Metabolic Syndrome

Calorie Restriction Attenuates Cardiac Remodeling and Diastolic Dysfunction in a Rat Model of Metabolic Syndrome

Miwa Takatsu, Chieko Nakashima, Keiji Takahashi, Tamayo Murase, Takuya Hattori, Hiromi Ito, Toyoaki Murohara, Kohzo Nagata

Abstract—Calorie restriction (CR) can modulate the features of obesity-related metabolic and cardiovascular diseases. We have recently characterized DahlS.Z-Lepr+/Lepr+/ (DS/obese) rats, derived from a cross between Dahl salt-sensitive and Zucker rats, as a new animal model of metabolic syndrome. DS/obese rats develop hypertension and manifest left ventricular remodeling and diastolic dysfunction, as well as increased cardiac oxidative stress and inflammation. We have now investigated the effects of CR on cardiac pathophysiology in DS/obese rats. DS/obese rats were fed either normal laboratory chow ad libitum or a calorie-restricted diet (65% of the average food intake for ad libitum) from 9 to 13 weeks. Age-matched homozygous lean (DahlS.Z-Lepr+/Lepr+ or DS/lean) littersmates served as controls. CR reduced body weight in both DS/obese and DS/lean rats, as well as attenuated the development of hypertension in DS/obese rats without affecting blood pressure in DS/lean rats. CR also reduced body fat content, ameliorated left ventricular hypertrophy, fibrosis, and diastolic dysfunction, and attenuated cardiac oxidative stress and inflammation in DS/obese rats. In addition, it increased serum adiponectin concentration, as well as downregulated the expression of angiotensin-converting enzyme and angiotensin II type 1A receptor genes in the heart of DS/obese rats. Our results thus show that CR attenuated obesity and hypertension, as well as left ventricular remodeling and diastolic dysfunction in DS/obese rats, with these latter effects being associated with reduced cardiac oxidative stress and inflammation. (Hypertension. 2013;62:957-965.) • Online Data Supplement

Key Words: calorie restriction • cardiac remodeling, ventricular • diastolic heart failure • hypertension • inflammation • metabolic syndrome • oxidative stress • renin–angiotensin system

Metabolic syndrome (MetS), a complex of disorders including hypertension, diabetes mellitus, and dyslipidemia, is associated with the development of visceral obesity.1 Adipocytes in visceral fat of obese humans secrete a variety of biological agents that are known as adipocytokines and include proinflammatory cytokines, such as tumor necrosis factor-α and interleukin-6, as well as angiotensinogen and leptin.2 Recent studies have also revealed intricate interactions among adipocytes, the sympathetic nervous system, and the renin–angiotensin system (RAS) that contribute to the disturbed metabolic state associated with obesity.3

Calorie restriction (CR) is the most effective nutritional intervention to slow aging and prevent chronic disease in rodents,4 and it reduces visceral fat accumulation and body weight in obese humans.5 It also ameliorates hyperglycemia and hyperlipidemia,6,7 2 major risk factors for ischemic heart disease, as well as attenuates atherosclerotic lesion formation,8 pathological cardiac hypertrophy,9 and ischemia-induced myocardial damage in animal models.9 In addition, CR was found to lower blood pressure, to reduce systemic inflammation and myocardial fibrosis, and to attenuate diastolic dysfunction in humans.8 These various observations suggest that CR counteracts the complications of obesity.10

We recently established a new animal model of MetS, the DahlS.Z-Lepr+/Lepr+ (DS/obese) rat, by crossing Dahl salt-sensitive (DS) rats with Zucker rats harboring a missense mutation in the leptin receptor gene (Lepr).11 When fed a normal diet, DS/obese rats develop a phenotype (including salt-sensitive hypertension) similar to MetS in humans. They also develop cardiac abnormalities, as well as renal and liver damage, conditions that may be responsible for their premature death. The heart abnormalities include left ventricular (LV) diastolic dysfunction, hypertrophy, and fibrosis, and these conditions are accompanied by increased cardiac oxidative stress and inflammation.12 We have now investigated the effects of CR on cardiac pathophysiology in DS/obese rats.

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From the Departments of Pathophysiological Laboratory Sciences (M.T., K.T., T.M., T.H., H.I., K.N.) and Cardiology (T.M.), Nagoya University Graduate School of Medicine, Nagoya, Japan; and Department of Medical Technology, Nagoya University School of Health Sciences, Nagoya, Japan (C.N.).

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Correspondence to Kohzo Nagata, Department of Pathophysiological Laboratory Sciences, Nagoya University Graduate School of Medicine, 1-1-20 Daikominami, Higashi-ku, Nagoya 461-8673, Japan. E-mail nagata@met.nagoya-u.ac.jp

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**Methods**

**Animals and Experimental Protocols**

Animal experiments were approved by the Animal Experiment Committee of Nagoya University Graduate School of Medicine (Daiko district, approval Nos. 022-031, 023-026, and 024-010). Animals were handled in accordance with the Regulations on Animal Experiments of Nagoya University, as well as with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health publication No. 85-23, revised 1996). Eight-week-old male inbred DS/obese and DahlS.Z-Lepr+/Lepr− (DS/lean) rats were obtained from Japan SLC (Hamamatsu, Japan). After weaning, the rats were fed normal laboratory chow containing 0.36% NaCl. DS/obese rats were fed normal laboratory chow ad libitum (AL-DS/obese rats) or a calorie-restricted diet (CR-DS/obese rats) from 9 weeks and were compared with homozygous lean (DS/lean) littermates. The CR rats received 65% of the average food intake of AL rats for 4 weeks. To ensure that sodium intake was similar for AL and CR groups, we provided salt daily by direct addition to the water bottle for the latter group. Tap water was provided AL throughout the experimental period. Body weight, food and water intake, and blood pressure were measured weekly. At 9, 11, and 13 weeks, AL-DS/obese and CR-DS/obese rats were anesthetized by intraperitoneal ketamine (50 mg/kg) and were subjected to echocardiographic analysis. At 13 weeks, the animals were also subjected to cardiac catheterization under anesthesia with intraperitoneal ketamine/xylazine injection. At 9, 10, 11, and 13 weeks, the animals were anesthetized by intraperitoneal injection of pentobarbital (50 mg/kg), the heart, liver, kidneys, and both visceral (retroperitoneal) and subcutaneous (inguinal) fats were removed and weighed, and LV tissue was separated for analysis.

**Blood Pressure Measurement, Echocardiography, and Cardiac Catheterization**

Systolic blood pressure (SBP) and heart rate were measured weekly in conscious animals by tail-cuff plethysmography (BP-98A; Softron, Tokyo, Japan). SBP was measured 5x at each time point for each rat, and the average value was calculated. Rats were subjected to trans-thoracic echocardiography at 9, 11, and 13 weeks and cardiac catheterization at 13 weeks, as previously described. For evaluation of macrophage infiltration into the myocardium, tissue sections were subjected to immunostaining as described.14,16 For evaluation of macrophage infiltration into the myocardium, tissue sections were subjected to immunostaining for the monocyte–macrophage marker CD68 as described previously (further details are provided in the online-only Data Supplement).14

**Histology and Immunohistochemistry**

LV tissue from rats at 13 weeks was fixed with ice-cold 4% paraformaldehyde for 48 hours, embedded in paraffin wax, and processed for histology as described. For evaluation of macrophage infiltration into the myocardium, tissue sections were subjected to immunostaining for the monocyte–macrophage marker CD68 as described previously (further details are provided in the online-only Data Supplement).14

**Other Methods**

Measurement of metabolic and hormonal parameters, assay of superoxide production, quantitative reverse transcription polymerase chain reaction analysis, and immunoblot analysis are described in the online-only Data Supplement.

**Statistical Analysis**

Data are presented as mean±SEM. Differences between AL and CR groups of DS/obese rats at various ages were assessed with the Mann–Whitney U test. The time course of body weight, SBP, or echocardiographic measurements was compared among groups by 2-way repeated-measures ANOVA. A P<0.05 was considered statistically significant.

**Results**

**Physiology, LV Geometry, and Cardiac Function**

Body weight was increased in DS/obese rats compared with DS/lean rats at 8 weeks, and thereafter it was reduced by CR in both DS/obese and DS/lean rats (Figure 1A). AL-DS/obese rats progressively developed hypertension during the experimental period, and this change was significantly attenuated by CR (Figure 1B). In contrast, DS/lean rats fed normal laboratory chow ad libitum and calorie-restricted diet maintained a normal SBP. Heart rate and tibia length did not differ between AL-DS/obese and CR-DS/obese rats during the experimental period (Table 1). The ratios of heart or LV weight to tibia length (indices of cardiac and LV hypertrophy, respectively) were significantly smaller in CR-DS/obese rats than in AL-DS/obese rats at 10 weeks and thereafter (Table 1). The ratio of liver weight to tibia length tended to be smaller in CR-DS/obese rats than in AL-DS/obese rats at 10 and 11 weeks and was significantly smaller in CR-DS/obese rats at 13 weeks (Table 1). The ratio of kidney weight to tibia length was significantly smaller in CR-DS/obese rats than in AL-DS/obese rats at 10 weeks and thereafter (Table 1). Visceral fat mass tended to be smaller in CR-DS/obese rats than in AL-DS/obese rats at 10 (P=0.076) and 11 (P=0.077) weeks and was significantly smaller in CR-DS/obese rats at 13 weeks (Table 1). Subcutaneous fat mass was significantly smaller in CR-DS/obese rats than in AL-DS/obese rats at 10 weeks and thereafter (Table 1). Water intake was significantly reduced in CR-DS/obese rats compared with AL-DS/obese rats at 10 weeks and thereafter (data not shown).

Echocardiography revealed that the LV end-diastolic dimension, interventricular septum thickness, LV posterior wall thickness, and LV mass were smaller in CR-DS/obese rats than in AL-DS/obese rats at 13 weeks (Figure 2A and 2B; Table 2). However, LV fractional shortening and relative wall thickness...
did not differ between AL-DS/obese and CR-DS/obese rats (Figure 2C and 2D; Table 2). The ratio of early to late ventricular filling velocities (E/A), isovolumic relaxation time, deceleration time, and time constant of isovolumic relaxation (τ), all of which are indices of LV relaxation, as well as the ratio of LV end-diastolic pressure to LV end-diastolic dimension (an index of LV diastolic stiffness) and the Tei index, were all decreased in CR-DS/obese rats compared with AL-DS/obese rats (Figure 2E–2H; Table 2). E/A is increased in CR-DS/obese rats as shown in Figure 2E and Table 2.

### Table 1. Time Course of Physiological Parameters in AL-DS/Obese and CR-DS/Obese Rats From 9 to 13 Weeks

<table>
<thead>
<tr>
<th>Parameter</th>
<th>9 wk</th>
<th>10 wk</th>
<th>11 wk</th>
<th>13 wk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart rate, bpm</td>
<td>340.9±8.2</td>
<td>367.0±23</td>
<td>356.0±11</td>
<td>376.0±15</td>
</tr>
<tr>
<td>TL, mm</td>
<td>30.9±0.3</td>
<td>31.0±0.2</td>
<td>32.8±0.3</td>
<td>32.3±0.3</td>
</tr>
<tr>
<td>Heart weight/TL, mg/mm</td>
<td>31.1±0.8</td>
<td>30.6±0.5</td>
<td>34.4±0.9</td>
<td>31.7±0.4*</td>
</tr>
<tr>
<td>LV weight/TL, mg/mm</td>
<td>23.1±0.7</td>
<td>22.8±0.4</td>
<td>26.4±0.8</td>
<td>23.6±0.5*</td>
</tr>
<tr>
<td>Liver weight/TL, mg/mm</td>
<td>499±16</td>
<td>492±35</td>
<td>590±23</td>
<td>540±17</td>
</tr>
<tr>
<td>Kidney weight/TL, mg/mm</td>
<td>93.1±3.3</td>
<td>90.2±2.6</td>
<td>99.8±2.0</td>
<td>86.6±3.2*</td>
</tr>
<tr>
<td>Visceral fat weight/TL, mg/mm</td>
<td>327±14</td>
<td>318±15</td>
<td>377±21</td>
<td>319±12</td>
</tr>
<tr>
<td>Subcutaneous fat weight/TL, mg/mm</td>
<td>547±13</td>
<td>535±22</td>
<td>706±9.3</td>
<td>600±27*</td>
</tr>
</tbody>
</table>

Data are mean±SEM (n=5 for each group). AL-DS/obese indicates Dahl salt-sensitive (DS)/obese rat fed normal laboratory chow ad libitum; CR-DS/obese, DS/obese rat fed calorie-restricted diet; LV, left ventricular; and TL, tibial length. *P<0.05 vs age-matched AL-DS/obese.

### Hormonal and Metabolic Parameters

Plasma renin activity tended to be higher in CR-DS/obese rats than in AL-DS/obese rats at 13 weeks, whereas there was no difference in plasma aldosterone concentration between the 2 groups (Table 2). The aldosterone/renin ratio thus tended to be lower in CR-DS/obese rats. Fasting plasma glucose levels were similar in the AL and CR groups of DS/obese rats between 9 and 11 weeks, but they tended to be lower (P=0.064) in CR-DS/obese rats at 13 weeks (Table 3). Fasting plasma insulin concentration and the homeostatic model assessment-insulin...
Table 2. Cardiac Morphological and Functional Parameters, As Well As Plasma Renin and Aldosterone Levels, in AL-DS/Obese and CR-DS/Obese Rats at 13 Weeks

<table>
<thead>
<tr>
<th>Parameter</th>
<th>AL-DS/Obese</th>
<th>CR-DS/Obese</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>LVST, mm</td>
<td>2.09±0.05</td>
<td>1.68±0.02</td>
<td>0.002</td>
</tr>
<tr>
<td>LVDD, mm</td>
<td>2.07±0.07</td>
<td>1.71±0.02</td>
<td>0.002</td>
</tr>
<tr>
<td>LVDD, mm</td>
<td>8.41±0.15</td>
<td>7.57±0.09</td>
<td>0.006</td>
</tr>
<tr>
<td>LV mass, mg</td>
<td>1446±33</td>
<td>919±19</td>
<td>0.002</td>
</tr>
<tr>
<td>LVFS, %</td>
<td>52.7±2.41</td>
<td>56.2±1.12</td>
<td>0.110</td>
</tr>
<tr>
<td>RWT</td>
<td>0.49±0.02</td>
<td>0.45±0.01</td>
<td>0.025</td>
</tr>
<tr>
<td>E/A</td>
<td>1.43±0.06</td>
<td>1.87±0.05</td>
<td>0.003</td>
</tr>
<tr>
<td>IRT, ms</td>
<td>34.3±2.3</td>
<td>18.1±0.59</td>
<td>0.004</td>
</tr>
<tr>
<td>DCt, ms</td>
<td>60.3±1.5</td>
<td>47.9±1.1</td>
<td>0.034</td>
</tr>
<tr>
<td>Tei index</td>
<td>0.49±0.03</td>
<td>0.29±0.004</td>
<td>0.004</td>
</tr>
<tr>
<td>tau, ms</td>
<td>34.3±4.2</td>
<td>26.0±1.8</td>
<td>0.034</td>
</tr>
<tr>
<td>LVEDP, mmHg</td>
<td>7.5±0.6</td>
<td>4.4±0.6</td>
<td>0.034</td>
</tr>
<tr>
<td>LVEDP/LVDD, mmHg/mm</td>
<td>0.95±0.12</td>
<td>0.57±0.08</td>
<td>0.0495</td>
</tr>
<tr>
<td>Plasma renin activity, ng · mL⁻¹ · h⁻¹</td>
<td>0.75±0.13</td>
<td>2.1±0.52</td>
<td>0.069</td>
</tr>
<tr>
<td>Plasma aldosterone, pg/mL</td>
<td>270±21.7</td>
<td>231±12.3</td>
<td>0.286</td>
</tr>
<tr>
<td>Aldosterone/renin ratio</td>
<td>404±93.0</td>
<td>149±41.1</td>
<td>0.055</td>
</tr>
</tbody>
</table>

Data are mean±SEM (n=7 and 8 for AL-DS/obese and CR-DS/obese groups, respectively). AL-DS/obese indicates Dahl salt-sensitive (DS)/obese rat fed normal laboratory chow ad libitum; CR-DS/obese, DS/obese rat fed calorie-restricted diet; DCt, deceleration time; E/A, the ratio of peak flow velocity at the mitral level during rapid filling (E) to that during atrial contraction (A); IRT, isovolumic relaxation time; IVST, interventricular septum thickness; LV, left ventricular; LVDD, LV end-diastolic dimension; LVEDP, LV end-diastolic pressure; LVFS, LV fractional shortening; LVPWT, LV posterior wall thickness; and RWT, relative wall thickness.

resistance did not differ significantly between the AL and CR groups of DS/obese rats during the experimental period, whereas the fasting plasma leptin level was markedly lower in CR-DS/obese rats at 11 and 13 weeks (Table 3). Serum adiponectin concentration was significantly higher in CR-DS/obese rats compared with AL-DS/obese rats at 13 weeks (Figure 3A–3C). The expression of growth hormone releasing hormone (GHRH) gene also tended to be downregulated in CR-DS/obese rats (Figure 3D). The expression of the mineralocorticoid receptor (MR) and serum/glucocorticoid-regulated kinase 1 (Sgk1) genes in the LV tended to be downregulated in CR-DS/obese rats (Figure 4C and 4D).

Cardiac Oxidative Stress

Superoxide production in myocardial tissue sections, as revealed by staining with dihydroethidium, and the activity of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase in LV homogenates were significantly decreased in CR-DS/obese rats compared with AL-DS/obese rats at 13 weeks (Figure 5A–5C). The expression of genes for the p22phox and gp91phox membrane components and for the p47phox and p67phox cytosolic components of NADPH oxidase in the LV was also downregulated in CR-DS/obese rats (Figure 5D–5G). The abundance of mRNA for the Rac1 cytosolic component of NADPH oxidase did not differ between the 2 groups (data not shown).

Cardiac Inflammation

Immunostaining for the monocyte–macrophage marker CD68 revealed that the extent of macrophage infiltration in the LV myocardium was decreased in CR-DS/obese rats at 13 weeks compared with AL-DS/obese rats (Figure 5H and 5I). The expression of monocyte chemoattractant protein-1 and osteopontin genes in the LV was also attenuated in CR-DS/obese rats (Figure 5J and 5K).

Table 3. Time Course of Hormonal Parameters in AL-DS/Obese and CR-DS/Obese Rats

<table>
<thead>
<tr>
<th>Parameter</th>
<th>9 wk</th>
<th>10 wk</th>
<th>11 wk</th>
<th>13 wk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma glucose, mg/dL</td>
<td>137±4.0</td>
<td>149±5.4</td>
<td>140±7.1</td>
<td>138±9.3</td>
</tr>
<tr>
<td>Plasma insulin, ng/mL</td>
<td>3.38±1.25</td>
<td>3.52±0.82</td>
<td>4.35±1.11</td>
<td>4.40±1.24</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>28.5±14.7</td>
<td>33.7±8.1</td>
<td>43.5±12.4</td>
<td>41.3±13.1</td>
</tr>
<tr>
<td>Plasma leptin, ng/mL</td>
<td>34.0±2.0</td>
<td>29.4±2.5</td>
<td>36.5±2.4</td>
<td>32.9±1.0</td>
</tr>
<tr>
<td>Serum adiponectin, ng/mL</td>
<td>6138±371</td>
<td>6488±189</td>
<td>5677±168</td>
<td>5928±79.4</td>
</tr>
<tr>
<td>Plasma TNF-α, pg/mL</td>
<td>2.86±0.94</td>
<td>2.79±0.29</td>
<td>3.00±0.75</td>
<td>3.36±0.62</td>
</tr>
</tbody>
</table>

Data are mean±SEM (n=5 for each group). AL-DS/obese indicates Dahl salt-sensitive (DS)/obese rat fed normal laboratory chow ad libitum; CR-DS/obese, DS/obese rat fed calorie-restricted diet; HOMA-IR, homeostatic model assessment-insulin resistance; and TNF, tumor necrosis factor-α.

*P<0.05 vs age-matched AL-DS/obese.
Here, we have shown that CR attenuated obesity, hypertension, as well as LV hypertrophy, fibrosis, and diastolic dysfunction in DS/obese rats, and that the beneficial cardiac effects of CR were associated with inhibition of cardiac oxidative stress and inflammation. Increased adiponectin, as well as downregulation of RAS-related gene expression in the myocardium, may have contributed to the amelioration of cardiac remodeling and diastolic dysfunction by CR in these animals.

Increased oxidative stress is associated with obesity in experimental animals and humans and may contribute to the development of MetS. We recently found that both NADPH-dependent superoxide generation and the expression of NADPH oxidase subunit genes are increased in the heart of DS/obese rats, consistent with previous results showing that oxidative damage plays a central role in endothelial dysfunction both during aging and in the setting of cardiovascular disease. We have now shown that these changes in the heart of DS/obese rats were attenuated by CR for 4 weeks, consistent with previous observations that CR attenuated cardiac oxidative damage and mitochondrial oxidant generation. Previously, CR of 40% for 12 months was also found...
to reduce mitochondrial generation of hydrogen peroxide, free radical leakage, and oxidative damage to mitochondrial DNA in the heart of aged rats, and 50% CR for 35 days reduced myocardial levels of lipoperoxidation in young rats.

Macrophages are implicated in fibrosis associated with various pathological conditions. We recently showed that macrophage infiltration into the interstitial space of the LV myocardium is accompanied by increased expression of genes for proinflammatory proteins such as monocyte chemoattractant protein-1 and osteopontin in the heart of DS/obese rats, consistent with previous observations showing the upregulation of proinflammatory factors in obese humans. These changes may thus have contributed to the development of myocardial fibrosis. CR of 35% for 1 month reduced macrophage infiltration into the interstitial space of the myocardium, as well as the expression of monocyte chemoattractant protein-1 and osteopontin genes in LV tissue of DS/obese rats, suggesting that CR attenuated the cardiac inflammatory response in these animals. With regard to the effect of CR on inflammation in the presence of cardiovascular disease, a lifelong reduction in food intake of 40% attenuated the myocardial inflammatory response to ischemia-reperfusion in rats. Furthermore, 15% CR reduced the plasma levels of interleukin-6 and tumor necrosis factor-α in salt-sensitive rats fed a high-salt diet. The plasma concentration of tumor necrosis factor-α did not differ significantly between the AL and CR groups of DS/obese rats during the experimental period although expression of genes for proinflammatory proteins was significantly reduced in the heart of CR-DS/obese rats at 13 weeks. It is thus possible that circulating levels of cytokines may have been confounded by differential expression of those cytokines in multiple tissues.

CR is thought to result in a general improvement in insulin sensitivity, leading to reduced plasma concentrations of glucose and insulin, as well as improved glucose tolerance. We recently showed that DS/obese rats develop insulin resistance and type II diabetes mellitus. In the present study, however, plasma glucose concentrations did not differ significantly between the AL and CR groups of DS/obese rats between 9 and 13 weeks although they tended to be lower in CR-DS/obese rats at 13 weeks. Plasma insulin concentrations and homeostatic model assessment-insulin resistance also did not differ significantly between the AL and CR groups of DS/obese rats. These data are consistent with the previous observation that CR did not fully ameliorate insulin resistance in Zucker fatty (fa/fa) rats. The accumulation of retroperitoneal and total body fat with aging leads to impaired glucose uptake in muscle and to a state of insulin resistance that is difficult to reverse. Subcutaneous fat mass was significantly reduced in CR-DS/obese rats compared with AL-DS/obese rats at 10 to 13 weeks. In contrast, visceral (retroperitoneal) fat mass tended to be smaller in CR-DS/obese rats at 10 and 11 weeks and was significantly reduced at 13 weeks. An insufficient reduction in visceral fat mass induced by CR in DS/obese rats may thus have limited any amelioration of insulin resistance.

Leptin is an adipokine with a pivotal role in regulation of food intake, energy expenditure, body weight, and neuroendocrine function, and serum levels of this hormone are proportional to fat mass. We previously showed that the serum concentration of leptin is markedly increased in DS/obese rats compared with DS/lean rats, suggesting that the obesity of the former animals is the result of leptin resistance. Hyperleptinemia and leptin resistance occur in animals with diet-induced obesity, as well as in obese humans. We have now found that plasma leptin levels were substantially reduced by CR in DS/obese rats at 11 and 13 weeks, likely as a result of an associated reduction in fat mass. However, leptin resistance itself was likely unaffected by CR because of the mutation in the leptin receptor in DS/obese rats.

CR markedly attenuated hypertension in DS/obese rats, but it did not affect SBP in DS/lean rats. CR was previously shown to reduce blood pressure in animals and humans. Given that CR attenuated oxidative stress in the heart of DS/obese rats, the antihypertensive effect of CR in these animals may result from a reduced superoxide-dependent conversion of vasodilatory nitric oxide to peroxynitrite. Insulin resistance and inflammation may alter vascular function and thereby lead to hypertension. In DS/
obese rats, CR inhibited cardiac inflammation but did not ameliorate insulin resistance. The lowering of blood pressure by CR in DS/obese rats may thus also be attributable, at least in part, to inhibition of vascular inflammation.

Obesity, especially when complicated by hypertension, is associated with changes in cardiac structure and function. CR attenuates obesity, hypertension, as well as LV hypertrophy, fibrosis, and diastolic dysfunction in DS/obese rats. CR may, therefore, alter LV structure and function, as well as blood pressure in hypertensive obese humans. In the present study, the beneficial effects of CR on LV remodeling and diastolic dysfunction could not be separated from its antihypertensive effect. However, CR has been found to reduce cardiovascular risk in experimental models and humans. CR prevents hypertension and cardiac hypertrophy in spontaneously hypertensive rats, ameliorates cardiac remodeling and diastolic dysfunction in DS rats, changes gene expression in a manner consistent with reduced cardiac remodeling and fibrosis and enhanced contractility and energy generation via lipid β-oxidation in mice, and protects cardiovascular tissue from oxidative stress in type II diabetic rats.

The RAS is implicated in the pathogenesis of MetS. We recently showed that plasma renin activity is markedly reduced by calorie restriction (CR) on oxidative stress and inflammation in the left ventricle of DS/obese rats at 13 weeks. A and B, Superoxide production in the left ventricular (LV) myocardium as revealed by dihydroethidium staining. Representative sections (scale bars, 200 µm) are shown in A, and relative dihydroethidium fluorescence intensity determined from similar sections is shown in B. C, Nicotinamide adenine dinucleotide phosphate (NADPH)-dependent superoxide production in LV homogenates. Results are expressed as relative light units (RLU) per milligram of protein. D–G, Quantitative reverse transcription polymerase chain reaction (RT-PCR) analysis of p22phox, gp91phox, p47phox, and p67phox mRNAs, respectively. H and I, Macrophage infiltration as revealed by immunohistochemical staining for CD68. Representative sections (scale bars, 100 µm) are shown in H, and the density of CD68-positive cells determined from similar sections is shown in I. J and K, Quantitative RT-PCR analysis of monocyte chemoattractant protein-1 (MCP-1) and osteopontin mRNAs, respectively. Data in B–G and I–K are mean±SEM (n=7 and 8 for DS/obese rats fed normal laboratory chow ad libitum [AL-DS/obese] and calorie-restricted diet [CR-DS/obese], respectively). *P<0.05 vs AL-DS/obese.
in DS/obese rats compared with DS/lean rats, whereas the serum aldosterone concentration does not differ between the 2 strains, indicative of inappropriate aldosterone secretion in DS/obese rats.11 Our present observations that CR tended to increase plasma renin activity but did not affect plasma aldosterone concentration in DS/obese rats seem somewhat inconsistent with previous results showing that weight loss is associated with a reduction in plasma renin activity and aldosterone concentration, as well as a lowering of blood pressure in hypertensive obese humans but not in normotensive obese individuals.41 The tendency of CR to reduce the aldosterone/renin ratio may have contributed to the amelioration of cardiac remodeling and dysfunction, as well as to the lowering of blood pressure induced by CR in DS/obese rats. We also previously found that the expression of angiotensin-converting enzyme and angiotensin II type 1A receptor genes is increased in the heart of DS/obese rats,13 consistent with previous data showing that the cardiac RAS is activated in response to pressure overload in rats with salt-sensitive hypertension.12,42 Although the serum aldosterone concentration is not altered in DS/obese rats, the expression of genes for the MR and the aldosterone effector kinase Sgrk1 is upregulated in the heart of these animals,13 consistent with a pathological role for aldosterone-independent MR activation.43 In the present study, CR downregulated the expression of RAS-related genes, as well as tended to attenuate MR signaling in the heart of DS/obese rats.

CR increased serum adiponectin concentration and attenuated cardiac remodeling and dysfunction in DS/obese rats, consistent with previous results that CR has the cardioprotective effects via increased production of adiponectin and the associated activation of AMP-activated protein kinase.9 Adiponectin is known as an anti-inflammatory adipocytokine and is regulated by renin–angiotensin–aldosterone system. Our data are consistent with previous results that osteopontin and adiponectin are related to each other, underlying the mechanisms of renin–angiotensin–aldosterone system and inflammation.44 It has proved difficult to determine whether the beneficial effect of CR on cardiac injury was mediated via the reduction of blood pressure or the direct effects on the myocardium. A recent study suggests that CR may have direct effects on cardiac growth in addition to the benefits of blood pressure lowering in spontaneously hypertensive rats.34 Together, these data suggest that CR increases circulating adiponectin levels and downregulated the cardiac RAS in DS/obese rats, contributing to the reduction of cardiac damage. Experimental CR and experimental and clinical RAS blockade manifest overlapping physiological and molecular effects.45

Perspectives
CR attenuated obesity, hypertension, as well as LV hypertrophy, fibrosis, and diastolic dysfunction in DS/obese rats. These effects were associated with inhibition of cardiac oxidative stress and inflammation. Increased adiponectin, as well as downregulation of the cardiac RAS by CR, may have contributed to its beneficial effects on cardiac remodeling and diastolic dysfunction in these animals. The tendency of CR to reduce relative aldosterone excess and cardiac MR signaling may also have contributed to the cardioprotective, as well as antihypertensive, effects of this dietary manipulation.

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Disclosures
None.

References


Novelty and Significance

What Is New?
- Calorie restriction attenuates obesity, hypertension, as well as cardiac hypertrophy, fibrosis, and diastolic dysfunction in Dahl S.Z-Lepr(fa)/Lepr(fa) rats, derived from a cross between Dahl salt-sensitive and Zucker rats as a new animal model of metabolic syndrome.
- The cardioprotective effects of calorie restriction are associated with inhibition of cardiac oxidative stress and inflammation.
- Calorie restriction increased serum adiponectin concentration, as well as downregulated the expression of renin-angiotensin system–related genes in the heart.

What Is Relevant?
- To date, calorie restriction is the most effective intervention for extending lifespan in a variety of species. Previous studies investigated the effects of calorie restriction on metabolism and cardiac pathophysiology in hypertensive and normal animals, as well as in humans. Also, there are plenty of data on the effects of calorie restriction on metabolism and adipose tissue pathophysiology in animals with obesity and metabolic syndrome. However, little information is available about the effects of calorie restriction on cardiac pathophysiology in animals with metabolic syndrome.

Summary
Calorie restriction attenuates obesity and hypertension, as well as cardiac remodeling and diastolic dysfunction in metabolic syndrome, with these latter effects being associated with reduced cardiac oxidative stress and inflammation.
Calorie Restriction Attenuates Cardiac Remodeling and Diastolic Dysfunction in a Rat Model of Metabolic Syndrome
Miwa Takatsu, Chieko Nakashima, Keiji Takahashi, Tamayo Murase, Takuya Hattori, Hiromi Ito, Toyoaki Murohara and Kohzo Nagata

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CALORIE RESTRICTION ATTENUATES CARDIAC REMODELING AND DIASTOLIC DYSFUNCTION IN A RAT MODEL OF METABOLIC SYNDROME

Miwa Takatsu,* Chieko Nakashima,† Keiji Takahashi,* Tamayo Murase,* Takuya Hattori,* Hiromi Ito,* Toyoaki Murohara,‡ Kohzo Nagata*

*Department of Pathophysiological Laboratory Sciences and ‡Department of Cardiology, Nagoya University Graduate School of Medicine, Nagoya, Japan; †Department of Medical Technology, Nagoya University School of Health Sciences, Nagoya, Japan

Address correspondence to: Kohzo Nagata, MD, PhD, Department of Pathophysiological Laboratory Sciences, Nagoya University Graduate School of Medicine, 1-1-20 Daikominami, Higashi-ku, Nagoya 461-8673, Japan. Tel./Fax: +81-52-719-1546. E-mail: nagata@met.nagoya-u.ac.jp
Supplemental Methods
Echocardiographic and hemodynamic analyses
M-mode echocardiography was performed with a 12.5-MHz transducer (Xario SSA-660A; Toshiba Medical Systems, Tochigi, Japan). LVDd, LV end-systolic dimension (LVDs), IVST, and LVPWT were measured, and LVFS, RWT, and LV mass were calculated as follows: LVFS (%) = [(LVDd – LVDs)/LVDd] × 100; RWT = (IVST + LVPWT)/LVDd; and LV mass (g) = ([1.04 × (IVST + LVDd + LVPWT)^3 – (LVDd)^3]) × 0.8) + 0.14. For assessment of Doppler-derived indices of LV function, both LV inflow and outflow velocity patterns were simultaneously recorded by pulsed-wave Doppler echocardiography. For evaluation of LV diastolic function, we calculated DcT and IRT. Both the isovolumic contraction time (ICT) and ejection time (ET) were also determined, and the Tei index, which reflects both LV diastolic and systolic function, was calculated as (ICT + IRT)/ET. After echocardiography, a 2F micromanometer-tipped catheter (SPR-407; Millar Instruments, Houston, TX) that had been calibrated relative to atmospheric pressure was inserted through the right carotid artery into the left ventricle. Tracings of LV pressure and the electrocardiogram were digitized to determine LVEDP. Tau was calculated by the derivative method of Raff and Glantz, as described previously.

Histology and immunohistochemistry
Transverse sections (thickness, 3 µm) of the left ventricle were stained either with hematoxylin-eosin for routine histological examination or with Azan-Mallory solution for evaluation of the extent of fibrosis. To evaluate macrophage infiltration into the myocardium, we performed immunostaining for the monocyte-macrophage marker CD68 with frozen sections (thickness, 5 µm) that had been fixed with acetone. Endogenous peroxidase activity was blocked by exposure of the sections to methanol containing 0.3% hydrogen peroxide. Sections were incubated at 4°C first overnight with mouse monoclonal antibodies to CD68 (diluted 1:100, clone ED1; Chemicon, Temecula, CA) and then for 30 min with Histofine Simple Stain Rat MAX PO (Nichirei Biosciences, Tokyo, Japan). Immune complexes were visualized with diaminobenzidine and hydrogen peroxide, and the sections were counterstained with hematoxylin. All image analysis was performed with the use of NIH Scion Image software (Scion, Frederick, MD).

Measurement of metabolic and hormonal parameters
Blood was collected from the right carotid artery of rats deprived of food overnight and was centrifuged at 1400 × g for 10 min at room temperature. The resultant plasma supernatant was maintained at −80°C until analysis. Plasma levels of glucose, creatinine, total cholesterol, low density lipoprotein (LDL)–cholesterol, high density lipoprotein (HDL)–cholesterol, triglyceride, free fatty acids, aspartate aminotransferase (AST), and alanine aminotransferase (ALT) were measured by routine enzymatic assays. The plasma concentrations of insulin and leptin were measured with the use of mouse/rat enzyme-linked immunosorbent assay kits (Morinaga Bioscience Institute, Yokohama, Japan). Insulin resistance was assessed from fasting insulin and glucose levels according to HOMA-IR [fasting glucose (mmol/L) × fasting insulin (µU/mL)/22.5]. The serum concentration of adiponectin was measured with the use of mouse/rat enzyme-linked immunosorbent assay kits (Otsuka Pharmaceutical, Tokyo, Japan). The plasma concentration of TNF-α was measured with the use of mouse/rat enzyme-linked immunosorbent assay kits (R&D systems, Inc. Minneapolis, USA). Urine volume, urinary protein, and the ratio of creatinine clearance to kidney weight or body weight were also determined. Creatinine clearance (Ccr) was calculated from the standard formula U/V/P, where U is the urinary creatinine concentration, V is the 24-h urine volume, and P is the plasma creatinine concentration. Plasma renin activity and aldosterone concentration were determined by radioimmunoassay with the use of renin RIA beads (Abbott Japan, Tokyo, Japan) and a DPC aldosterone kit (Mitsubishi Chemical Medience, Tokyo, Japan),
respectively.

**Superoxide production**

NADPH-dependent superoxide production by homogenates prepared from freshly frozen LV tissue was measured with an assay based on lucigenin-enhanced chemiluminescence as described previously.\(^5\) The chemiluminescence signal was sampled every minute for 10 min with a microplate reader (Wallac 1420 Arvo MX/Light, Perkin-Elmer, Waltham, MA), and the respective background counts were subtracted from experimental values. Lucigenin chemiluminescence was expressed as relative light units per milligram of protein. Superoxide production in tissue sections was examined by staining with dihydroethidium (Sigma, St. Louis, MO) as described.\(^6\) Dihydroethidium is rapidly oxidized by superoxide to yield fluorescent ethidium, and the sections were examined with a fluorescence microscope equipped with a 585-nm long-pass filter. As a negative control, we performed dihydroethidium staining after incubation of sections with superoxide dismutase (300 U/mL), and we confirmed that this procedure abolished the fluorescence (data not shown). The average of dihydroethidium fluorescence intensity values was calculated with the use of NIH Image software (ImageJ).\(^7\)

**Quantitative RT-PCR analysis**

Total RNA was extracted from LV tissue and treated with DNase with the use of a spin-vacuum total RNA isolation kit (Promega, Madison, WI). Portions of the RNA (2 µg) were subjected to RT with the use of random primers (Invitrogen, Carlsbad, CA) and MuLV Reverse Transcriptase (Applied Biosystems, Foster City, CA). The resulting cDNA was subjected to real-time PCR analysis with the use of a Prism 7000 Sequence Detector (Perkin-Elmer), as previously described,\(^8\) and with primers and TaqMan probes specific for ANP,\(^8\) BNP,\(^8\) collagen types I or III,\(^9\) TGF-β1,\(^8\) CTGF,\(^5\) ACE,\(^8\) the AT\(_{1A}\) receptor,\(^8\) the MR,\(^10\) Sgk1,\(^10\) MCP-1,\(^5\) osteopontin,\(^5\) or the p22\(^{phox}\),\(^11\) gp91\(^{phox}\),\(^11\) p47\(^{phox}\),\(^10\) p67\(^{phox}\),\(^10\) and Rac1\(^10\) subunits of NADPH oxidase. Reagents for detection of human GAPDH mRNA (Applied Biosystems) were used to quantify rat GAPDH mRNA as an internal standard.

**Immunoblot analysis**

Total protein was isolated from LV tissue and quantitated with the use of the Bradford reagent (Bio-Rad, Hercules, CA). Equal amounts of protein were subjected to SDS-polyacrylamide gel electrophoresis, and the separated proteins were transferred to a polyvinylidene difluoride membrane as described previously.\(^12\) The membrane was incubated first with a 1:1000 dilution of rabbit polyclonal antibodies to the AT\(_1\) receptor (Santa Cruz Biotechnology, Santa Cruz, CA) and then with a 1:2000 dilution of horseradish peroxidase–conjugated goat antibodies to rabbit immunoglobulin G (Medical and Biological Laboratories, Nagoya, Japan). Antibodies to GAPDH (Santa Cruz Biotechnology) were used to confirm equal loading of samples. Detection and quantification of immune complexes were performed as described previously.\(^12\)

**Supplemental Results**

Plasma concentrations of total cholesterol, LDL-cholesterol, and triglyceride as well as the ratio of LDL-cholesterol to HDL-cholesterol levels were reduced in CR-DS/obese rats compared with AL-DS/obese rats (Supplemental Table S1). Plasma AST and ALT levels did not differ between the two groups (Supplemental Table S1). Plasma creatinine levels were also similar in both groups, whereas urinary protein concentration tended to be lower in CR-DS/obese rats. The ratio of creatinine clearance to either body or kidney weight did not differ between the two groups.
Supplemental References


**Supplemental Table S1.** Plasma and urinary parameters in AL-DS/obese and CR-DS/obese rats at 13 weeks of age.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>AL-DS/obese</th>
<th>CR-DS/obese</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol (mg/dL)</td>
<td>445.6±89.8</td>
<td>212.4±18.5</td>
<td>0.028</td>
</tr>
<tr>
<td>LDL-cholesterol (mg/dL)</td>
<td>82.8±13.5</td>
<td>45.9±7.6</td>
<td>0.038</td>
</tr>
<tr>
<td>HDL-cholesterol (mg/dL)</td>
<td>88.8±9.4</td>
<td>106.1±6.0</td>
<td>0.123</td>
</tr>
<tr>
<td>LDL-cholesterol/HDL-cholesterol</td>
<td>1.08±0.36</td>
<td>0.42±0.05</td>
<td>0.014</td>
</tr>
<tr>
<td>Triglyceride (mg/dL)</td>
<td>2667.2±546.9</td>
<td>811.6±143.5</td>
<td>0.005</td>
</tr>
<tr>
<td>Free fatty acids (mEq/L)</td>
<td>1.63±0.08</td>
<td>1.68±0.26</td>
<td>0.570</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>41.8±7.3</td>
<td>46.2±3.3</td>
<td>0.141</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>43.6±6.0</td>
<td>37.3±3.2</td>
<td>0.357</td>
</tr>
<tr>
<td>Plasma creatinine (mg/dL)</td>
<td>0.22±0.04</td>
<td>0.26±0.02</td>
<td>0.289</td>
</tr>
<tr>
<td>Urinary protein (mg/day)</td>
<td>1652±108</td>
<td>1362±71.3</td>
<td>0.062</td>
</tr>
<tr>
<td>Ccr (mL min(^{-1}) per g kidney weight)</td>
<td>0.61±0.09</td>
<td>0.67±0.05</td>
<td>0.345</td>
</tr>
<tr>
<td>Ccr (mL min(^{-1}) per 100 g body weight)</td>
<td>0.48±0.07</td>
<td>0.45±0.03</td>
<td>0.345</td>
</tr>
</tbody>
</table>

Data are means ± SEM (n = 7 and 8 for AL-DS/obese and CR-DS/obese groups, respectively).