by substantially increased type 1 collagen and type 1/3 ratio was accompanied by an increased trend on light microscopy for peri-arterial in female Ren2 rats (Fig. 2C and E).

Four Percent Salt Diet Induced Increases in Myocardial Oxidant Stress in Female Ren2 Rats

Myocardial oxidative stress is associated with heart failure through direct links to fibrosis and alterations in myofibroblast responses [21–24]. In this context, nitrosylation of tyrosine residues can be evaluated by 3-NT staining as an indirect marker of increased peroxynitrite formation and oxidant stress. There were salt-induced increases in 3-NT content in both female SD and Ren2 rats (Fig. 3) with a minimal strain effect. The dietary salt-induced increases in 3-NT content parallels the observation of salt-induced increases in type 1 collagen content suggesting an imbalance in redox homeostasis that contributes to fibrosis and myocardial tissue remodeling.

Four Percent Salt Induces Hypertrophy and Mitochondrial Biogenesis in Female Ren2 Rats

Early in myocardial tissue remodeling, cardiomyocyte hypertrophy in response to injury are accompanied by ultrastructural changes consistent with increases in cytoplasm, nuclei and mitochondria [25]. In the current work, there was a significant strain effect for hypertrophy and cardiomyocyte size in female Ren2 rats compared to SD counterparts indicated by an increase in the LV plus septal (S) (LV+S)/BW ratios and by ultrastructural observations (Table 1). Moreover, there was also a significant effect of high dietary salt on LV+S/BW ratios manifested in both SD and Ren2 rats. Specifically, LV hypertrophy (LVH) was significantly augmented in the SD-HS group compared to SD-C and LVH with a trend in Ren2-HS group compared to Ren2-C.

Consistent with the increases in hypertrophy and cardiomyocyte size, there were ultrastructural changes consistent with salt-induced increases in cardiomyocyte size in the female Ren2 4% salt (Ren2-HS) rat (Fig. 4, lower right panel). This occurred in parallel with increased numbers of mitochondria in the subsarcolemma regions and not the inter-myofibrillar regions (Fig. 5A Ren2-HS). This salt-induced increase in mitochondria content in the subsarcolemma region of female Ren2 rat occurred in parallel with increases in Cox IV content (Fig. 5B).

Discussion

Our current observation of accelerated cardiac oxidative stress, mitochondrial content, and diastolic dysfunction in female Ren2 rats due to increases in dietary salt, suggests that preconditioning by an activated renin-angiotensin-aldosterone system (RAAS) may have contributed to these structural and functional abnormalities. In this regard, there has been a significant amount of work to suggest that Ang II and aldosterone contribute to cardiac and vascular tissue fibrosis with stimulation of fibroblast formation and collagen synthesis through reactive oxygen species production [20,26]. Indeed, it has been previously demonstrated in female rats that Ang II infusion over two weeks was associated with substantial increases in cardiac type 1 collagen and diastolic dysfunction [27]. Previous work done in our laboratory in the Ren2 suggests that there are sex-specific effects whereby increases in reactive oxygen species production promote perivascular fibrosis and cardiomyocyte hypertrophy [18]. This would support our observation that 3-NT content was increased in the high salt-fed female Ren2 rat that occurred contemporaneously with the increases in type 1 collagen content, pericapillary fibrosis and diastolic dysfunction. 3-NT as a product for peroxynitrite is considered a nitric oxide (NO)-dependent species wherein inhibition of the Ang II type 1 receptor can lead to reductions in fibrosis and improve LVH and remodeling [28].

The results of this study underscore the fact that there are sex-specific differences in LV structural and functional adaptation to diet and other stressors such as salt. Women develop a specific pattern of concentric hypertrophy in response to alterations in afterload that place women at particular risk for development of heart failure compared to men. In this context, studies support that obese women suffer disparately from higher prevalent rates of heart failure [7,8,29,30], especially that characterized by diastolic dysfunction relative to men [3–5,29,30]. Diabetic women also have a higher mortality post myocardial infarction [1,2,6], and the gender-specific alterations in LV remodeling place women at higher risk for wall rupture [31]. Collectively these data suggest that women are particularly susceptible to diastolic dysfunction in response to stressors such as increased caloric intake that create conditions of obesity, insulin resistance and excess RAAS activity. In this regard, our group has reported that the Ren2 rat manifests insulin resistance which is characterized by increased oxidative stress in skeletal muscle, heart and other insulin sensitive tissue [13–18]. Data from the present investigation support that the Ren2 heart manifests increased oxidative stress, and that three weeks of 4% salt in the diet induces greater oxidative stress and diastolic dysfunction in this model with overexpression of the murine renin transgene. The female Ren2 rat displays increases in SBP and insulin resistance [14–19] and increases in LV systolic pressures (Table 1) that provide underlying load-dependent alterations that precondition for salt-inducible diastolic dysfunction. Indeed, three weeks of 4% salt accentuated diastolic dysfunction contemporaneous with increases in oxidant stress, type 1 collagen content, LV remodeling and mitochondrial biogenesis in the female Ren2 rat. This occurred despite the fact that the high salt diet did not further worsen insulin resistance or augment LV systolic pressures.

There is increasing interest in the observation that obese or diabetic women are at increased risk for diastolic dysfunction relative to male counterparts. Population studies support that >75% of prevalent cases of heart failure with preserved ejection fraction, or isolated diastolic dysfunction, occur in women [32]. There are fundamental structural differences in hearts of women that include smaller LV chamber size and preservation of LV mass that contribute to fractional shortening as a marker for diastolic compliance and wall thickness [33,34]. This, coupled with differences in sex-specific biomechanical properties such as pulsatile arterial loading and increases in wave reflection due to increases in body size contribute directly to differences in adaptation to increases in afterload on LVH in obese women [35,36]. Work done in the Dahl salt-sensitive rat suggests that development of diastolic dysfunction in female rats was dependent on a hypertrophied left ventricle, whereas males under the same conditions did not exhibit any impairments in the pressure-volume relationship [37]. This suggests sex-specific effects on LVH and remodeling that precondition for diastolic dysfunction. Our data corroborate a similar maladaptation in female Ren2 hearts wherein delays in IFR were accompanied by increases in LV systolic pressure and LVH and fibrosis.

Others have shown similar findings in regards to sex-specific effects on hypertrophy in models of pressure overload, post-ischemic models, and Ang II-infused models [37–39]. In the model of ascending aortic banding of gradually induced pressure overload, female rats demonstrated a concentric hypertrophy with preservation of LV mass and systolic function not seen in males [38]. The observation that females preserve LV mass and systolic function compared to men support the finding that women are at higher risk for the development of isolated diastolic dysfunction [27].

Our most significant new observation was that salt induced further delays in IFR and relaxation times in the female Ren2 rat without further increases in LV systolic pressures or additional increases in measures of hypertrophy. This would suggest a load-independent effect of salt on the female heart in the development of diastolic dysfunction. The work is consistent with findings that female salt-loaded Dahl rats developed concentric hypertrophy and diastolic dysfunction while males manifested an eccentric hypertrophy without diastolic dysfunction [37]. Findings from previous work in the congenic Ren2-Lewis rat would corroborate our findings that female Ren2 rats develop hypertrophy and impairments in LV filling suggestive of diastolic dysfunction [40]. Importantly, a previous study showed a significant correction of diastolic dysfunction by BH4 supplementation, an essential NOS cofactor, in surgically oophorectomized m[Ren2]27-Lewis rats [26]. In our current work with 4% salt we demonstrate by cine-MRI, additional reductions in IFR and increases in relaxation times. This likely represents a mild or early transition time of diastolic abnormalities that are precipitated by increases in salt intake.

It is clear from other rodent models that salt-induced LV remodeling impairs LV relaxation and diastolic function through increases in fibrosis and cardiac stiffness [41–43]. In this regard, alterations in the composition of collagen isoforms within the myocardial extracellular matrix can determine the extent of ventricular stiffness and impaired relaxation. There are two predominant isoforms of collagen, types 1 and 3 in the myocardium wherein increases in type 1 represent increases in tensile strength and stiffness whereas type 3 promotes elasticity [21]. Our observations demonstrated increases in type 1 collagen and ultrastructural observation of pericapillary fibrosis suggest an early stage of fibrosis and consistent with a reactive fibrosis wherein alterations in collagen content occur in the perivascular space and then spread into the interstitium. The current observation, that salt induced a trend to reduced type 3 collagen and increased the ratio of type 1 to type 3, suggests one potential mechanism for development of diastolic dysfunction. Differences in the ratio of type 1 and type 3 alter the passive mechanical properties of the left ventricle. Col

3 is the more compliant isoform while Col 1 is stronger and stiffer. In this regard, an increase in the Col 1 to Col 3 ratio denotes increased LV wall stiffness, such as would occur in a heart with diastolic dysfunction with preserved ejection fraction. Our findings would be consistent with prior work in that salt induces fibrosis and diastolic dysfunction in various models; however, to our knowledge this would be a first report of a sex-specific response to salt-induced diastolic dysfunction through increases in type 1 collagen in the early stages of fibrosis.

Considering that pericoronary fibroblasts are known to synthesize collagens which promote fibrosis and possess the mineralocorticoid receptor (MR) [44,45], our ultrastructural findings that salt-induced increases in pericapillary fibrosis in the Ren2 would suggest an aldosterone dependent mechanism. Previous work supports that salt requires aldosterone action on the MR to induce perivascular fibrosis in the heart, directly related to ventricular stiffness and progressive heart failure [45]. Work in the Ren2 would suggest that antagonizing the MR improves perivascular fibrosis and diastolic dysfunction independent of blood pressure changes in the Ren2 rat [46]. Our current work, suggests that salt may augment MR-dependent actions on perivascular fibrosis in females that may relate to the inability for females to adapt to cardiac stressors and a role for sex hormones in cardiac protection. In this regard there is one study that suggests agonism of the estrogen receptor, either α or β , protects against aldosterone/salt dependent perivascular fibrosis in the heart [47]. Treatment with 16 α -LE2 and 8 β -VE2 improved remodeling and led to improvements in perivascular collagen accumulation as well as indices of hypertrophy such as the cardiomyocyte area. Our current ultrastructural findings would complement these observations that in female Ren2 rats with increases in aldosterone, salt-induced increases in cardiomyocyte hypertrophy, as well as pericapillary fibrosis.

Early in the course of LV remodeling there are structural adaptations within the cardiomyocyte and the extracellular matrix that include increases in cardiomyocyte size, loss of cytoskeletal arrangement, and accumulation of extracellular proteins [20]. In the hypertrophied myocardium there are adaptive increases in myocyte size but also loss of myofibrils and grouping of increased numbers of smaller-sized mitochondria. The morphologic changes suggest increased mitochondrial content and thus biogenesis to compensate for increased energy demands of the myocardium to sustain cardiac contractility [48,49]. Our ultrastructural observations suggest there was cardiomyocyte hypertrophy in the female salt-treated Ren2 with corresponding increases in cytoplasm and increased subsarcolemma mitochondria as well as disorganization of the extracellular matrix. Additionally the increases in subsarcolemmal mitochondria were corroborated on immunostaining with increased Cox IV content. These observations would further suggest the female heart is susceptible to salt-induced mitochondrial biogenesis.

In summary, data from the present investigation suggests that salt induces diastolic dysfunction without increasing insulin resistance or load-dependent changes under preconditioning of excess cardiac Ang II and plasma aldosterone in a female transgenic model that overexpresses the murine renin transgene, the Ren2 rat. Further, the salt-induced changes occurred in relation to increases in oxidant stress, increased mitochondrial biogenesis and alterations in collagen composition with pericapillary fibrosis. Our finding that oxidant stress was temporally related to the development of diastolic dysfunction would suggest a mechanism but our lack of an anti-oxidant arm negate definitive conclusions. Further, it should be noted that while our results in a transgenic rodent model provide insight into the influences that dictate diastolic dysfunction, this study may not recapitulate the human condition. However, our results underscore the notion there are sex-specific differences in LV structural and functional adaptation to dietary stressors such as salt.

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Abbreviations

Ren2	transgenic (mRen2)27
SD	Sprague-Dawley
LV	left ventricular
Ang II	angiotensin II
SBP	systolic blood pressure
QUICKI	quantitative insulin sensitivity check index
BW	body weight
HR	heart rate
MRI	magnetic resonance imaging
LVV	left ventricular volume
IFR	initial filing rate
DRT	diastolic relaxation time
3-NT	3-nitrotyrosine
Cox IV	cytochrome oxidase IV
Ren2-HS	Ren2 4% salt
TEM	transmission electron microscopy
S	septal
LVH	left ventricular hypertrophy
RAAS	renin-angiotensin-aldosterone system
MR	mineralocorticoid receptor

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Figure 1. Salt induces diastolic dysfunction in female transgenicRen2 rats

A) Representative images from cine Magnetic Resonance Imaging of female rats fed a normal chow diet Sprague-Dawley and Ren2 (SD-C and Ren2-C, respectively) as well as high salt fed SD and Ren2 rats (SD-HS and Ren2-HS, respectively). The images represent end-systolic, early diastolic, and end-diastolic phases from a total of 16 cine MRI frames recorded in 1 entire cardiac cycle for each rat. The frames shown here are frames 7, 10, and 16. Histograms depict diastolic indices of **B)** initial filling rate and **C)** diastolic relaxation. Values are means $\pm$ SE. Post hoc comparisons for strain effect: * <0.05 and for intervention effect: †, $p<0.05$.

Figure 2. Salt induces collagen shift in pericapillary and periarterial fibrosis in the left ventricle (LV) of female transgenic Ren2 rats

A) Representative images of immunohistochemical analysis of type 1 collagen content with corresponding measures of intensity below of rats fed a normal chow diet Sprague-Dawley and Ren2 (SD-C and Ren2-C, respectively) as well as high salt fed SD and Ren2 rats (SD-HS and Ren2-HS, respectively). Values are means $\pm$ SE. Adventitia are marked “A” and lumen “L” for orientation. Post hoc comparisons for intervention effect: †, $p < 0.05$. **B)** Ultrastructural analysis depicts pericapillary, periarterial, and early stages of interstitial fibrosis in Ren2-HS. **Panel A and B** depicts interstitial fibrosis (white arrows) with panel B depicting periarterial fibrosis extending into the interstitium. Panel A magnification X800; scale bar = 5 μ m. Panel B magnification X2000; scale bar = 2 μ m. **Panels C and D** depict pericapillary fibrosis at x2000 magnification in panel C (scale bar = 2 μ m) and at higher

magnification X4000 (scale bar =1 μm). Insert illustrates the banded morphology of organized collagen fibers (including whorls of collagen) in contrast to loose areolar collagen found in SDF-C, SDF-4%S, and R2-C 4% S in figures 1 and 2. Magnification X10,000; (scale bar =0.2 μm). **C)** Representative images of immunohistochemical analysis of type 3 collagen content with corresponding measures of intensities below. Adventitia are marked “A” and lumen “l” for orientation. Post hoc comparisons for intervention effect: †, $p<0.05$. **D)** Representative light micrographs of Verhoeff-van Gieson stained LV sections, which stains collagen pink, with measures below of rats fed a normal chow diet Sprague-Dawley and Ren2 (SD-C and Ren2-C, respectively) as well as high salt fed SD and Ren2 rats (SD-HS and Ren2-HS, respectively). Values are means $\pm$ SE. **E)** Depicts the ratio of type 1 to type 3 collagen with individual measures of type 1 and 3 below.

Figure 3. Salt induces oxidant stress in the LV of female SD and Ren2

A) Representative sections for 3-nitrotyrosine, a marker of peroxynitrite (ONOO^-) formation with corresponding measures of intensity below. Values are means $\pm$ SE. Post hoc comparisons for intervention effect: †, $p < 0.05$. Normal chow diet fed Sprague-Dawley and Ren2 (SD-C and Ren2-C, respectively) as well as high salt fed SD and Ren2 rats (SD-HS and Ren2-HS, respectively).

Figure 4. Salt induces increases in ultrastructural cardiomyocyte size in the left ventricle (LV) of female transgenic Ren2 rats

There is similar morphological size of cardiomyocytes in normal chow diet fed Sprague-Dawley and Ren2 (SD-C and Ren2-C, respectively) female rats as well as high salt fed SD rats (SD-HS). Analysis of the high salt fed female Ren2 (Ren2-HS) suggest cardiomyocyte hypertrophy when compared to panels A–C. Magnification X800; scale bar = 5 μ m.

Figure 5. Salt-induced mitochondrial biogenesis in the left ventricle (LV) of female transgenic Ren2 rats

A) On ultrastructural analysis using TEM, there are a few mitochondria scattered throughout the subsarcolemma regions (white arrows) in normal chow diet fed Sprague-Dawley and Ren2 (SD-C and Ren2-C, respectively) female rats as well as high salt fed SD rats (SD-HS). The high salt fed female Ren2 (Ren2-HS) depict a marked increase in subsarcolemma mitochondria. Magnification X2000; scale bar = 2 μ m. **B)** Representative images of immunohistochemical analysis of cytochrome oxidase IV (Cox IV) with corresponding measures of intensity to the right. Values are means $\pm$ SE. Post hoc comparisons for strain effect: * <0.05 and for intervention effect: †, p<0.05.

Mean ($\pm$ SEM) hemodynamic parameters, body and ventricular weights in Sprague-Dawley (SD) and Ren2 (R2) rats. Statistics are by Two-way ANOVA.

Table 1

Parameter	Significant Main Effects; P<0.05	SDC	SD-HS	Ren2-C	Ren2-HS
		(4)	(5)	(4)	(6)
LV systolic pressure, mmHg	Strain	141 $\pm$ 5	148 $\pm$ 5	187 $\pm$ 7 [*]	182 $\pm$ 7 [#]
	Tx				
	Interaction				
Heart Rate, bpm	Strain	341 $\pm$ 14	350 $\pm$ 13	336 $\pm$ 15	318 $\pm$ 20
	Tx				
	interaction				
Body weight, g	Strain	(8)	(8)	(11)	(10)
	Tx	215 $\pm$ 7	205 $\pm$ 6	220 $\pm$ 7	197 $\pm$ 11
	interaction				
LV+S•BW ⁻¹ , mg/g	Strain	505 $\pm$ 36	536 $\pm$ 24	667 $\pm$ 26 [*]	635 $\pm$ 32 [#]
	Tx				
	interaction				
LV+S•BW ⁻¹ , mg/g	Strain	2.33 $\pm$ 0.11	2.62 $\pm$ 0.09 [§]	3.03 $\pm$ 0.07 [*]	3.25 $\pm$ 0.08 [‡]
	Tx				
	interaction				
Cardiomyocyte size, average gray scale intensities	Strain	(5)	(5)	(5)	(5)
	Tx	350	459.1	712.9	589.4
	interaction				

^{*} P<0.05 SDC vs R2C;

[‡] Ren2-C vs Ren2-HS;

[§] SDC vs SD-HS;

[#] SD-HS vs Ren2-HS.

Samples sizes are in parentheses. Tx, treatment.