Restraint stress exacerabtes cardiac and adipose tissue pathology via β-adrenergic signaling in rats with metabolic syndrome

Natsumi Matsuura,1 Kai Nagasawa,1 Yuji Minagawa,2 Shogo Ito,1 Yusuke Sano,1 Yuichiro Yamada,1 Takuya Hattori,1 Shogo Watanabe,1 Toyoaki Murohara,3 and Kohzo Nagata1

1Department of Pathophysiological Laboratory Sciences, Nagoya University Graduate School of Medicine, Nagoya, Japan; 2Department of Medical Technology, Nagoya University School of Health Sciences, Nagoya, Japan; and 3Department of Cardiology, Nagoya University Graduate School of Medicine, Nagoya, Japan

Submitted 18 December 2014; accepted in final form 10 March 2015

METABOLIC SYNDROME (MetS) is a complex of highly debilitating disorders, including hypertension, diabetes mellitus, and dyslipidemia, and is characterized by both chronic low-grade inflammation and insulin resistance/hyperinsulinemia associated with abdominal obesity (35). The increase in the prevalence of MetS worldwide highlights the need to identify underlyng factors that may render an individual susceptible to this condition. Stress and obesity have similar effects on MetS, but the relation between stress and obesity remains unclear.

Although evidence suggests that stress and obesity are related to the hypothalamic control of hypertension and metabolism, little is known of the mechanisms by which stress affects adiposity.

Chronic psychological stress has been linked to various negative health consequences, including effects on cardiovascular, endocrine, and immune systems. Major stress-related events (47) as well as chronic work stress (1) have been associated with MetS. Stress stimulates both the sympathetic nervous system (SNS) and hypothalamic-pituitary-adrenal (HPA) axis, which together account for the major components of the stress response. Cold stress induces the release of cortisol (corticosterone) via activation of the HPA axis as well as the release of the adrenergic co-transmitter neuropeptide Y (NPY), the latter of which stimulates angiogenesis as well as predisapycyte proliferation, differentiation, and lipid-filling (adipogenesis) by activating NPY Y2 receptors (16). On the other hand, restraint stress preferentially induces the release of noradrenaline via activation of the SNS (without a substantial increase in NPY release), resulting in increased β-adrenergic thermogenesis in brown adipose tissue and lipolysis in white adipose tissue, which together lead to weight loss (17).

We recently established a new animal model of MetS, the DahlS.Z-Leprfa+/Leprfa (DS/obese) rat, by crossing Dahl salt-sensitive rats with Zucker rats harboring a missense mutation of the leptin receptor gene (Lepr). When fed a normal diet, DS/obese rats develop a phenotype, including hypertension, that resembles MetS in humans. They also develop cardiac abnormalities as well as fat-induced liver damage (7). The left ventricular (LV) hypertrophy, fibrosis, and diastolic dysfunction manifested in these animals are associated with increased cardiac oxidative stress and inflammation as well as with activation of the cardiac renin-angiotensin-aldosterone system (RAAS) (24). In the present study, we investigated the effects of restraint stress on cardiac and adipose tissue pathology as well as on metabolic disorders in DS/obese rats.

MATERIALS AND METHODS

Animals and experimental protocols. Animal experiments were approved by the Animal Experiment Committee of Nagoya University Graduate School of Medicine (Duiko district, approval nos. 025-025 and 026-008). Eight-week-old male inbred DS/obese rats were obtained from Japan SLC (Hamamatsu, Japan) and were handled in accordance with guidelines of the Nagoya University Graduate School of Medicine as well as with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (NIH Pub. No. 85-23, Revised 1996). Animals were fed normal laboratory chow containing 0.36% NaCl, and both the diet and tap water were provided ad libitum throughout the experimental period. DS/obese rats were

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exposed to restraint stress (restraint cage, 2 h/day) for 4 wk from 9 wk of age either with (RS + Prop group) or without (RS group) administration of the β-adrenergic receptor (β-AR) blocker propranolol (2 mg/kg body wt). Rats were stressed in the morning from 09:00 to 11:00 hours, and propranolol or saline vehicle was administered subcutaneously at 08:30 hours every day. DS/obese rats not subjected to daily stress or injection served as the MetS group. Age-matched male homozygous lean littermates of DS/obese rats, designated DahlS.Z-Lepr+/Lepr+ (DS/lean) rats, served as control animals (Cont group). Body weight as well as food and water intake were measured weekly. At 13 wk of age, rats were placed in metabolic cages for the collection of 24-h urine specimens. For the oral glucose tolerance test (OGTT), glucose (2 g/kg) was administered orally early in the morning to animals that had been deprived of food overnight (48). For the insulin tolerance test (ITT), rats were injected with human insulin (0.75 U/kg) intraperitoneally after deprivation of food for 6 h beginning at 09:00 hours (48). Blood glucose concentrations were measured with the use of a glucose analyzer (Glutest Neo Super, Sanwa Kagaku Kenkyusho, Nagoya, Japan). Blood glucose responses during the OGTT and ITT were calculated by the area under the curve using the trapezoidal method (48). Rats were anesthetized by an intraperitoneal injection of ketamine (50 mg/kg) and xylazine (10 mg/kg) for echocardiographic and hemodynamic analyses. They were subsequently killed by an intraperitoneal injection of an overdose of pentobarbital sodium (50 mg/kg), and the heart, liver, and both visceral (retroperitoneal, epididymal, and mesenteric) and subcutaneous (inguinal) fat tissue were removed and weighed. LV tissue was also separated for analysis.

Echocardiographic and hemodynamic analyses. Systolic blood pressure (SBP) and heart rate were measured weekly in conscious animals by tail-cuff plethysmography (BP-98A, Softron, Tokyo, Japan). At 13 wk of age, rats were subjected to transthoracic echocardiography, as previously described (29). M-mode echocardiography was performed with a 12.5-MHz transducer (Xario SSA-660A, Toshiba Medical Systems, Tochigi, Japan). LV end-diastolic (LVEDd) and end-systolic (LVESd) dimensions as well as the thickness of the interventricular septum (IVST) and LV posterior wall (LVPWT) were measured, and LV fractional shortening (LVFS), relative wall thickness (RWT), and LV mass were calculated as follows: LVFS (in %) = [(LVEDd − LVESd)/LVEDd] × 100; RWT = [(IVST + LVPWT)/LVEDd]; and LV mass (in g) = [1.04 × [(IVST + LVEDd + LVPWT)3 − (LVESd)3] − 0.8] + 0.14. LV ejection fraction (LVEF) was calculated with the formula of Teichholz (37). For assessment of Doppler-derived indexes of LV function, both LV inflow and outflow velocity patterns were simultaneously recorded by pulsed-wave Doppler echocardiography. For assessment of LV diastolic function, we calculated the deceleration time during the OGTT and ITT were calculated by the area under the curve, as previously described (14). Tracings of LV pressure and the ECG were digitized to Houston, TX) that had been calibrated relative to atmospheric pressure (SBP) and heart rate were measured weekly in conscious (inguinal) fat tissue were removed and weighed. LV tissue was subsequently killed by an intraperitoneal injection of an overdose of pentobarbital sodium (50 mg/kg), and the heart, liver, and both visceral (retroperitoneal, epididymal, and mesenteric) and subcutaneous (inguinal) fat tissue were removed and weighed. LV tissue was also separated for analysis.

Histology and immunohistochemistry. LV and visceral (retroperitoneal) fat tissue was fixed in 4% paraformaldehyde for 48 h, embedded in paraffin, and processed for histology, as previously described (14, 23). In brief, transverse sections (thickness: 5 μm for LV tissue and 5 μm for fat tissue) were stained either with hematoxylin and eosin for routine histological examination or with Azan-Mallory solution for evaluation of the extent of fibrosis. To evaluate macrophage infiltration into the myocardium or fat tissue, we performed immunostaining for the monocyte-macrophage marker CD68 with paraffin-embedded sections. Endogenous peroxidase activity was blocked by exposure of the sections to methanol containing 0.3% H2O2. Sections were incubated at 4°C first overnight with mouse monoclonal antibodies to CD68 (clone ED1, diluted 1:100, Chemicon, Temecula, CA) and then for 30 min with Histone Simple Stain Max PO (Nichirei Biosciences, Tokyo, Japan). The number of immunoreactive myocardial interstitial macrophages was counted, and the adipocyte cross-sectional area was measured as previously described (8). All image analysis was performed with the use of NIH Image software (Scion, Frederick, MD).

Metabolic analysis. Blood was collected from the right carotid artery of rats that had been deprived of food overnight and then anesthetized by an intraperitoneal injection of pentobarbital sodium (50 mg/kg). Blood was centrifuged at 1,400 g for 10 min at 4°C, and the resultant serum or plasma supernatants were maintained at −80°C until analysis. The concentration of insulin in serum was measured with the use of a mouse/rat ELISA kit (Morinaga Bioscience Institute, Yokohama, Japan). The concentration of corticosterone in plasma was measured with the use of an ELISA kit (Assaypro, St. Charles, MO). Serum levels of total cholesterol, LDL-cholesterol, HDL-cholesterol, triglyceride, and free fatty acids (FFAs) were measured by routine enzymatic assays. Urinary catecholamine concentrations were measured by HPLC, and urinary catecholamine excretion over 24 h was calculated as previously described (12).

Superoxide production. NADPH-dependent superoxide production by homogenates prepared from freshly frozen LV tissue was measured by the use of an assay based on lucigenin-enhanced chemiluminescence, as previously described (28). 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 minute per milligram of protein. Superoxide production in tissue sections was examined by staining with dihydroethidium (Sigma, St. Louis, MO), as previously described (4). Dihydroethidium is rapidly oxidized by superoxide to yield fluorescent ethidium, and sections were examined with a fluorescence microscope equipped with a 585-nm long-pass filter. As a negative control, we performed staining with dihydroethidium after incubation of sections with SOD (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 (19).
Time courses of body weight, food intake, SBP, or heart rate were compared among groups by two-way repeated-measures ANOVA. P values of <0.05 were considered statistically significant.

RESULTS

Physiological analysis, LV geometry, and cardiac function. Both body weight (Fig. 1A) and food intake (Fig. 1B) were increased in the MetS group compared with the Cont group at 9 wk of age and thereafter. Body weight was decreased in the RS group compared with the MetS group, and this effect of stress tended to be attenuated by propranolol ($P = 0.0682$) without a corresponding effect on food intake. SBP was significantly increased in the MetS group compared with the Cont group, and restraint stress induced a further increase in SBP in a manner sensitive to propranolol treatment (Fig. 1C). Heart rate was significantly lower in the MetS group than in the Cont group and was reduced further in the RS and RS + Prop groups (Fig. 1D). At 13 wk of age, the ratios of both heart and LV weight to tibial length, indexes of cardiac and LV hypertrophy, respectively, were significantly increased in the MetS group compared with the Cont group, and restraint stress further increased these parameters in a manner sensitive to propranolol administration. The ratio of liver weight to tibial length as well as those of visceral (retroperitoneal, epididymal, and mesenteric) and subcutaneous (inguinal) fat mass to tibial length were all similarly increased in the MetS, RS, and RS + Prop groups compared with the Cont group (Table 1).

Echocardiography revealed that IVST and LVPWT were significantly increased in the MetS group compared with the Cont group, that restraint stress further increased these parameters, and that these effects of stress were attenuated by propranolol (Table 2). Both LVFS and LVEF were significantly increased in the MetS group but were not further affected by restraint stress or propranolol. LV mass and RWT...
were significantly increased in the MetS group and were increased further by restraint stress in a manner sensitive to propranolol treatment. Deceleration time, isovolumic relaxation time, and time constant of isovolumic relaxation were significantly prolonged, and both LVEDP and the ratio of LVEDP to LVDd were significantly increased in the MetS group compared with the Cont group; all of these changes were further increased in the RS group compared with the MetS group, and propranolol attenuated all of these effects of stress. Thus, these data indicated that restraint stress exacerbated LV remodeling and diastolic dysfunction in DS/obese rats in a manner sensitive to propranolol administration.

Glucose tolerance, insulin sensitivity, and metabolic parameters. The fasting serum glucose concentration was similar in the four experimental groups (Table 3). However, the fasting serum insulin level was significantly greater in the MetS group than in the Cont group, and it was further increased in the RS group in a manner sensitive to propranolol. Moreover, OGTT and ITT revealed that restraint stress exacerbated the glucose intolerance and insulin resistance, respectively, apparent in the MetS group and that this exacerbation was sensitive to propranolol treatment (Fig. 1, E and F).

The plasma corticosterone concentration was similar in the four experimental groups. Serum levels of total cholesterol, LDL-cholesterol, HDL-cholesterol, triglyceride, and FFAs were increased in DS/obese rats. With the exception of HDL-cholesterol, these parameters were further increased by restraint stress in a manner sensitive to propranolol treatment. Restraint stress significantly reduced the serum HDL-cholesterol concentration, and this effect was attenuated by propranolol ($P = 0.069$). Urinary dopamine, norepinephrine, and epinephrine excretion did not differ between Cont and MetS groups; restraint stress increased all of these parameters, and propranolol attenuated these effects of stress, although those on dopamine and epinephrine excretion did not achieve statistical significance ($P = 0.078$ and $P = 0.092$, respectively).

Cardiomyocyte hypertrophy as well as cardiac fibrosis and gene expression. Microscopic analysis revealed that the cross-sectional area of cardiac myocytes was increased in the MetS group compared with the Cont group. This increase was further augmented in the RS group, and this effect of stress was abrogated by propranolol (Fig. 2, A and B). Hemodynamic overload also resulted in a significant upregulation of the expression of atrial natriuretic peptide and brain natriuretic peptide genes in the hearts of DS/obese rats, and this upregulation was enhanced by restraint stress in a propranolol-sensitive manner (Fig. 2, C and D). Azan-Mallory staining revealed that fibrosis in perivascular and interstitial regions of the LV myocardium was increased in the MetS group compared with the Cont group and was increased further in the RS group in a manner sensitive to propranolol (Fig. 2, I–L). Amounts of collagen types I and III, connective tissue growth factor, and transforming growth factor-$\beta_1$ mRNAs were also upregulated in the MetS group, this upregulation was further augmented in the RS group, and these effects of stress were attenuated by propranolol (Fig. 2, I–L).

Cardiac oxidative stress and inflammation. Superoxide production in myocardial tissue sections, as revealed by staining

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Cont Group</th>
<th>MetS Group</th>
<th>RS Group</th>
<th>RS + Prop Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tibial length, mm</td>
<td>37.6 ± 0.2</td>
<td>34.8 ± 0.2*</td>
<td>34.6 ± 0.2*</td>
<td>34.7 ± 0.2*</td>
</tr>
<tr>
<td>Heart weight/tibial length, mg/mm</td>
<td>30.4 ± 0.3</td>
<td>37.6 ± 0.9*</td>
<td>39.9 ± 1.0†</td>
<td>35.6 ± 0.4‡</td>
</tr>
<tr>
<td>LV weight/tibial length, mg/mm</td>
<td>21.8 ± 0.3</td>
<td>27.7 ± 0.8*</td>
<td>30.6 ± 0.8†</td>
<td>25.1 ± 0.3‡</td>
</tr>
<tr>
<td>Liver weight/tibial length, mg/mm</td>
<td>293.2 ± 6.0</td>
<td>587.7 ± 25.6*</td>
<td>609.5 ± 28.1*</td>
<td>532.3 ± 21.2*</td>
</tr>
<tr>
<td>Retropertioneal fat weight/tibial length, mg/mm</td>
<td>102.5 ± 5.3</td>
<td>490.3 ± 21.9*</td>
<td>480.3 ± 14.3*</td>
<td>497.6 ± 17.4*</td>
</tr>
<tr>
<td>Epididymal fat weight/tibial length, mg/mm</td>
<td>118.6 ± 4.9</td>
<td>380.5 ± 18.9*</td>
<td>364.1 ± 16.3*</td>
<td>373.2 ± 15.0*</td>
</tr>
<tr>
<td>Mesenteric fat weight/tibial length, mg/mm</td>
<td>82.2 ± 5.2</td>
<td>401.4 ± 19.0*</td>
<td>396.9 ± 21.3*</td>
<td>397.4 ± 25.2*</td>
</tr>
<tr>
<td>Intraperitoneal fat weight/tibial length, mg/mm</td>
<td>121.9 ± 6.2</td>
<td>847.4 ± 37.9*</td>
<td>803.1 ± 45.7*</td>
<td>802.0 ± 31.2*</td>
</tr>
</tbody>
</table>

Data are means ± SE. The following four groups were used: control (Cont; DahlS.Z-Lepr+/Lepr+ rats, $n = 7$), metabolic syndrome (MetS; DahlS.Z-Lepr+/Lepr+ rats, $n = 7$), DahlS.Z-Lepr+/Lepr+ rats exposed to restraint stress (RS; $n = 14$), and DahlS.Z-Lepr+/Lepr+ rats exposed to restraint stress plus propranolol (RS + Prop; $n = 14$). LV, left ventricular. *$P < 0.05$ vs. the Cont group; †$P < 0.05$ vs. the MetS group; ‡$P < 0.05$ vs. the RS group.

Cardiac oxidative stress and inflammation. Superoxide production in myocardial tissue sections, as revealed by staining

Table 2. Cardiac morphological and functional parameters for rats from the four experimental groups at 13 wk of age

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Cont Group</th>
<th>MetS Group</th>
<th>RS Group</th>
<th>RS + Prop Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interventricular septal thickness, mm</td>
<td>1.75 ± 0.07</td>
<td>2.06 ± 0.23*</td>
<td>2.46 ± 0.06†</td>
<td>1.95 ± 0.08‡</td>
</tr>
<tr>
<td>LV posterior wall thickness, mm</td>
<td>1.75 ± 0.10</td>
<td>2.14 ± 0.09*</td>
<td>2.44 ± 0.05†</td>
<td>1.97 ± 0.02‡</td>
</tr>
<tr>
<td>LVDd, mm</td>
<td>7.73 ± 0.15</td>
<td>7.64 ± 0.20</td>
<td>7.64 ± 0.14</td>
<td>7.89 ± 0.06</td>
</tr>
<tr>
<td>LV end-systolic dimension, mm</td>
<td>4.72 ± 0.22</td>
<td>3.97 ± 0.23*</td>
<td>3.98 ± 0.21*</td>
<td>4.10 ± 0.14*</td>
</tr>
<tr>
<td>LV fractional shortening, %</td>
<td>39.3 ± 2.2</td>
<td>48.3 ± 2.7*</td>
<td>48.1 ± 2.2*</td>
<td>49.3 ± 1.8*</td>
</tr>
<tr>
<td>LV ejection fraction, %</td>
<td>76.2 ± 2.2</td>
<td>84.7 ± 2.0*</td>
<td>85.1 ± 1.7*</td>
<td>86.2 ± 1.5*</td>
</tr>
<tr>
<td>LV mass, mg</td>
<td>978.2 ± 42.3</td>
<td>1268.4 ± 62.6*</td>
<td>1589.6 ± 44.2†</td>
<td>1164.6 ± 27.7‡</td>
</tr>
<tr>
<td>Relative wall thickness</td>
<td>0.47 ± 0.03</td>
<td>0.56 ± 0.03*</td>
<td>0.65 ± 0.02‡</td>
<td>0.46 ± 0.04‡</td>
</tr>
<tr>
<td>Deceleration time, ms</td>
<td>46.5 ± 1.8</td>
<td>54.9 ± 2.3*</td>
<td>73.4 ± 1.8†</td>
<td>48.6 ± 1.4‡</td>
</tr>
<tr>
<td>Isovolumic relaxation time, ms</td>
<td>27.3 ± 1.6</td>
<td>33.6 ± 1.8*</td>
<td>50.9 ± 1.8†</td>
<td>34.4 ± 1.2‡</td>
</tr>
<tr>
<td>Time constant of isovolumic relaxation, ms</td>
<td>25.2 ± 1.3</td>
<td>34.3 ± 2.3*</td>
<td>58.9 ± 6.8†</td>
<td>28.9 ± 3.3‡</td>
</tr>
<tr>
<td>LVEDP, mmHg</td>
<td>4.11 ± 0.59</td>
<td>8.99 ± 0.42*</td>
<td>12.86 ± 1.78†</td>
<td>7.51 ± 0.70‡</td>
</tr>
<tr>
<td>LVEDP/LVDd, mmHg/mm</td>
<td>0.48 ± 0.08</td>
<td>0.94 ± 0.21*</td>
<td>1.68 ± 0.25†</td>
<td>0.93 ± 0.09‡</td>
</tr>
</tbody>
</table>

Data are means ± SE; $n = 7, 7, 14,$ and 14 for Cont, MetS, RS, and RS + Prop groups, respectively. LVDd, LV end-diastolic dimension; LVEDP, LV end-diastolic pressure. *$P < 0.05$ vs. the Cont group; †$P < 0.05$ vs. the MetS group; ‡$P < 0.05$ vs. the RS group.
Table 3. Metabolic parameters for rats from the four experimental groups at 13 wk of age

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Cont Group</th>
<th>MetS Group</th>
<th>RS Group</th>
<th>RS + Prop Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fasting serum glucose, mg/dl</td>
<td>123.4 ± 4.1</td>
<td>133.6 ± 28.4</td>
<td>132.3 ± 7.4</td>
<td>139.7 ± 10.4</td>
</tr>
<tr>
<td>Fasting serum insulin, ng/dl</td>
<td>0.38 ± 0.04</td>
<td>2.66 ± 0.08</td>
<td>6.65 ± 1.06</td>
<td>2.78 ± 0.40</td>
</tr>
<tr>
<td>Plasma corticosterone, ng/ml</td>
<td>567.7 ± 85.1</td>
<td>556.3 ± 76.7</td>
<td>546 ± 77.8</td>
<td>526.8 ± 94.5</td>
</tr>
<tr>
<td>Total cholesterol, mg/dl</td>
<td>67.4 ± 1.4</td>
<td>251.5 ± 16.4</td>
<td>396.4 ± 53.8</td>
<td>232.8 ± 31.2</td>
</tr>
<tr>
<td>LDL-cholesterol, mg/dl</td>
<td>18.0 ± 0.9</td>
<td>43.9 ± 8.0</td>
<td>83.4 ± 8.8</td>
<td>50.0 ± 43.2</td>
</tr>
<tr>
<td>HDL-cholesterol, mg/dl</td>
<td>35.6 ± 1.2</td>
<td>108.3 ± 14.0</td>
<td>64.6 ± 8.6</td>
<td>97.2 ± 58.8</td>
</tr>
<tr>
<td>Triglyceride, mg/dl</td>
<td>62.8 ± 6.8</td>
<td>925.3 ± 123.8</td>
<td>2061.8 ± 426.7</td>
<td>1010.4 ± 208.2</td>
</tr>
<tr>
<td>Free fatty acids, mEq/l</td>
<td>0.71 ± 0.07</td>
<td>1.02 ± 0.04</td>
<td>1.50 ± 0.13</td>
<td>1.09 ± 0.14</td>
</tr>
<tr>
<td>Urinary dopamine, ng/day</td>
<td>133.2 ± 2.0</td>
<td>143.6 ± 34.0</td>
<td>342.0 ± 63.1</td>
<td>249.0 ± 73.4</td>
</tr>
<tr>
<td>Urinary norepinephrine, ng/day</td>
<td>17.81 ± 4.51</td>
<td>30.86 ± 12.52</td>
<td>70.74 ± 8.59</td>
<td>47.26 ± 2.76</td>
</tr>
<tr>
<td>Urinary epinephrine, ng/day</td>
<td>31.76 ± 5.87</td>
<td>27.4 ± 5.40</td>
<td>58.68 ± 11.41</td>
<td>38.33 ± 8.46</td>
</tr>
</tbody>
</table>

Data are means ± SE; n = 7, 7, 14, and 14 for Cont, MetS, RS, and RS + Prop groups, respectively. *P < 0.05 vs. the Cont group; †P < 0.05 vs. the MetS group; ‡P < 0.05 vs. the RS group.

Discussion

In the present study, we have shown that chronic restraint stress exacerbated hypertension as well as LV hypertrophy, fibrosis, and diastolic dysfunction in DS/obese rats in a manner sensitive to propranolol treatment. Restraint stress also attenuated body weight gain in DS/obese rats, and propranolol tended to restore body weight in stressed animals. Neither restraint stress nor propranolol significantly affected visceral or subcutaneous fat mass. However, cardiac and visceral adipose tissue inflammation as well as cardiac oxidative stress were enhanced by restraint stress, and these effects were attenuated by propranolol. Restraint stress also exacerbated glucose intolerance, insulin resistance, and dyslipidemia, with all of these effects also being limited by propranolol. In addition, restraint stress increased urinary norepinephrine excretion in a manner sensitive to propranolol. As far as we are aware, our study is the first to show the effects of restraint stress on cardiovascular and metabolic disorders in MetS as well as to shed light on the mechanism of these effects.

Stress strongly affects the pathophysiology of MetS. In addition, obesity is often accompanied by sympathetic nerve activation, and mediators of stress systems (such as corticosterone, catecholamines, and neuropeptides) influence the immune system and disease states. Norepinephrine and epinephrine act at three subtypes of β-ARs expressed in the heart, kidney, skeletal muscle, and adipose tissue. We have now shown that restraint stress increased urinary catecholamine excretion but did not affect plasma corticosterone levels in DS/obese rats. Restraint stress is a mild psychological stress that induces activation of the SNS, resulting in the release of catecholamines from sympathetic nerve terminals and the adrenal glands. Restraint stress may therefore have contributed to elevation of sympathetic nerve activity throughout the body of the DS/obese rats in our study. As a key part of the autonomic nervous system, the SNS is a major pathway connecting the brain to the periphery and is responsible for the regulation of many homeostatic mechanisms. We found that the nonselective β-AR blocker propranolol attenuated the stress-induced increase in urinary norepinephrine excretion in DS/obese rats.

The relation between stress and obesity remains elusive. In response to stress, some people lose weight, whereas others gain it. Chronic restraint stress in male Wistar rats reduced body weight gain compared with nonstressed animals (50). In the present study, neither visceral nor subcutaneous fat mass was significantly affected by restraint stress, whereas body weight was significantly reduced by such stress in a manner sensitive to propranolol. Given that food intake and obesity (fat mass) were not influenced by either restraint stress or propranolol, the reduced weight gain apparent in stressed rats may have been due to a decrease in lean body mass, such as muscle...
or bone mass, rather than an increase in energy expenditure as a result of lipolysis. Activation of the SNS and the consequent binding of norepinephrine to β-ARs on white fat cells triggers lipolysis and the consequent release of FFAs. Systemic inhibition of lipolysis or disturbance of lipolysis in adipose tissue has been previously shown to reduce plasma FFA levels (6). Consistent with previous observations (46), we found that serum FFA levels were significantly increased in rats exposed
to restraint stress, suggesting that stress induced lipolysis in adipose tissue. The β-AR-induced accumulation of cAMP has been shown to activate lipase enzymes in adipocytes (9). Restraint stress therefore likely induced weight loss in DS/obese rats by increasing norepinephrine release and thereby inducing lipolysis and the consequent production of FFAs in white adipose tissue. In addition, the binding of norepinephrine to β2-ARs on brown fat cells upregulates the expression of uncoupling protein 1, and energy consumption is promoted via a similar system in white adipose tissue cells that acquire a brown adipose phenotype (beige adipocytes) (18, 49). However, the size of adipocytes was not significantly affected by restraint stress in DS/obese rats, in contrast to a previous finding with lean mice (46). Although the reason for this apparent discrepancy is unclear, it may be due to the fact that restraint stress further increased insulin resistance and hyperinsulinemia in DS/obese rats. Insulin inhibits apoptosis and increases lipid droplet formation in adipocytes (13). This effect of insulin on adipocyte hypertrophy might also have contributed to the stress-induced dyslipidemia observed in DS/obese rats, with high triglyceride levels being a frequent consequence of insulin resistance (34).

Both obesity and diabetes are associated with activation of inflammatory pathways in key metabolic tissues as well as macrophages. Adipocyte size was significantly increased in DS/obese rats, and enlargement of adipocytes promotes the production of proinflammatory adipokines and a consequent shift in macrophage polarization from the anti-inflammatory M2 type to the proinflammatory M1 type (44). The accumulation of M1 macrophages in white adipose tissue and the release of FFAs during lipolysis contribute to chronic inflammation in this tissue (15). FFAs thus act through Toll-like receptor 4 expressed on the surface of macrophages and adipocytes to induce inflammatory signaling and to suppress insulin signaling including insulin-mediated regulation of glucose metabolism (42). Moreover, sympathetic denervation has been shown to downregulate p53 expression and to attenuate inflammation in adipose tissue, thereby ameliorating insulin resistance (43). Restraint stress augmented macrophage accumulation in visceral adipose tissue of DS/obese rats, and this effect was accompanied by upregulation of the expression of genes for proinflammatory proteins in this tissue. Our results are also consistent with the recent observation that restraint stress evoked the expression of MCP-1 and other inflammatory adipokines in adipose tissue of lean mice (46). Adipose tissue inflammation is closely linked to the development of insulin resistance and type 2 diabetes mellitus (3a). We found that insulin resistance and glucose intolerance were further exacerbated by restraint stress in DS/obese rats, consistent with previous results showing that restraint stress augments insulin resistance in male C57BL/6J mice (46). Since skeletal muscle plays an important role in the determination of insulin sensitivity, augmentation of skeletal muscle inflammation by restraint stress might have contributed to the stress-induced exacerbation of insulin resistance in our rats (40).

Obesity, especially when complicated by hypertension, is associated with changes in cardiac structure and function (32). Although stress is a risk factor for high blood pressure and heart disease, it remains unclear how stress increases heart disease risk. We now show that restraint stress exacerbated hypertension as well as LV hypertrophy, fibrosis, and diastolic dysfunction in DS/obese rats in a manner sensitive to β-AR blockade. Given that hypertension in obesity is accompanied by central sympathetic overactivity (26), restraint stress may increase blood pressure via activation of the SNS in the brain. Our results are also consistent with a previous finding that restraint stress increased blood pressure in male Wistar rats (11). In addition, cardiac hypertrophy and insulin resistance are closely associated conditions (39), and many cardiovascular disorders associated with MetS are explained by the presence of insulin resistance (33). Our data thus suggest that restraint stress promoted LV remodeling and diastolic dysfunction through augmentation of both hypertension and insulin resistance.

Abnormalities in adipokine production, insulin resistance, and chronic inflammation are all thought to be important factors in MetS. Increased oxidative stress contributes to the development these conditions (5) and is implicated in both the stress response (52) and pathogenesis of neurological and psychiatric diseases (30). NADPH oxidase is a major source of ROS in the central nervous system. Both NADPH-dependent generation of superoxide and the expression of NADPH oxidase subunit genes were increased in the heart of DS/obese rats by restraint stress, and these effects were attenuated by propranolol. Repeated restraint stress has been previously shown to increase the expression of NADPH oxidase subunits p47phox and p67phox in the brains of C57BL/6J mice (41). The production of BDNF, a member of the neurotrophin family of growth factors, is associated with oxidative stress (3). Psychological stress, including restraint stress, affects BDNF levels in various brain regions as well as the coronary circulation, so that a heightened stress response might contribute to increased oxidative stress (3). Indeed, we found that expression of the BDNF gene was significantly increased in the heart of DS/obese rats and that restraint stress further increased this expression in a manner sensitive to propranolol. Oxidative stress activates intracellular stress signaling pathways, such as those mediated by MAPKs and NF-κB, and it thereby induces the secretion of TNF-α in streptozotocin-induced diabetic kidneys (31). Restraint stress augmented macrophage infiltration into...
Fig. 3. NADPH oxidase activity and gene expression as well as macrophage infiltration and inflammation-related gene expression in the LV of rats from the four experimental groups at 13 wk of age. **A**: superoxide production as revealed by dihydroethidium (DHE) staining in interstitial regions of the LV myocardium. Scale bars = 100 μm. **B**: relative DHE fluorescence intensity determined from sections similar to those in A. **C**: NADPH-dependent superoxide production in LV homogenates. Results are expressed as relative light units (RLU) per minute per milligram of protein. **D–F**: quantitative RT-PCR analysis of p22<sub>phox</sub> (**D**), gp91<sub>phox</sub> (**E**), and Rac1 (**F**) mRNAs. The amount of each mRNA was normalized by that of GAPDH mRNA and then expressed relative to the mean value for the Cont group. **G**: immunohistochemical analysis with antibodies to the monocyte-macrophage marker CD68. Scale bars = 100 μm. **H**: density of CD68-positive cells determined from sections similar to those in G. **I–K**: quantitative RT-PCR analysis of monocyte chemoattractant protein (MCP)-1 (**I**), osteopontin (**J**), and cyclooxygenase (COX)-2 (**K**) mRNAs. All quantitative data are means ± SE; n = 7, 7, 14, and 14 for Cont, MetS, RS, and RS + Prop groups, respectively. *P < 0.05 vs. the Cont group; †P < 0.05 vs. the MetS group; ‡P < 0.05 vs. the RS group.
the myocardium as well as the upregulation of MCP-1, TNF-α, and COX-2 gene expression in the hearts of DS/obese rats, indicating that restraint stress exacerbated cardiac inflammation. Acute restraint stress also induced stress-related coronary inflammation via a mast cell-dependent increase in serum IL-6 levels in apolipoprotein E knockout mice (10). The expression of cardiac RAAS genes in DS/obese rats was also further increased by restraint stress, and propranolol inhibited this upregulation. Enhanced MR signaling in the myocardium may thus have contributed to the augmentation of cardiac inflammation and oxidative stress and to the consequent progression of cardiac remodeling and dysfunction induced by restraint stress (28).

Although animals respond to stress with several types of reaction, the major pathways include activation of the central SNS and HPA axis, both of which are closely connected at several levels. The activity of the HPA axis is controlled by negative feedback mechanisms mediated by central GRs. Under stressful conditions, corticotropin-releasing hormones secreted from the hypothalamus triggers the release of adrenocorticotropic hormone from the pituitary gland. Adrenocorticotropic hormone then triggers the secretion of glucocorticoids from the adrenal cortex. Previous studies have shown that restraint stress increases (20, 21) or has no effect on (17) corticosterone levels. In the present study, the plasma corticosterone concentration was not affected by restraint stress or

Fig. 4. Cardiac renin-angiotensin-aldosterone system- and glucocorticoid-related gene expression in the LV of rats from the four experimental groups at 13 wk of age. The amounts of angiotensin-converting enzyme (ACE; A), ANG II type 1A (AT1A) receptor (B), mineralocorticoid receptor (MR; C), serum- and glucocorticoid-regulated kinase 1 (Sgk1; D), 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1; E), glucocorticoid receptor (GR; F), and brain-derived neurotrophic factor (BDNF; G) mRNAs were determined by quantitative RT-PCR analysis. Data were normalized by the abundance of GAPDH mRNA and then expressed relative to the mean value for the CONT group. Values are means ± SE; n = 7, 7, 14, and 14 for CONT, MetS, RS, and RS + Prop groups, respectively. *P < 0.05 vs. the CONT group; †P < 0.05 vs. the MetS group; ‡P < 0.05 vs. the RS group.
propranolol. However, restraint stress further upregulated the expression of 11β-HSD1 and GR genes in both the heart and visceral fat of DS/obese rats. Increased expression of 11β-HSD1 can lead to glucocorticoid excess (45), and tissue-specific amplification of glucocorticoid signaling by stress has been found to exacerbate the pathophysiology of MetS without affecting obesity (36).

Heart rate was lower in DS/obese rats than in DS/lean rats, probably because of the decrease in cardiac sympathetic nerve activity resulting from leptin receptor deficiency in the hypothalamus (19). Indeed, urinary norepinephrine excretion was not increased in DS/obese rats despite the presence of hypertension. We speculate that insulin resistance/hyperinsulinemia may importantly contribute to hypertension and that baroreflex function may be preserved in this model of MetS. Mental stress, such as restraint stress, can stimulate sympathetic nerve activity in the hypothalamus and thus exacerbate insulin resistance/hyperinsulinemia to further increase blood pressure. This increase in blood pressure can reduce heart rate by baroreflex. Since propranolol is able to penetrate across the blood-brain barrier, treatment with propranolol could inhibit sympathetic nerve activity in the hypothalamus and attenuate insulin resistance/hyperinsulinemia as well as attenuate baroreflex function in the central and/or peripheral nervous systems. Therefore, propranolol treatment attenuated the restraint stress-induced increase in blood pressure but did not affect heart rate. Since DS/obese rats developed hypertension and LV diastolic dysfunction as well as LV hypertrophy and fibrosis, they can show nearly normal, normal, or supranormal LV systolic function (LVFS and LVEF), depending on the breeding environment.

Fig. 5. Macrophage infiltration as well as inflammatory and glucocorticoid-related gene expression in retroperitoneal adipose tissue of rats from the four experimental groups at 13 wk of age. A: Immunohistochemical staining for the monocyte-macrophage marker CD68. Scale bars = 100 μm. B: cross-sectional area of adipocytes determined from sections similar to those in A. C: number of nuclei for CD68-positive cells as a percentage of total nuclei as determined from sections similar to those in A. D–H: quantitative RT-PCR analysis of MCP-1 (D), osteopontin (E), COX-2 (F), 11β-HSD1 (G), and GR (H) mRNAs. The amount of each mRNA was normalized by that of GAPDH mRNA and then expressed relative to the mean value for the Cont group. All quantitative data are means ± SE; n = 7, 7, 14, and 14 for Cont, MetS, RS, and RS + Prop groups, respectively. *P < 0.05 vs. the Cont group; †P < 0.05 vs. the MetS group; ‡P < 0.05 vs. the RS group.
and age. Under our experimental conditions, DS/obese rats at 13 wk of age developed a hypercontractile state likely to maintain LV pump function. Indexes of LV systolic function were not significantly affected by restraint stress or propranolol, probably as a result of the net effects on LV contractility, load, and heart rate.

In conclusion, restraint stress attenuated body weight gain without significantly affecting food intake in DS/obese rats, and propranolol tended to restore body weight in stressed animals. Restraint stress also augmented hypertension, LV hypertrophy, fibrosis, and diastolic dysfunction, and cardiac oxidative stress as well as inflammation in a manner sensitive to propranolol treatment. In addition, abnormal glucose and lipid metabolism as well as insulin resistance were exacerbated by restraint stress, and these effects were attenuated by propranolol. Restraint stress or propranolol did not significantly affect visceral or subcutaneous fat mass. However, propranolol attenuated the stress-induced increase in visceral adipose tissue inflammation. Our results thus implicate β-ARs in the stress-induced exacerbation of cardiac and adipose tissue pathology and metabolic disorders characteristic of MetS. Further investigations are required to clarify the molecular mechanisms of these actions of restraint stress.

ACKNOWLEDGMENTS

The authors thank Yuuri Takeshita and Sae Ohura for technical assistance.

GRANTS

This work was supported by Ministry of Education, Culture, Sports, Science, and Technology of Japan Grant 24590690 (to K. Nagata).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: N.M., K. Nagasawa, Y.M., S.I., Y.S., Y.Y., T.H., and S.W. performed experiments; N.M., K. Nagasawa, T.M., and K. Nagata interpreted results of experiments; N.M. prepared figures; N.M. drafted manuscript; N.M. and K. Nagata revised the manuscript; N.M., K. Nagasawa, Y.M., S.I., Y.S., Y.Y., T.H., and S.W. prepared figures; N.M., K. Nagata edited and revised manuscript; N.M., K. Nagasawa, Y.M., S.I., Y.S., Y.Y., T.H., and S.W. conceived and designed research. K. Nagata was responsible for obtaining funding.

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