Salt Excess Causes Left Ventricular Diastolic Dysfunction in Rats With Metabolic Disorder

Hiromitsu Matsui, Katsuyuki Ando, Hiroo Kawarazaki, Ai Nagae, Megumi Fujita, Tatsuo Shimosawa, Miki Nagase, Toshiro Fujita

Abstract—Metabolic syndrome is a highly predisposing condition for cardiovascular disease and could be a cause of excess salt–induced organ damage. Recently, several investigators have demonstrated that salt loading causes left ventricular diastolic dysfunction associated with increased oxidative stress and mineralocorticoid receptor activation. We, therefore, investigated whether excess salt induces cardiac diastolic dysfunction in metabolic syndrome via increased oxidative stress and upregulation of mineralocorticoid receptor signals. Thirteen-week-old spontaneously hypertensive rats and SHR/NDmcr-cps, the genetic model of metabolic syndrome, were fed a normal salt (0.5% NaCl) or high-salt (8% NaCl) diet for 4 weeks. In SHR/NDmcr-cps, salt loading induced severe hypertension, abnormal left ventricular relaxation, and perivascular fibrosis. Salt-loaded SHR/NDmcr-cps also exhibited overproduction of reactive oxygen species and upregulation of mineralocorticoid receptor–dependent gene expression, such as Na+/H+ exchange-1 and serum-and glucocorticoid-inducible kinase-1 in the cardiac tissue. However, in spontaneously hypertensive rats, salt loading did not cause these cardiac abnormalities despite a similar increase in blood pressure. An antioxidant, tempol, prevented salt-induced diastolic dysfunction, perivascular fibrosis, and upregulation of mineralocorticoid receptor signals in SHR/NDmcr-cps. Moreover, a selective mineralocorticoid receptor antagonist, eplerenone, prevented not only diastolic dysfunction but also overproduction of reactive oxygen species in salt-loaded SHR/NDmcr-cps. These results suggest that metabolic syndrome is a predisposed condition for salt-induced left ventricular diastolic dysfunction, possibly via increased oxidative stress and enhanced mineralocorticoid receptor signals. (Hypertension. 2008;52:287-294.)

Key Words: metabolic syndrome ■ salt intake ■ cardiac diastolic function ■ oxidative stress ■ mineralocorticoid receptor

Metabolic syndrome (MetS) is associated with a high rate of cardiovascular disease morbidity.1,2 Patients with MetS have been reported to exhibit left ventricular (LV) diastolic dysfunction,3,4 which eventually leads to diastolic heart failure with a poor prognosis.5,6 However, LV diastolic dysfunction has not always been seen in a MetS animal model. For example, in obesity-prone Sprague-Dawley rats, a moderate fat diet induced metabolic abnormalities but did not cause LV diastolic dysfunction.7 This discrepancy indicates a possibility that some exogenous factors deteriorate cardiac function in MetS. In patients with MetS, high salt intake increased blood pressure (BP) greater than in those without MetS.8 In an MetS rat model, evident renal injury was induced by salt loading.9 These findings suggest that the MetS model is highly susceptible to salt-induced organ damage. Salt loading induced LV hypertrophy or LV diastolic dysfunction in several hypertension models.10-14 Therefore, it is speculated that LV diastolic impairment could be accelerated with excess salt in an MetS model.

Reactive oxygen species (ROS) are important elements associated with an increase in oxidative stress,17-19 possibly through enhanced ROS-inducible adipocytokines.20-22 Also, salt loading increased oxidative stress in several salt-sensitive hypertension models.10,23-25 Given that those models with MetS are predisposed to salt-induced ROS overproduction, salt loading is expected to induce ROS augmentation and resultant LV diastolic dysfunction in MetS.

The beneficial effect of a mineralocorticoid receptor (MR) antagonist has been proven by clinical trials in patients with severe heart failure.26-27 MR antagonists showed a cardioprotective effect despite a low circulating aldosterone level in salt-loaded animal models.13,14,28,29 Furthermore, we reported recently that MR blockade prevented salt-induced renal damage in a rat MetS model.9 These results suggest that an MR signal is involved in the pathogenesis of salt-induced organ damage, including cardiac dysfunction in MetS.

In the present study, therefore, we investigated whether salt loading induces LV diastolic dysfunction in a MetS model, and, if so, whether ROS or MR signaling is involved in the mechanisms of salt-induced diastolic dysfunction. As a genetic rat model of MetS, we used the SHR/NDmcr-cp...
(SHR/cp), a derivative of the spontaneously hypertensive rat (SHR) with leptin receptor deficiency, characterized by obesity, hyperinsulinemia, dyslipidemia, and hypertriglyceridemia, all of which are consistent with MetS.30

Methods

Animal Preparation

All of the animals were handled in an accredited facility in accordance with the institutional animal care guidelines, and all of the research protocols conformed to the guiding principles for animal experimentation as outlined by the ethics committee on animal research of the University of Tokyo.

Thirteen-week-old male SHR/cps (SLC, Hamamatsu, Japan) were randomized into 4 groups: a normal salt (0.3% NaCl) diet group (NS-SHR/cps, n = 10); high-salt (8% NaCl) diet group (HS-SHR/cps, n = 12); high-salt diet with a superoxide dismutase mimetic, 4-hydroxy-2,2,6,6-tetramethyl-piperidine-N-oxyl (tempol; 1 mmol/L in drinking water) group (HS+TE-SHR/cps, n = 12); or high-salt diet with a selective MR antagonist (eplerenone; 1.25 g/kg of chow: 50 to 80 mg/kg per day) group (HS+EP-SHR/cps, n = 12). The groups were treated for 4 weeks. SHRs (Eisai, Tokyo, Japan) with or without salt loading (HS-SHRs: n = 4; NS-SHRs: n = 6) were used as controls. The animals were maintained in a regulated environment at 26±2°C with a 12-hour:12-hour light-dark cycle.

BP Monitoring

Systolic BP (SBP) was monitored every week with the tail-cuff method (BP-98A, Softron). In each examination, we measured SBP 5 times at each time point for each rat and calculated the average.

Evaluation of Excretion of Urinary 8-Hydroxy-2'-Deoxyguanosine

The urinary 8-hydroxy-2'-deoxyguanosine (8-OHdG) concentration was evaluated using a commercially available 8-OHdG ELISA kit (Japanese Institute for the Control of Aging).

Echocardiography

Transthoracic echocardiographic studies were performed as described previously.10 For details, see the data supplement (available online at http://hyper.ahajournals.org).

Measurement of Metabolic Parameters and Serum Aldosterone

For more information, please see the data supplement.

Hemodynamic Study

Hemodynamic parameters were recorded from a catheter placed in the cavity of the left ventricle, and maximal negative slope (−dp/dt max) and time constant (T) at the isovolumic relaxation phase were calculated as reported previously.10 For details, see the data supplement.

ROS Production Induced by Addition of Nicotinamide-Adenine Dinucleotide Phosphate in Cardiac Tissue

ROS production in the cardiac tissue was evaluated by lucigenin chemiluminescence, as described previously.10 For details, see the data supplement.

Histological Studies

For details see the data supplement.

Real-Time Quantitative PCR

The mRNA was extracted by the guanidinium method (Isogen, Nippon Gene). After the procedure of reverse transcription real-time quantitative PCR was performed using an ABI PRISM 7000 (Applied Biosystems).31 Commercially available, ready-

| Table 1. Body Weight, Cardiac Weight, and Cardiac Weight:Body Weight |
|---------------------------------|-----------------|-----------------|-----------------|
| Experimental Group             | Body Weight, g  | Cardiac Weight, g | CW/BW ×10³     |
| NS-SHRs (n=6)                  | 375.8±3.0       | 1.21±0.03        | 3.21±0.09       |
| HS-SHRs (n=4)                  | 535.8±6.3       | 1.49±0.04*       | 4.21±0.12*      |
| NS-SHR/cps (n=10)              | 471.5±8.7*      | 1.15±0.06        | 2.43±0.10       |
| HS-SHR/cps (n=12)              | 457.4±10.0†     | 1.51±0.06‡       | 3.29±0.10†      |
| HS+TE-SHR/cps (n=12)           | 491.0±6.2†      | 1.57±0.06‡       | 3.21±0.10‡      |
| HS+EP-SHR/cps (n=12)           | 461.3±9.7†      | 1.40±0.06‡       | 3.06±0.10‡      |

Data are means±SEMs. CW indicates cardiac weight; BW, body weight. *P<0.01 vs NS-SHRs by 1-way ANOVA. †P<0.01 vs HS-SHRs by 1-way ANOVA. ‡P<0.01 vs NS-SHR/cps by 1-way ANOVA.

made primers were used for the real-time quantitative-PCR (Applied Biosystems).

Statistical Analysis

All of the values are expressed as means±SEMs. Comparisons were made with 1-way ANOVA followed by Scheffe’s method in all of the groups of rats. P values of <0.05 were considered to indicate significance. Furthermore, we analyzed the data using 2-way ANOVA to evaluate the independent and interactive influence of strains and salt loading on various parameters, including cardiac diastolic function and oxidative stress, in the 4 groups of rats (NS-SHR, HS-SHR, NS-SHR/cp, and HS-SHR/cp).

Results

Body Weight, BP, and Cardiac Weight

Body weight was significantly higher in SHR/cps than in SHRs (Table 1). Body weight was not affected by salt loading, with or without tempol or eplerenone. Salt loading elevated SBP in both SHRs and SHR/cps, although this effect was slightly weak in SHR/cps at the early phase (Figure 1). Concomitantly, salt loading increased cardiac weight and LV wall thickness, as shown by the echocardiography (Tables 1 and 2 and Figure S1). In HS-SHR/cps, tempol did not affect SBP. On the other hand, eplerenone decreased SBP slightly but not significantly in HS-SHR/cps. The cardiac weight was not affected by tempol in HS-SHR/cps, whereas eplerenone decreased it slightly but not significantly.

Metabolic Parameters

Metabolic parameters such as plasma leptin, serum insulin, and free fatty acids were significantly higher in SHR/cps than in SHRs, with or without salt loading (Table 3). The levels of serum triglyceride and fasting glucose were also higher in SHR/cps than in SHRs. These metabolic parameters in salt-loaded SHR/cps were not altered by treatment with tempol or eplerenone. Large SEs were detected in triglyceride and free fatty acid levels in SHR/cps.

LV Systolic and Diastolic Function

LV systolic function in echocardiography was preserved among all of the groups (Table 2 and Figure S1). NS-SHR/cps rats exhibited elongation in the deceleration time of early mitral inflow (E/E′) and in T in the hemodynamic study compared with NS-SHRs; however, these elongations were very small (Figures 2A through 2C, S2, and S3). Actually,
Coronary Perivascular Fibrosis in the SHR/cp Group
The histological study showed that salt loading caused evident perivascular fibrosis of the coronary artery in SHR/cps (2.8 ± 0.2 versus 2.1 ± 0.04; P < 0.01) but not in SHRs (2.2 ± 0.1 versus 2.1 ± 0.1; Figures 3A and S4). The fibrotic change was restricted to the perivascular region of the heart. Concomitantly, salt loading significantly increased cardiac mRNA expression of connective tissue growth factor and collagen type III in SHR/cps but not in SHRs (Figure 3B and S3).

ROS Generation
Without salt loading, markers of ROS, such as urinary excretion of 8-OHdG, superoxide production induced by addition of NADPH in cardiac tissue, and mRNA expression of NADPH oxidase components p22phox, p47phox and gp91phox, were equivalent between SHRs and SHR/cps (Figure 4). Tempol significantly increased these ROS markers in SHR/cps but did not in SHRs.

Serum Aldosterone and MR Signals
Serum aldosterone level was significantly higher in NS-SHR/cps than in SHRs (Figure 5A). Salt loading decreased the serum aldosterone level, but serum aldosterone was still slightly but not significantly higher in HS-SHR/cps as compared with HS-SHRs. In SHR/cps, salt loading enhanced cardiac mRNA expression of Na+/H+ exchanger-1 (NHE-1) and serum- and glucocorticoid-inducible kinase-1 (Skg-1), the markers of MR signals (Figure 5B and S4). However, SHRs did not show any salt-induced upregulation of MR signals. Concomitantly, gene expression of angiotensin-converting enzyme (ACE) in the cardiac tissue was enhanced by salt loading in SHR/cps but not in SHRs (Figure 5).

Table 2. Echocardiographic Findings

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>IVSd, mm</th>
<th>LVd, mm</th>
<th>PW, mm</th>
<th>LVEF, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>NS-SHRs (n = 6)</td>
<td>1.8 ± 0.1</td>
<td>8.2 ± 0.2</td>
<td>1.9 ± 0.0</td>
<td>72.8 ± 1.0</td>
</tr>
<tr>
<td>HS-SHRs (n = 4)</td>
<td>2.3 ± 0.1*</td>
<td>7.6 ± 0.4</td>
<td>2.3 ± 0.1†</td>
<td>76.2 ± 2.1</td>
</tr>
<tr>
<td>NS-SHR/cps (n = 8)</td>
<td>1.9 ± 0.1</td>
<td>8.5 ± 0.3</td>
<td>1.7 ± 0.1</td>
<td>71.7 ± 1.7</td>
</tr>
<tr>
<td>HS-SHR/cps (n = 8)</td>
<td>2.6 ± 0.1‡</td>
<td>7.6 ± 0.4</td>
<td>2.1 ± 0.0§</td>
<td>81.4 ± 1.8‡</td>
</tr>
<tr>
<td>HS+TE-SHR/cps (n = 8)</td>
<td>2.3 ± 0.1</td>
<td>8.1 ± 0.1</td>
<td>2.2 ± 0.1§</td>
<td>78.9 ± 1.7</td>
</tr>
<tr>
<td>HS+EP-SHR/cps (n = 8)</td>
<td>2.2 ± 0.2‡</td>
<td>8.1 ± 0.3</td>
<td>2.1 ± 0.1§</td>
<td>76.6 ± 2.1</td>
</tr>
</tbody>
</table>

IVSd indicates interventricular septal dimension; LVd, LV end-diastolic dimension; PW, posterior wall dimension; LVEF, LV ejection fraction. Data are means ± SEMs.

*P < 0.05 vs NS-SHRs by 1-way ANOVA.
†P < 0.01 vs NS-SHRs by 1-way ANOVA.
‡P < 0.05 vs NS-SHR/cps by 1-way ANOVA.
§P < 0.01 vs NS-SHR/cps by 1-way ANOVA.
||P < 0.05 vs HS-SHR/cps by 1-way ANOVA.
Eplerenone Inhibited LV Diastolic Dysfunction and Inhibited ROS Overproduction in Salt-Loaded SHR/cps Rats

In HS-SHR/cps, a selective MR antagonist, eplerenone, not only downregulated MR signals (Figure 5B and 5C) but also prevented perivascular fibrosis (2.0 ± 0.3 vs NS-SHR/cps, †P < 0.05 vs NS-SHR/cps). In addition, eplerenone inhibited the overexpression of ACE in HS-SHR/cps (Figure 5D).

Influences of Strain and Salt Loading: Analysis by 2-Way ANOVA

Obesity and metabolic abnormalities were marked in SHR/cps as compared with SHR/SHRs, whereas SBP and cardiac weight were increased by salt loading (Table S1). However, there was no interaction between strains and salt loading from these data.

With regard to LV diastolic dysfunction, EDcT was significantly influenced by strains or salt loading, and the interaction between both was significant. Also, T was influenced by these 2 factors, and the interaction was marginally significant. Although −dp/dt max was not significantly influenced by either of them, the interaction was marginally significant.

Cardiac ROS production induced by the addition of NADPH was significantly influenced by strain or salt loading, and the interaction was marginally significant. Urinary 8-OHdG showed a similar tendency but was not significant, suggesting that redox changes occur mainly in cardiac tissue of salt-loaded SHR/cps. Although the expression of NADPH oxidase components was not influenced by either of them because of large SEs, the huge increase in these parameters was compatible with the data of nicotinamide-adenine dinucleotide phosphate (NADPH)–induced cardiac ROS production. The changes in MR signal expression also showed similar tendencies to those in NADPH oxidase components. Thus, the data of LV diastolic function analyzed by 2-way ANOVA were compatible with the original results analyzed by 1-way ANOVA.

Discussion

In the present study, SHR/cp, a rat model of MetS, showed salt-induced LV diastolic dysfunction, as indicated by either elongated EDcT by Doppler echocardiography or blunted −dp/dt max and prolonged T in the hemodynamic study. This abnormal LV relaxation may be regarded as an early func-

![Figure 2](http://hyper.ahajournals.org/)

**Figure 2.** Semiquantification of LV active relaxation. A, Deceleration time of early mitral inflow obtained with Doppler echocardiography (NS-SHRs: n = 6; HS-SHRs: n = 4; NS-SHR/cps: n = 8; HS-SHR/cps: n = 8). The −dp/dt max by direct LV pressure monitoring (NS-SHRs: n = 6; HS-SHRs: n = 4; NS-SHR/cps: n = 8; HS-SHR/cps: n = 8). B, The −dp/dt max by direct LV pressure monitoring (NS-SHRs: n = 6; HS-SHRs: n = 4; NS-SHR/cps: n = 8; HS-SHR/cps: n = 8). C, Time constant at isovolumic relaxation phase obtained by the direct LV pressure monitoring (NS-SHRs: n = 6; HS-SHRs: n = 4; NS-SHR/cps: n = 8; HS-SHR/cps: n = 8). For abbreviations, see Table 1. One-way ANOVA: †P < 0.05 vs NS-SHRs. †P < 0.01 vs HS-SHRs. †P < 0.01 vs NS-SHR/cps. §P < 0.05 vs HS-SHR/cps. |P| < 0.01 vs HS-SHR/cps.
Table 4. LV Peak Systolic Pressure and LV End-Diastolic Pressure

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>LVSP, mm Hg</th>
<th>LVEDP, mm Hg</th>
</tr>
</thead>
<tbody>
<tr>
<td>NS-SHRs (n=6)</td>
<td>154.3±10.1</td>
<td>10.2±1.0</td>
</tr>
<tr>
<td>HS-SHRs (n=4)</td>
<td>184.4±6.5*</td>
<td>10.7±0.7</td>
</tr>
<tr>
<td>NS-SHR/cps (n=8)</td>
<td>156.1±3.4</td>
<td>11.1±0.8</td>
</tr>
<tr>
<td>HS-SHR/cps (n=9)</td>
<td>181.2±2.7†</td>
<td>11.4±0.4</td>
</tr>
<tr>
<td>HS+TE-SHR/cps (n=10)</td>
<td>181.2±11.2‡</td>
<td>11.4±0.6</td>
</tr>
<tr>
<td>HS+EP-SHR/cps (n=9)</td>
<td>178.3±6.1</td>
<td>11.7±0.5</td>
</tr>
</tbody>
</table>

LVSP indicates LV peak systolic pressure; LVEDP, LV end-diastolic pressure. Data are means±SEMs.

*P<0.05 vs NS-SHRs by 1-way ANOVA.
†P<0.05 vs NS-SHR/cps by 1-way ANOVA.
§P<0.01 vs HS-SHR/cps by 1-way ANOVA.

Excess Salt Causes Diastolic Dysfunction in SHR/cp

Matsui et al

Excess salt may play a pivotal role in the progression of organ damage in MetS. Therefore, excess salt may play a pivotal role in the progression of organ damage in MetS.

In other words, the degree of susceptibility to salt-related organ damage may be an important factor. In SHR/cps, LV diastolic dysfunction or the upregulation of MR signals was induced by long-term (6 to 12 weeks) salt loading.11–13,34 These previous findings are compatible with the present ones in that short-term (4 weeks) salt loading was not sufficient to induce LV diastolic dysfunction in SHR/cps. However, short-term salt loading caused diastolic dysfunction associated with ROS overproduction and MR promotion in SHR/cps. Similarly, in salt-sensitive rats, such as Dahl salt-sensitive rats and stroke-prone SHR/cps, cardiac damage or an increase in oxidative stress was induced by a relatively short period (4 to 6 weeks) of salt loading.10,14-24,25,35 Taken together, this shows, for the first time, that the SHR/cp is a more predisposed model to salt-induced organ abnormalities, such as LV diastolic dysfunction, oxidative stress, and MR promotion, compared with the SHR.

Salt-related ROS augmentation might be explained partly by increased ROS-inducible adipocytokines, such as tumor necrosis factor-α or nonesterified fatty acids in MetS.20–22 However, further investigation is required to evaluate the response of adipocytokines to salt loading. On the other hand, it is hypothesized that MR could be activated by excess salt in an aldosterone-independent manner in MetS. Even with a reduced level of circulating aldosterone, MR blockers were found to be protective against salt-induced cardiac dysfunction or proteinuria.9,28,31 Similarly, in SHR/cps, high salt intake promoted MR signals in the heart despite reduced circulating aldosterone, and eplerenone prevented LV diastolic dysfunction. It should be noted that NS-SHR/cps exhibited a significantly higher level of serum aldosterone than NS-SHRs, as reported previously.30 Although salt decreased circulating aldosterone, aldosterone was slightly higher in

Figure 3. Evaluation of perivascular fibrosis and cardiac fibrotic markers. A, The ratio of perivascular fibrosis area/vessel area (NS-SHRs: n=6; HS-SHRs: n=4; NS-SHR/cps: n=6; HS-SHR/cps: n=6; HS+TE-SHR/cps: n=6; HS+EP-SHR/cps: n=6). B and C, Expression of connective tissue growth factor (CTGF) or collagen type III (Col 3) in the cardiac tissue (NS-SHRs: n=6; HS-SHRs: n=4; NS-SHR/cps: n=8; HS-SHR/cps: n=10; HS+TE-SHR/cps: n=10; HS+EP-SHR/cps: n=10). For abbreviations, see Table 1. One-way ANOVA: *P<0.05 vs HS-SHRs. †P<0.05 vs NS-SHR/cps. ‡P<0.01 vs NS-SHR/cps. §P<0.05 vs HS-SHR/cps. ||P<0.01 vs HS-SHR/cps.
HS-SHR/cps than in HS-SHRs. Indeed, this higher level of aldosterone might affect salt-induced renal injury in SHR/cp rats, but this might not be the case in the heart. With the small increase in serum aldosterone in HS-SHR/cps, local aldosterone does not seem to overwhelm the huge amount of corticosterone in the heart. 11-β-Hydroxysteroid dehydrogenase type 2 was undetectable in the heart, and thus corticosterone in the cardiac tissue. Thus, MR might be occupied mostly by corticosterone in the cardiac tissue. Therefore, MR should be occupied mostly by corticosterone in the cardiac tissue. Thus, MR might be stimulated by an aldosterone-independent mechanism in the heart of HS-SHR/cps.

It is questioned whether ROS augmentation and MR signal promotion emerged merely in parallel or whether they interacted with each other in salt-loaded SHR/cps. A causal linkage between MR activation and ROS has been proposed. First, administration of aldosterone augments ROS production via NADPH oxidase in vascular endothelial cells, which was abolished by an MR antagonist. Consistently, in our salt-loaded SHR/cps, eplerenone inhibited oxidative stress. Therefore, MR activation can induce ROS overproduction.

Second, ROS could be speculated to be an upstream element in MR activation. This hypothesis is compatible with our finding that tempol inhibited salt-induced upregulation of NHE-1 and SgK-1. Thus, the involvement of ROS is one possible explanation for aldosterone-independent MR activation. However, the possibility of MR activation by ROS is still speculative and should be further evaluated.

The upregulation of ACE might be involved in the proposed association between ROS and MR signals. It has been reported that aldosterone increases the expression of ACE in cardiomyocytes. In SHR/cps, along with the increased expression of ROS and MR signals, ACE expression was upregulated by salt loading and, in turn, tempol or eplerenone inhibited the overexpression of ACE. These results suggest a feed-forward pathway from ROS overproduction or MR signal stimulation to ACE upregulation, which stimulates production of angiotensin II, resulting in further ROS overproduction or MR activation. However, this should also be evaluated by further experiments.

It is known that elevated BP accelerates LV diastolic dysfunction. In eplerenone-treated SHR/cps, a small reduc-
tion in BP might contribute to the preservation of LV function. However, LV relaxation was apparently impaired in HS-SHR/cps but not in HS-SHRs, even with a slightly lower BP elevation, and tempol prevented salt-induced LV abnormal relaxation with suppressed MR signals and without a reduction in BP. Therefore, a BP-independent mechanism may be at least partly involved in salt-induced diastolic dysfunction in MetS, as mentioned above. However, we could not clarify the extent to which BP contributes to eplerenone-induced amelioration of LV dysfunction because of the limitations involved in measuring BP.

As expected, metabolic parameters such as insulin and lipids were increased in SHR/cps compared with SHRs. Large SEs were detected in serum triglyceride and free fatty acid in SHR/cps but not in SHRs, although the blood specimens were collected after overnight fasting in both. Therefore, the fluctuation of lipids should be involved in the characteristics of SHR/cps. However, salt-induced diastolic dysfunction was not accompanied by additive exacerbation of metabolic parameters in SHR/cps. Consistently, tempol or eplerenone prevented salt-induced diastolic dysfunction without amelioration of these metabolic parameters. Thus, our results do not indicate that this metabolic disorder directly affects salt-induced LV diastolic dysfunction.

In conclusion, the present study demonstrates that SHR/cp, a model of MetS, is a highly predisposed condition for salt-induced LV diastolic dysfunction. It also suggests that increased oxidative stress and enhanced MR signals are involved in accelerating the pathogenesis of salt-induced diastolic dysfunction in MetS.

Perspectives
MetS is a highly predisposing condition for cardiovascular disease, and one of the early cardiac involvements is LV diastolic dysfunction. Thus, an appropriate interventional strategy should be prescribed to preserve LV function in MetS. We demonstrated that high salt intake may be one exacerbating factor in the progression of LV diastolic dysfunction in an MetS animal model. Renal damage is also accelerated with salt loading in the same model. Moreover, we demonstrated the possible involvement of oxidative stress and MR signals in the pathogenesis of salt-induced LV diastolic dysfunction in MetS. Therefore, it is also suggested that treatment with an antioxidant or a selective MR blocker could be highly effective in preventing cardiac dysfunction in patients with MetS.

Sources of Funding
This work was supported by grants from the Daiichi Sankyo Company, Ltd.

Disclosures
None.

References


Salt Excess Causes Left Ventricular Diastolic Dysfunction in Rats With Metabolic Disorder
Hiromitsu Matsui, Katsuyuki Ando, Hiroo Kawarazaki, Ai Nagae, Megumi Fujita, Tatsuo Shimosawa, Miki Nagase and Toshiro Fujita

*Hypertension*. 2008;52:287-294; originally published online July 7, 2008;
doi: 10.1161/HYPERTENSIONAHA.108.111815

*Hypertension* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2008 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/52/2/287

Data Supplement (unedited) at:
http://hyper.ahajournals.org/content/suppl/2008/07/07/HYPERTENSIONAHA.108.111815.DC1

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

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

**Subscriptions:** Information about subscribing to *Hypertension* is online at:
http://hyper.ahajournals.org/subscriptions/
Salt excess causes left ventricular diastolic dysfunction in rats with metabolic disorder

Hiromitsu Matsui, MD, Ph. D, Katsuyuki Ando, MD, Ph. D, Hiroo Kawarazaki, MD, Ai Nagae, MD, Ph. D, Megumi Fujita, MD, Ph. D, Tatsuo Shimosawa, MD, Ph. D, Miki Nagase, MD, Ph. D, and Toshiro Fujita, MD, Ph. D

Running title: Excess salt causes diastolic dysfunction in SHR/cp

Department of Internal Medicine, Faculty of Medicine, the University of Tokyo, Bunkyo-ku, Tokyo, Japan.

All correspondence to Toshiro Fujita, MD, Department of Nephrology and Endocrinology, Faculty of Medicine, the University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo, 113-8655, Japan.
Phone: +81-3-5800-9735
Fax  : +81-3-5800-9736
E-mail fujita-dis@h.u-tokyo.ac.jp
Expanded Methods

Echocardiography

Trans-thoracic echocardiographic studies were performed with a 12.0 MHz phased-array ultrasound system (Aplio, Toshiba, Tokyo, Japan). Rats were injected intraperitoneally with ketamine HCl (25 to 50 mg/kg) and xylazine (5 to 10 mg/kg) to achieve the appropriate heart rate (/min) control under light anesthesia for Doppler echocardiography evaluation (NS-SHRs: 284.5±15.5, HS-SHRs: 282.3±9.4, NS-SHR/cps: 275.9±14.6, HS-SHR/cps: 271.2±8.1, HS+TE-SHR/cps: 283.2±4.0, HS+EP-SHR/cps: 298.3±6.9). M-mode tracings of the left ventricle were recorded at the papillary muscle level to measure inter-ventricular septal dimension (IVSd), left ventricular (LV) end-diastolic dimension (LVDd), LV end-systolic dimension (LVDs), and posterior wall dimension (PW). LV ejection fraction (LVEF) was calculated with the formula from Teich Holz. Pulse-wave Doppler echocardiography was performed on the apical four-chamber view and, subsequently, deceleration time of early mitral inflow (EDcT) was recorded. These echocardiographic parameters were recorded for four cardiac cycles in each rat, and the average was calculated.

Measurement of metabolic parameters and serum aldosterone

After overnight fasting, a blood specimen was collected. Serum triglycerides,
free fatty acid, blood glucose, and aldosterone were measured by routine enzymatic assays. Serum insulin concentration was measured by routine enzyme-linked immunosorbent assay (ELISA). The plasma leptin level was measured using a commercially available leptin ELISA Kit (Morinaga Bioscience Institute, Yokohama, Japan).

**Hemodynamic study**

LV pressure (LVP) curve, peak LV systolic pressure (LVSP), and LV end-diastolic pressure (LVEDP) were recorded from a catheter placed in the LV cavity under light ether anesthesia. The heart rate (/min) in each group was controlled to the same level (NS-SHRs: 413.6±4.3, HS-SHRs: 385.4±17.1, NS-SHR/cps: 382.4±6.7, HS-SHR/cps: 389.1±8.9, HS+TE-SHR/cps: 382.2±9.3, HS+EP-SHR/cps: 382.4±5.0). Maximal negative slope (–dp/dt\(_{\text{max}}\)) and time constant (\(T\)) at the isovolumic relaxation phase were calculated as reported previously.\(^2\) In brief, the LVP curve was digitized every 2 ms and the steepest slope was evaluated. The LVP was plotted and fitted by the method of least squares to the function \(P=e^{At+B}\) (where \(P\) was LVP at the isovolumic relaxation time; \(A\) was a negative number which represented the slope of \(\ln P\) vs. time in s\(^{-1}\); \(B\) was y-intercept for \(\ln P\); and \(T\) was calculated by \(-1/A\)).\(^3,4\) These hemodynamic parameters were evaluated averaging over three beats for each rat.
Evaluation of ROS production in cardiac tissue by lucigenin chemiluminescence

Superoxide (O$_2^-$) production by LV tissue was measured using bis-N-methylacridinium nitrate (lucigenin) chemiluminescence in a microplate luminometer (LB 9507, Berthold Technologies, Germany). Approximately 100 mg of LV was sectioned, homogenized, incubated in 1 ml of Krebs buffer containing 10 mmol/L HEPES-NaOH (pH 7.4) for 20 min at 37°C, and then lucigenin was added into the sample to a final concentration of 10 µmol/L. The O$_2^-$ level was measured as relative light units (RLU) by chemiluminescence, and subsequently standardized by tissue weight. In some experiments, nicotinamide-adenine dinucleotide phosphate (NADPH) (100 µmol/L final concentration) was added just before the luminescence measurement. NADPH oxidase was included in flavoenzymes, and diphenileneiodonium (DPI) is an inhibitor of flavoenzymes. Therefore, DPI can inhibit NADPH oxidase, and we used DPI (100 µmol/L final concentration) to confirm the inhibition of oxidative burst.

Histological studies

LVs of rats were fixed in 4% paraformaldehyde, embedded in paraffin, and cut into 3 µm sections at the level of the papillary muscle. Azan staining was performed
for evaluation of fibrotic changes. For evaluation of perivascular fibrosis, histology photographs were uploaded into image software (Meta Imaging Series version 7, Molecular Devices, Pennsylvania, USA), and subsequently the ratio of the fibrotic area surrounding the vessel wall to the total vessel area was calculated. The analysis of perivascular fibrosis was performed for 4-5 arteries for each rat and the average was used.
References


Table S 1. Results of two-way ANOVA in four groups of rats (NS-SHR, HS-SHR, NS-SHR/cp and HS-SHR/cp).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Influence of strain</th>
<th>Influence of salt loading</th>
<th>Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>f value</td>
<td>p value</td>
<td>f value</td>
</tr>
<tr>
<td>SBP 1wk</td>
<td>9.956</td>
<td>0.0065</td>
<td>21.813</td>
</tr>
<tr>
<td>SBP 2wk</td>
<td>9.813</td>
<td>0.0068</td>
<td>13.714</td>
</tr>
<tr>
<td>SBP 3wk</td>
<td>0.193</td>
<td>0.6665</td>
<td>119.262</td>
</tr>
<tr>
<td>SBP 4wk</td>
<td>0.803</td>
<td>0.3842</td>
<td>23.244</td>
</tr>
<tr>
<td>Body weight (BW)</td>
<td>124.378</td>
<td>&lt;0.0001</td>
<td>4.106</td>
</tr>
<tr>
<td>Cardiac weight (CW)</td>
<td>0.187</td>
<td>0.6707</td>
<td>30.85</td>
</tr>
<tr>
<td>CW/BW</td>
<td>3.269</td>
<td>0.0873</td>
<td>13.844</td>
</tr>
<tr>
<td>IVSd</td>
<td>4.743</td>
<td>0.0521</td>
<td>61.869</td>
</tr>
<tr>
<td>Fasting blood glucose</td>
<td>3.778</td>
<td>0.0617</td>
<td>0.547</td>
</tr>
<tr>
<td>Leptin</td>
<td>526.356</td>
<td>&lt;0.0001</td>
<td>18.33</td>
</tr>
<tr>
<td>Insulin</td>
<td>30.744</td>
<td>&lt;0.0001</td>
<td>0.035</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>9.552</td>
<td>0.0063</td>
<td>0.08</td>
</tr>
<tr>
<td>Free fatty acids</td>
<td>14.397</td>
<td>0.0013</td>
<td>0.56</td>
</tr>
<tr>
<td>LVSP</td>
<td>0.015</td>
<td>0.9036</td>
<td>22.294</td>
</tr>
<tr>
<td>EDcT</td>
<td>32.31</td>
<td>0.0001</td>
<td>27.004</td>
</tr>
<tr>
<td>-dp/dt_max</td>
<td>1.887</td>
<td>0.1855</td>
<td>4.341</td>
</tr>
<tr>
<td>T</td>
<td>4.810</td>
<td>0.0397</td>
<td>6.132</td>
</tr>
<tr>
<td>Perivascular fibrosis</td>
<td>2.583</td>
<td>0.1118</td>
<td>7.882</td>
</tr>
<tr>
<td>CTGF</td>
<td>1.343</td>
<td>0.2626</td>
<td>1.355</td>
</tr>
<tr>
<td>Col 3</td>
<td>1.362</td>
<td>0.2592</td>
<td>1.32</td>
</tr>
<tr>
<td>8-OHdG</td>
<td>4.031</td>
<td>0.0699</td>
<td>2.79</td>
</tr>
<tr>
<td>Cardiac ROS production by addition of NADPH</td>
<td>10.449</td>
<td>0.0056</td>
<td>7.942</td>
</tr>
<tr>
<td>p22phox</td>
<td>1.47</td>
<td>0.242</td>
<td>1.466</td>
</tr>
<tr>
<td>p47phox</td>
<td>2.463</td>
<td>0.135</td>
<td>1.635</td>
</tr>
<tr>
<td>gp91phox</td>
<td>2.148</td>
<td>0.161</td>
<td>2.046</td>
</tr>
<tr>
<td>Serum aldosterone</td>
<td>10.009</td>
<td>0.0075</td>
<td>18.551</td>
</tr>
<tr>
<td>NHE-1</td>
<td>1.315</td>
<td>0.2674</td>
<td>1.255</td>
</tr>
<tr>
<td>SgK-1</td>
<td>1.632</td>
<td>0.2186</td>
<td>1.56</td>
</tr>
<tr>
<td>ACE</td>
<td>24.95</td>
<td>0.0002</td>
<td>17.88</td>
</tr>
</tbody>
</table>
SBP: systolic blood pressure measured by tail-cuff method. IVSd: interventricular septal dimension measured by echocardiography. LVEF: left ventricular (LV) ejection fraction. LVSP: directly measured LV peak systolic pressure. EDcT: deceleration time of early mitral inflow obtained with Doppler echocardiography. –dp/dt\text{max} : maximal negative slope of the isovolumic relaxation phase obtained by the direct LV pressure monitoring. T: time constant of isovolumic relaxation phase obtained by the direct LV pressure monitoring. CTGF: cardiac mRNA expression of connective tissue growth factor. Col 3: cardiac mRNA expression of collagen type III. 8-OHdG: excretion of urinary 8-hydroxy-2’-deoxyguanosine. p22\text{phox}, p47\text{phox} and gp91\text{phox} : cardiac mRNA expression of NADPH oxidase components. NHE-1, SgK-1 and ACE: cardiac mRNA expression of Na\textsuperscript{+}/H\textsuperscript{+} exchanger-1, serum- and glucocorticoid-inducible kinase-1 and angiotensin-converting enzyme, respectively.
Figure legends

Figure S 1. Representative M-mode tracing of the LV chamber after 4 weeks of treatment. SHRs: spontaneously hypertensive rats. SHR/cps: SHR/NDmcr-cps. NS: normal salt (0.5% NaCl) diet. HS: high salt (8% NaCl) diet. TE: 4-hydroxy-2,2,6,6-tetramethyl-piperidine-N-oxyl (temol) (1 mmol/L in drinking water). EP: eplerenone (1.25 g/kg chow; 50-80 mg/kg/day). IVSd: inter ventricular septal dimension. LVDd: LV end-diastolic dimension. LVDs: LV end systolic dimension. PW: posterior wall dimension.

Figure S 2. Representative mitral inflow image obtained with Doppler echocardiography after 4 weeks of treatment. For abbreviations, see legend to Figure S 1.

Figure S 3. Representative pressure curve of the left ventricle after 4 weeks of treatment. For abbreviations, see legend to Figure S 1.

Figure S 4. Representative LV histopathological photomicrographs. (Azan staining: original magnification X200) After 4 weeks of treatment. For abbreviations, see legend to Figure S 1.