Effects of Estrogen on Cardiovascular Injury in Ovariectomized Female DahlS.Z-Lepr<sup>fa</sup>/Lepr<sup>fa</sup> Rats as a New Animal Model of Metabolic Syndrome

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See Editorial Commentary, pp 552–554

Abstract—Although recent clinical trials have found an increased incidence of cardiovascular disease in women on estrogen replacement therapy, the underlying mechanism remains unclear. We have recently characterized DahlS.Z-Lepr<sup>fa</sup>/Lepr<sup>fa</sup> (DS/obese) rats, derived from a cross between Dahl salt-sensitive and Zucker rats, as a new animal model of metabolic syndrome. We have now examined the effects of estrogen replacement on cardiac pathophysiology in ovariectomized female DS/obese (Ovx-DS/obese) rats. Animals subjected to ovariectomy at 7 weeks of age were implanted subcutaneously with a 60-day release pellet containing 0.5 mg of 17β-estradiol (E<sub>2</sub>) or placebo at 8 weeks. Age-matched female homozygous lean littermates (DahlS.Z-Lepr<sup>+</sup>/Lepr<sup>-</sup> or DS/lean rats) of DS/obese rats served as controls. Animals were maintained on a normal diet and were subjected to echocardiography followed by various pathological analyses at 13 weeks of age. Ovx-DS/obese rats manifested hypertension at 7 weeks of age and thereafter and showed left ventricular (LV) fibrosis and diastolic dysfunction at 13 weeks. Treatment with E<sub>2</sub> attenuated hypertension in Ovx-DS/obese rats but had no effect on blood pressure in ovariectomized female DS/lean (Ovx-DS/lean) rats. E<sub>2</sub> treatment exacerbated LV fibrosis and diastolic dysfunction, as well as further increased cardiac oxidative stress and inflammation in Ovx-DS/obese rats, and it elicited similar effects in Ovx-DS/lean rats. E<sub>2</sub> reduced food intake, body weight, and visceral fat content in both Ovx-DS/obese and Ovx-DS/lean rats. E<sub>2</sub> treatment attenuated hypertension and obesity but exacerbated LV fibrosis and diastolic dysfunction in Ovx-DS/obese rats, with these latter effects being associated with increased cardiac oxidative stress and inflammation. (Hypertension. 2012;59:694-704.)

Key Words: metabolic syndrome ■ estrogen ■ hypertension ■ myocardial fibrosis ■ diastolic dysfunction ■ oxidative stress ■ inflammation

Metabolic syndrome (MetS), a complex of highly debilitating disorders including hypertension, diabetes mellitus, and dyslipidemia, is associated with the development of visceral obesity. MetS afflicts both men and women and increases the risk of heart disease in both sexes, although it appears to inflict a greater burden in women. The incidence of cardiovascular disease among women is low before menopause but steadily increases thereafter. This increase is thought to result in part from the loss of endogenous estrogen and its associated cardioprotective effects. A key issue faced by most postmenopausal women is the potential impact of estrogen replacement therapy on the prevalence of cardiovascular disease. Estrogen replacement in postmenopausal women has been associated with a reduced risk of cardiovascular disease. However, the Heart and Estrogen/Progestin Replacement Study and the Women’s Health Initiative Study do not support the notion that hormone replacement therapy protects the cardiovascular system but rather suggest the opposite view, that such therapy may increase the risk of cardiovascular disease. Further analysis of the Women’s Health Initiative study data suggested that estrogen-plus-progesterone therapy was beneficial in healthy young postmenopausal women but that it increased cardiovascular risk when initiated in older postmenopausal women with established coronary artery disease. The reason for the disparate results regarding the cardiovascular effects of estrogen are

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unclear in part because of an incomplete understanding of the mechanism of such effects. Experimental studies in animals have provided evidence that estrogen protects the cardiovascular system. Animal studies have shown beneficial effects of 17β-estradiol (E2) on atherosclerosis, inflammation, and endothelial or vascular function, and estrogen modulation of endothelial nitric oxide (NO) synthase has been implicated as a mechanism of cardiac protection. However, other studies suggest that estrogen activates the renin-angiotensin-aldosterone system (RAAS), an action that might contribute to cardiac injury. In humans, estrogen increases circulating levels of angiotensin II (Ang II), as well as intrarenal Ang II activity, with the latter effect being associated with a decrease in renal blood flow. In animal models of cardiovascular injury attributed to an activated RAAS, estrogen increases the incidence of stroke and renal injury.

We recently established a new animal model of MetS, the DahlS.Z-Leprfa (DS/obese) rat, by crossing Dahl salt-sensitive (DS) rats with Zucker rats harboring a missense mutation in the leptin receptor gene (Lepr). When fed a normal diet, male DS/obese rats develop a phenotype similar to MetS in humans, including hypertension. In addition, they develop cardiac hypertrophy, as well as renal and liver damage, conditions that may be responsible for their premature death. Observations suggested that salt sensitivity of blood pressure and target organ damage are enhanced in MetS. We have also shown that female DS/obese rats develop salt-sensitive hypertension, as well as left ventricular (LV) diastolic dysfunction, hypertrophy, and fibrosis, and that these conditions are accompanied by increased cardiac oxidative stress and inflammation. We have now investigated the effects of estrogen replacement on cardiac pathophysiology in ovariectomized female DS/obese (Ovx-DS/obese) rats.

**Methods**

**Animals and Experimental Protocols**

Female inbred DS/obese rats were fed normal laboratory chow containing 0.36% NaCl and were compared with homozygous lean littermates, DahlS.Z-Lepr+/Lepr+ (DS/lean) rats. Rats subjected to bilateral ovariectomy at 7 weeks of age were implanted subcutaneously with an E2 pellet (60-day release pellet containing 0.5 mg of E2; Innovative Research of America, Sarasota, FL) or a placebo at 8 weeks. Body weight, as well as food and water intake, was measured weekly. At 13 weeks of age, rats were anesthetized by intraperitoneal injection of ketamine (50 mg per kilogram of body weight) and xylazine (10 mg/kg) and were subjected to echocardiographic and hemodynamic analyses. The animals were subsequently killed, the heart and both visceral (retroperitoneal) and subcutaneous (inguinal) fat were removed and weighed, and LV tissue was separated for analysis. Extended details can be found in the online-only Data Supplement.

**Echocardiographic and Hemodynamic Analyses**

Systolic blood pressure (SBP) was measured weekly in conscious animals by tail-cuff plethysmography (BP-98A; Softron, Tokyo, Japan). The rate-pressure product (RPP), an index of cardiac work, was calculated by multiplying SBP and heart rate. At 13 weeks of age, rats were subjected to transthoracic echocardiography followed by cardiac catheterization, as described previously. Details are available in the online-only Data Supplement.

**Histology and Immunohistochemistry**

LV tissue was fixed in ice-cold 4% paraformaldehyde for 48 to 72 hours, embedded in paraffin, and processed for histology, as described. For evaluation of macrophage infiltration into the LV myocardium, tissue sections were subjected to immunostaining for the monocyte-macrophage marker CD68, as described previously. Details are available in the online-only Data Supplement.

**Superoxide Production**

Please see the online-only Data Supplement.

**Quantitative RT-PCR Analysis**

Please see the online-only Data Supplement.

**Immunoblot Analysis**

Please see the online-only Data Supplement.

**Statistical Analysis**

Data are presented as mean±SEM. Differences among groups of rats at 13 weeks of age were assessed by 1-way factorial ANOVA; if a significant difference was detected, intergroup comparisons were performed with a Fisher multiple-comparison test. Furthermore, we analyzed the data using 2-way factorial ANOVA to evaluate the independent and interactive influence of strains and E2 treatment in the 4 groups of rats (Ovx-DS/lean + placebo, Ovx-DS/lean + E2, Ovx-DS/obese + placebo, and Ovx-DS/obese + E2 groups). The time course of body weight, SBP, or food intake was compared among groups by 2-way repeated-measures ANOVA. A P value of <0.05 was considered statistically significant.

**Results**

**Cardiac Geometry and Function and Metabolic Characteristics**

Body weight was significantly increased in Ovx-DS/obese rats compared with ovariectomized female DS/lean (Ovx-DS/lean) rats at 7 weeks and thereafter, and food intake was significantly increased in Ovx-DS/obese rats compared with Ovx-DS/DS/lean rats between 7 and 11 weeks of age (Figure 1A and 1B and Table 1). The ratios of visceral or subcutaneous fat weight to tibial length were also greatly increased in Ovx-DS/obese rats compared with Ovx-DS/lean rats at 13 weeks (Table 1). Treatment with E2 resulted in a significant decrease in body weight, food intake, and the ratio of visceral fat mass:tibial length in both Ovx-DS/obese and Ovx-DS/lean rats (Figure 1A and 1B and Table 1), whereas E2 also reduced the ratio of subcutaneous fat mass:tibial length significantly in Ovx-DS/obese rats but failed to do so in Ovx-DS/lean rats (P=0.19). SBP was significantly higher in Ovx-DS/obese rats than in Ovx-DS/lean rats at 7 weeks and thereafter (Figure 1C and Table 1). Treatment with E2 attenuated the blood pressure elevation in Ovx-DS/obese rats but had no effect on SBP in Ovx-DS/lean rats. Rate-pressure product showed a pattern similar to that of SBP in the 4 experimental groups (Figure 1D and Table 1). At 13 weeks of age, the ratios of both heart and LV weight to tibial length, indices of cardiac and LV hypertrophy, respectively, were significantly increased in Ovx-DS/obese rats compared with Ovx-DS/lean rats; treatment with E2 did not significantly affect these parameters in Ovx-DS/obese rats but reduced them in Ovx-DS/lean rats (Table 1).

Echocardiography revealed that interventricular septal thickness, LV posterior wall thickness, LV mass, and relative
wall thickness were significantly increased in Ovx-DS/obese rats compared with Ovx-DS/lean rats (Table 2). Although E2 treatment did not affect these parameters in Ovx-DS/obese rats, it reduced interventricular septal thickness, LV posterior wall dimension, and LV mass (but not relative wall thickness) in Ovx-DS/lean rats. LV fractional shortening was greater in Ovx-DS/obese rats than in Ovx-DS/lean rats and was increased in both groups with treatment with E2 (Table 2). Deceleration time, isovolumic relaxation time, and the time constant of isovolumic relaxation (τ), all of which are indices of LV relaxation, as well as LV end-diastolic pressure and the ratio of LV end-diastolic pressure to LV end-diastolic dimension, both of which are indices of LV diastolic stiffness, were significantly increased in Ovx-DS/obese rats compared with Ovx-DS/lean rats, indicating that LV diastolic function was impaired in Ovx-DS/obese rats (Table 2). E2 increased the

**Table 1. Physiological and Metabolic Parameters of Ovx-DS/Lean and Ovx-DS/Obese Rats in the 4 Experimental Groups at 13 wk of Age**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Ovx-DS/Lean + Placebo</th>
<th>Ovx-DS/Lean + E2</th>
<th>Ovx-DS/Obese + Placebo</th>
<th>Ovx-DS/Obese + E2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>294 ± 6</td>
<td>220 ± 4*</td>
<td>393 ± 6†</td>
<td>339 ± 13‡†</td>
</tr>
<tr>
<td>Tibial length, mm</td>
<td>35.8 ± 0.4</td>
<td>33.6 ± 0.2*</td>
<td>32.5 ± 0.9†</td>
<td>31.8 ± 0.6†</td>
</tr>
<tr>
<td>SBP, mm Hg</td>
<td>138 ± 1</td>
<td>141 ± 4</td>
<td>221 ± 5†</td>
<td>202 ± 6†</td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td>380 ± 8</td>
<td>392 ± 5</td>
<td>339 ± 11†</td>
<td>366 ± 8</td>
</tr>
<tr>
<td>RPP, mm Hg × bpm</td>
<td>52 376 ± 929</td>
<td>55 132 ± 1722</td>
<td>73 513 ± 3980†</td>
<td>74 675 ± 3263†</td>
</tr>
<tr>
<td>Heart weight/tibial length, mg/mm</td>
<td>26.3 ± 0.6</td>
<td>23.1 ± 0.4*</td>
<td>35.0 ± 1.2†</td>
<td>35.1 ± 1.0†</td>
</tr>
<tr>
<td>LV weight/tibial length, mg/mm</td>
<td>20.0 ± 0.5</td>
<td>17.6 ± 0.3*</td>
<td>28.0 ± 0.7†</td>
<td>28.2 ± 0.9†</td>
</tr>
<tr>
<td>Visceral fat weight/tibial length, mg/mm</td>
<td>105 ± 9</td>
<td>49 ± 3*</td>
<td>482 ± 18†</td>
<td>377 ± 33††</td>
</tr>
<tr>
<td>Subcutaneous fat weight/tibial length, mg/mm</td>
<td>32 ± 3</td>
<td>19 ± 1</td>
<td>209 ± 12†</td>
<td>155 ± 14††</td>
</tr>
</tbody>
</table>

SBP indicates systolic blood pressure; RPP, rate-pressure product; LV, left ventricular; Ovx-DS/lean, ovariectomized Dahl S.Z-Lepr+/Lepr+; Ovx-DS/obese, ovariectomized Dahl S.Z-Lepr+/Lepr+/E2, 17β-estradiol. Data are mean ± SEM (n = 9, 12, 8, and 7 for Ovx-DS/lean + placebo, Ovx-DS/obese + E2, Ovx-DS/obese + placebo, and Ovx-DS/obese + E2 groups, respectively).

* P < 0.05 vs Ovx-DS/lean + placebo.
† P < 0.05 vs Ovx-DS/lean + E2.
‡ P < 0.05 vs Ovx-DS/obese + placebo.
Cardiomyocyte Hypertrophy, Cardiac Fibrosis, and Gene Expression

Microscopic analysis revealed that the cross-sectional area of cardiac myocytes was increased in Ovx-DS/obese rats compared with Ovx-DS/lean rats. Hemodynamic overload resulted in marked upregulation of the expression of fetal-type cardiac genes, including those for atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and β-myosin heavy chain (β-MHC), in Ovx-DS/obese rat hearts, and such upregulation was not significantly affected by treatment with E2 (Figure 2C through 2E).

Azan-Mallory staining revealed that fibrosis in perivascular and interstitial regions of the LV myocardium was increased in Ovx-DS/obese rats compared with Ovx-DS/lean rats, and this effect was exacerbated by treatment with E2 (Figure 3A through 3C). The abundance of collagen type I mRNA, which correlates with myocardial stiffness,21 as well as the amounts of transforming growth factor-β1 (TGF-β1) and connective tissue growth factor (CTGF) mRNAs, which correlate with cardiac fibrosis and growth,24 were also increased in Ovx-DS/obese rats compared with Ovx-DS/lean rats, and treatment with E2 enhanced these effects (Figure 3D through 3F). Treatment with E2 also increased both the extents of perivascular and interstitial fibrosis, as well as the amounts of collagen type I, TGF-β1, and CTGF mRNAs in Ovx-DS/lean rats (Figure 3B through 3F).

Cardiac Oxidative Stress

Superoxide production in myocardial tissue sections revealed by staining with dihydroethidium, as well as the activity of NADPH oxidase in LV homogenates, was significantly increased for Ovx-DS/obese rats compared with Ovx-DS/lean rats (Figure 4A through 4C). The expressions of genes for the p22phox and gp91phox membrane components and for the Rac1 cytoplasmic component of NADPH oxidase in the heart were also upregulated in Ovx-DS/obese rats compared with Ovx-DS/lean rats (Figure 4D through 4F). E2 treatment exacerbated all of these effects in Ovx-DS/obese rats and mimicked them in Ovx-DS/lean rats.

Cardiac Inflammation

Immunostaining for the monocyte-macrophage marker CD68 revealed that macrophage infiltration in the LV myocardium was increased in Ovx-DS/obese rats compared with Ovx-DS/lean rats and that E2 treatment increased such infiltration in both Ovx-DS/obese and Ovx-DS/lean rats (Figure 5A and 5B). The expressions of monocyte chemoattractant protein-1 (MCP-1), osteopontin, and cyclooxygenase-2 (COX-2) genes in the left ventricle were also increased in Ovx-DS/obese rats compared with Ovx-DS/lean rats, and treatment with E2 increased such gene expression in both types of rats (Figure 5C through 5E).

Cardiac RAAS

The abundance of mRNAs for angiotensin-converting enzyme (ACE), the Ang II type 1A (AT1A) receptor, mineralocorticoid receptor (MR), and serum/glucocorticoid-regulated kinase 1 (Sgk1) in the left ventricle was increased in Ovx-DS/obese rats compared with Ovx-DS/lean rats, and these effects were increased further in Ovx-DS/obese rats and mimicked in Ovx-DS/lean rats by treatment with E2 (Figure 6A through 6D). The abundance of the Ang II type 1 (AT1) receptor protein in the left ventricle showed a pattern similar to that of the AT1A receptor mRNA in the 4 experimental groups (Figure 6E).

Influence of Strain and E2 Treatment: Analysis by 2-Way Factorial ANOVA

Please see the online-only Data Supplement.

Discussion

Our results show that Ovx-DS/obese rats fed a normal diet develop obesity as well as hypertension and LV hypertrophy, fibrosis, and diastolic dysfunction compared with Ovx-DS/lean rats. Treatment with E2 attenuated hypertension and obesity but exacerbated LV fibrosis and diastolic dysfunction in Ovx-DS/obese rats, the latter effects being associated with increased cardiac oxidative stress and inflammation. E2-induced activation of the cardiac RAAS may have contrib-
uted to the cardiac remodeling and diastolic dysfunction in these animals.

Cross-talk between estrogen and leptin has been suggested, with both of these hormones acting as signals related to nutritional state.25 In the present study, E2 treatment reduced body weight, food intake, and visceral fat content in both Ovx-DS/obese and Ovx-DS/lean rats. The estrogen receptor (ER) contributes to the modulation and distribution of body fat mass, and its expression in adipose tissue appears to be responsible for the lipolytic effect of estrogen.26 E2 treatment was found to enhance pro-opiomelanocortin tone, decreasing food intake and body weight, in both wild-type and leptin-mutant obese animals.3,25 E2 may, therefore, reduce obesity under conditions of failed leptin receptor signaling, in part by rewiring of melanocortin signaling.

We showed recently that SBP was higher in female DS/obese rats than in DS/lean females,17 suggesting that the presence of the ja allele of Lepr on the DahlS background is associated with increased salt sensitivity of blood pressure. In the present study, Ovx-DS/obese rats manifested hypertension at 7 weeks of age and thereafter compared with Ovx-DS/lean rats, and treatment with E2 attenuated this increase in blood pressure in Ovx-DS/obese rats without affecting SBP in Ovx-DS/lean rats. E2 lowers SBP in several animal models of hypertension, including spontaneously hypertensive rats (SHRs),27 stroke-prone SHRs,28 Dahl salt-sensitive rats,29 and rats with pulmonary hypertension.30 Administration of E2 (oral or transdermal patches) in normotensive postmenopausal women either reduced or had no effect on SBP.31 SBP was also found to be similar in E2- or placebo-treated ovariectomized Wistar rats fed a high-sodium diet.32 Moreover, sublingual administration of E2 lowered SBP in hypertensive but not in normotensive postmenopausal women.33 Hypertensive individuals showed higher peak concentrations of E2 in plasma compared with normotensive controls after administration of the hormone,33 suggesting that hypertension might affect the bioavailability of estrogen during E2 treatment. Our results suggest that the beneficial effects of estrogen replacement with regard to an improvement in vascular endothelial function and reduction in arterial stiffness3,10 were induced in Ovx-DS/obese rats. Of note, however, E2 lowered SBP only by ~20 mm Hg in Ovx-DS/obese rats, and these rats were still severely hypertensive. It was also reported that E2 increased blood pressure robustly associated with reduced arterial compliance in postmenopausal women.34 We speculate that the competing influences of estrogen replacement on vascular tone cancelled out

Figure 2. Cardiomyocyte size and expression of fetal-type cardiac genes in the left ventricle of ovariectomized DahlS.Z-Lepr ja/Lepre ja (Ovx-DS/lean) and ovariectomized DahlS.Z-Lepr ja/Leps −/− (Ovx-DS/obese) rats in the 4 experimental groups at 13 weeks of age. A, Hematoxylin-eosin staining of transverse sections of the left ventricular (LV) myocardium. Scale bars, 50 μm. B, Cross-sectional area of cardiomyocytes determined from sections similar to those in A, C through E. Quantitative RT-PCR analysis of atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and β-myosin heavy chain (β-MHC) mRNAs, respectively. The amount of each mRNA was normalized by that of GAPDH mRNA and then expressed relative to the mean value for the Ovx-DS/lean + placebo group. Data in B through E are mean ± SEM (n = 9, 12, 8, and 7 for Ovx-DS/lean + placebo, Ovx-DS/lean + 17β-estradiol [E2], Ovx-DS/obese + placebo, and Ovx-DS/obese + E2 groups, respectively). *P < 0.05 vs Ovx-DS/lean + placebo; †P < 0.05 vs Ovx-DS/lean + E2.
potential changes in arterial compliance, resulting in a modest attenuation of high blood pressure in Ovx-DS/obese rats. Obesity, especially when complicated with hypertension, is associated with changes in cardiac structure and function.35 Our observations that both the ratio of LV weight to tibial length (or echocardiography-derived LV mass) and relative wall thickness, as well as the expression of fetal-type cardiac genes, were significantly increased in Ovx-DS/obese rats, indicate that these animals develop of fetal-type cardiac genes, were significantly increased in Ovx-DS/obese rats. In contrast, the ratio of LV weight to tibial length (or echocardiography-derived LV mass) and relative wall thickness, as well as the expression of fetal-type cardiac genes, were significantly increased in Ovx-DS/obese rats. These data are consistent with previous observations showing that these growth factors are involved in the development of LV remodeling in a rat model of heart failure24 and in SHR-NDmcr-cp rats fed a high-salt diet.37 E2 enhanced these profibrotic effects in Ovx-DS/obese rats and induced similar changes in Ovx-DS/obese rats. These data are consistent with previous observations showing that these growth factors are involved in the development of LV remodeling in a rat model of heart failure24 and in SHR-NDmcr-cp rats fed a high-salt diet.37 E2 enhanced these profibrotic effects in Ovx-DS/obese rats and induced similar changes in Ovx-DS/obese rats.

Cardiac fibrosis is a pathological feature associated with hypertension and is responsible for LV diastolic dysfunction, likely as a result of increased LV diastolic stiffness.22 Ovx-DS/obese rats developed hypertension and impaired LV relaxation, as well as an increase in the extents of LV perivascular and interstitial fibrosis compared with Ovx-DS/lean rats, and these changes in Ovx-DS/obese rats were accompanied by the upregulation of TGF-β1 and CTGF mRNAs in the heart. These data are consistent with previous observations showing that these growth factors are involved in the development of LV remodeling in a rat model of heart failure24 and in SHR-NDmcr-cp rats fed a high-salt diet.37 E2 enhanced these profibrotic effects in Ovx-DS/obese rats and induced similar changes in Ovx-DS/obese rats. E2 treatment enhanced myocardial infarction–induced cardiac fibrosis in mice.40 Whether estrogen exerts protective or detrimental effects on the heart may thus depend on the dose and on animal species and strain.

Macrophages have been implicated in fibrosis associated with various diseases. In the present study, macrophage infiltration into the myocardial interstitial space was ac-

Figure 3. Cardiac fibrosis and gene expression in the left ventricle of ovariectomized DahlS.Z-Lepr+/Lepr− (Ovx-DS/lean) and ovariectomized DahlS.Z-Lepr+/Lepr− (Ovx-DS/obese) rats in the 4 experimental groups at 13 weeks of age. A, Collagen deposition as revealed by Azan-Mallory staining in perivascular (top) and interstitial (bottom) regions of the left ventricular (LV) myocardium. Scale bars, 200 μm. B and C, Relative extents of perivascular and interstitial fibrosis, respectively, in the LV myocardium as determined from sections similar to those in A, D through F. Quantitative RT-PCR analysis of collagen type I, transforming growth factor-β1 (TGF-β1), and connective tissue growth factor (CTGF) mRNAs, respectively. The amount of each mRNA was normalized by that of GAPDH mRNA and then expressed relative to the mean value for the Ovx-DS/lean + placebo group. Data in B through F are mean ± SEM (n=9, 12, 8, and 7 for Ovx-DS/lean + placebo, Ovx-DS/lean + 17β-estradiol [E2], Ovx-DS/obese + placebo, and Ovx-DS/obese + E2 groups, respectively). *P<0.05 vs Ovx-DS/lean + placebo; †P<0.05 vs Ovx-DS/lean + E2; ‡P<0.05 vs Ovx-DS/obese + placebo.
compounded by upregulation of the expression of proinflammatory genes, including those for MCP-1, osteopontin, and COX-2, in the heart of Ovx-DS/obese rats, effects that may have triggered myocardial fibrosis.41 E2 treatment induced further increases in macrophage infiltration and the expression of MCP-1, osteopontin, and COX-2 genes in Ovx-DS/obese rats, and elicited similar effects in Ovx-DS/lean rats. In postmenopausal women, hormone replacement therapy is associated with increases in the circulating concentration of C-reactive protein, as well as in other markers of inflammation.42 In stroke-prone SHRs, ovariectomy reduced stroke and renal injury, whereas estrogen treatment promoted cardiac inflammation in Ovx-DS/obese rats, and elicited similar effects in Ovx-DS/lean rats. 

Figure 4. NADPH oxidase activity and gene expression in the left ventricle of ovariectomized Dahl.S-Z-Lepr+/Lepr+ (Ovx-DS/lean) and ovariectomized Dahl.S-Z-Lepr+/Lepr− (Ovx-DS/obese) rats in the 4 experimental group at 13 weeks of age. A, Superoxide production as revealed by dihydroethidium staining in interstitial regions of the left ventricular (LV) myocardium. Scale bars, 200 μm. B, Dihydroethidium fluorescence intensity determined from sections similar to those in A. Data are expressed as the relative differences compared with Ovx-DS/lean + placebo rats. C, NADPH-dependent superoxide production in LV homogenates. Results are expressed as relative light units (RLU) per milligram of protein. D through F, Quantitative RT-PCR analysis of p22phox, gp91phox, and Rac1 mRNAs, respectively. The amount of each mRNA was normalized by that of GAPDH mRNA and then expressed relative to the mean value for the Ovx-DS/obese group. Data in B through F are mean ± SEM (n = 9, 12, 8, and 7 for Ovx-DS/lean + placebo, Ovx-DS/obese + placebo, Ovx-DS/obese + E2 groups, respectively). *P < 0.05 vs Ovx-DS/obese + placebo; †P < 0.05 vs Ovx-DS/lean + E2; ‡P < 0.05 vs Ovx-DS/obese + E2.
blunts the decay of diastolic Ca\(^{2+}\) transients and impairs myocyte relaxation.\(^4^8\) In contrast, estrogen is reported to have antioxidant effects and to exert direct effects on the vessel wall, such as an increase in vascular NO production and modulation of endothelial NO synthase expression.\(^4^9\) These observations suggest that additional oxidative stress induced by E\(_2\) may play a role in the enhancement of LV diastolic dysfunction by E\(_2\) in Ovx-DS/obese rats.

E\(_2\) also has effects on components of the RAAS. The expression of genes for the AT\(_1\)A receptor, ACE, MR, and the aldosterone effector kinase Sgk1 was upregulated in the heart of DS/obese rats,\(^1^7\) consistent with a causative role for RAAS activation in the development of cardiac injury associated with MetS,\(^3^7\) and these effects were increased further by E\(_2\) treatment. Enhanced MR signaling in the myocardium results in increased oxidative stress and inflammation, leading to the development of cardiac remodeling and dysfunction.\(^2^2,^5^0\) Our observation that E\(_2\) increased the abundance of AT\(_1\) receptor protein in the heart of both Ovx-DS/lean and Ovx-DS/obese rats is consistent with the previous finding that E\(_2\) increased AT\(_1\) receptor expression in the heart of healthy rats, as well as in the renal cortex of rats treated with both Ang II and the NO synthase inhibitor N\(^G\)-nitro-L-arginine methyl ester.\(^1^5,^3^2\) Ang II increases oxidative stress in the cardiovascular system through AT\(_1\) receptor–mediated activation of NADPH oxidase.\(^5^1\) E\(_2\)-induced RAAS activation may, therefore, play a role in the pathophysiology of cardiac oxidative stress in Ovx-DS/obese rats. In contrast to our observation, estrogen replacement was found to downregulate cardiac AT\(_1\) receptors and to attenuate cardiac remodeling in 1-year–old ovariectomized Sprague-Dawley rats.\(^5^2\) It is likely that the effects of estrogen on AT\(_1\) receptor expression and RAAS activity differ in a manner dependent on experimental conditions, including the animal model, age, genotype, and dietary sodium intake. Together, these data suggest that E\(_2\)-induced additional activation of the cardiac RAAS may contribute to LV remodeling and diastolic dysfunction in Ovx-DS/obese rats.

There is evidence that estradiol inhibits the sympathetic nervous system both in animal models of hypertension and in postmenopausal women.\(^3^1\) Nevertheless, careful examination of the literature revealed conflicting results regard-
The effects of estradiol therapy on the autonomic tone in women. Although we have no data on the sympathetic activity, it is possible that E2-induced modulation of the cardiac sympathovagal balance might have contributed to LV remodeling and diastolic dysfunction in Ovx-DS/obese rats.

**Perspectives**

E2 treatment attenuated hypertension and obesity but exacerbated LV fibrosis and diastolic dysfunction in Ovx-DS/obese rats, the latter effects being associated with increased cardiac oxidative stress and inflammation. E2-induced activation of the cardiac RAAS may have contributed to cardiac injury in these animals and may also help explain the increased risk of cardiovascular disease caused by hormone replacement therapy in postmenopausal women. Further investigations are required to clarify the molecular mechanisms of these actions of E2.

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**Disclosures**

None.
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Effects of Estrogen on Cardiovascular Injury in Ovariectomized Female DahlS.Z-Lepr<sup>fa</sup>/Lepr<sup>fa</sup> Rats as a New Animal Model of Metabolic Syndrome

Tamayo Murase, Takuya Hattori, Masafumi Ohtake, Chieko Nakashima, Miwa Takatsu, Toyoaki Murohara and Kohzo Nagata

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EFFECTS OF ESTROGEN ON CARDIOVASCULAR INJURY IN OVARIECTOMIZED FEMALE DahlS.Z-Lept/*Lept/* RATS AS A NEW ANIMAL MODEL OF METABOLIC SYNDROME

Short title: Estrogen and cardiac injury

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Expanded Materials and Methods

Animals and experimental protocols
Six-week-old female inbred DahlS.Z-Lepr<sup>fa</sup>/Lepr<sup>fa</sup> (DS/obese) rats were obtained from Japan SLC (Hamamatsu, Japan) and were handled in accordance with the guidelines of Nagoya University Graduate School of Medicine as well as with the Guide for the Care and Use of Laboratory Animals (NIH publication no. 85-23, revised 1996). After weaning, the rats were fed normal laboratory chow containing 0.36% NaCl. Both the diet and tap water were provided ad libitum throughout the experimental period. Age-matched female homozygous lean littermates (DahlS.Z-Lepr<sup>+/-</sup>/Lepr<sup>+/-</sup>, or DS/lean) of DS/obese rats served as control animals.

Echocardiographic Analysis
M-mode echocardiography was performed with a 12.5-MHz transducer (Xario SSA-660A; Toshiba Medical Systems, Tochigi, Japan). Left ventricular (LV) end-diastolic (LVDd) and end-systolic (LVDs) dimensions and the thickness of the interventricular septum (IVST) and LV posterior wall (LVPWT) were measured, and fractional shortening (LVFS), relative wall thickness (RWT), and LV mass were calculated as follows: LVFS (%) = v × 100; RWT = (IVST + LVPWT)/LVDd; LV mass (g) = [(1.04 × [(IVST + LVDd + LVPWT)<sup>3</sup> – (LVDd)<sup>3</sup>]] × 0.8) + 0.14. 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 (DcT) and isovolumic relaxation time (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, USA) 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 LV end-diastolic pressure (LVEDP). The time constant of isovolumic relaxation (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. Image analysis was performed with NIH Scion Image software. 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, USA) 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 Corp., Frederick, MD, USA).

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. The chemiluminescence signal was sampled every minute for 10 min with a microplate reader (Wallac 1420 Arvo MX/Light; Perkin Elmer, Waltham, MA, USA), and the respective background counts were subtracted from experimental values. Lucigenin chemiluminescence was expressed as relative light units (RLU) per milligram of protein.
Superoxide production in tissue sections was examined by staining with dihydroethidium (Sigma, St. Louis, MO, USA) as described. 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. For negative controls, we performed dihydroethidium staining after incubation of the 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).

**Quantitative RT-PCR Analysis**

Total RNA was extracted from LV tissue and subjected to quantitative reverse transcription (RT)-polymerase chain reaction (PCR) analysis, as described, with primers and TaqMan probes specific for rat complementary DNAs encoding atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), β-myosin heavy chain (β-MHC), collagen type I, transforming growth factor–β1 (TGFBβ1), connective tissue growth factor (CTGF), monocyte chemoattractant protein–1 (MCP-1), osteopontin, cyclooxygenase (COX)–2, (5′-CCAACCTCTCTCTACTACACCAG-3′, 5′-GAAGTTCCTTATTTCCTTACACC-3′, and 5′-CTCTCTCTCTTGCGCTGATGACTG-3′ as the forward primer, reverse primer, and TaqMan probe, respectively; GenBank accession no. NM_017232), angiotensin-converting enzyme (ACE), Ang II type 1A (AT1A) receptor, mineralocorticoid receptor (MR), serum/glucocorticoid–regulated kinase (Sgk) 1, as well as p22phox, gp91phox, and Rac1 subunits of NADPH oxidase. TaqMan rodent glyceraldehyde-3-phosphate dehydrogenase (GAPDH) control reagents (Applied Biosystems) were used for detection of 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, USA). 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. The membrane was then incubated first with a 1:200 dilution of rabbit polyclonal antibodies to the Ang II type 1 (AT1) receptor (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and a 1:200 dilution of goat polyclonal antibodies to GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and then with a 1:3000 dilution of horseradish peroxidase–conjugated goat antibodies to rabbit immunoglobulin G (Medical and Biological Laboratories, Nagoya, Japan). Detection and quantification of immune complexes were performed as described previously.

**Expanded Results**

**Influence of strain and E2 treatment: analysis by two-way factorial ANOVA**

Body weight was influenced by strains or E2 treatment. However, there was no interaction between strains and E2 treatment. Both SBP and RPP were influenced by strains, but not by E2 treatment, and there were no interactions. Both heart and LV weights were influenced significantly by strains and insignificantly by E2 treatment, and the interactions were both significant. Both visceral and subcutaneous fat weights were influenced by strains or E2 treatment. The interaction was significant in subcutaneous fat weight, but not in visceral fat weight. IVST, LVPWT, and LV mass were influenced by strains, but not by E2 treatment, and the interactions were all significant. With regard to LV diastolic dysfunction, IRT, LVEDP, Tau, and LVEDP/LVDD were influenced by these 2 factors, but there were no interactions. Myocyte cross-sectional area was influenced by strains or E2 treatment, and the interaction was significant. However, the expression of fetal-type cardiac genes was influenced only by strains and there was no interaction. Perivascular and interstitial fibrosis of LV myocardium and cardiac expression of collagen type I and growth factors were influenced by these 2
factors but there were no interactions. Cardiac oxidative stress, the expression of NADPH oxidase components, cardiac inflammation (except for osteopontin), and the expression of RAAS genes also showed similar tendencies to those in fibrosis. Taken together, these data suggest that attenuation of LV (cardiomyocyte) hypertrophy with E₂ occurs mainly in Ovx-DS/lean rats, and that changes in body composition, LV diastolic function, fibrosis, oxidative stress, inflammation, and RAAS gene expression with E₂ are observed similarly in both Ovx-DS/lean and Ovx-DS/obese rats. Thus, the data analyzed by two-way factorial ANOVA were compatible with the original results analyzed by one-way factorial ANOVA.

Supplemental references


Table S 1. Results of two-way factorial ANOVA in four groups of rats (Ovx-DS/lean + placebo, Ovx-DS/lean + E2, Ovx-DS/obese + placebo, and Ovx-DS/obese + E2).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Influence of strain</th>
<th>Influence of E&lt;sub&gt;2&lt;/sub&gt; treatment</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>Body weight (g)</td>
<td>246.968</td>
<td>&lt;0.0001</td>
<td>86.129</td>
</tr>
<tr>
<td>Tibial length (mm)</td>
<td>23.795</td>
<td>&lt;0.0001</td>
<td>6.969</td>
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<tr>
<td>SBP (mmHg)</td>
<td>240.712</td>
<td>&lt;0.0001</td>
<td>0.580</td>
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<tr>
<td>Heart rate (beats/min)</td>
<td>15.777</td>
<td>0.0004</td>
<td>5.427</td>
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<tr>
<td>RPP (mmHg x beats/min)</td>
<td>69.217</td>
<td>&lt;0.0001</td>
<td>0.642</td>
</tr>
<tr>
<td>Heart weight/tibial length (mg/mm)</td>
<td>168.559</td>
<td>&lt;0.0001</td>
<td>3.941</td>
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<tr>
<td>LV weight/tibial length (mg/mm)</td>
<td>247.733</td>
<td>&lt;0.0001</td>
<td>3.484</td>
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<tr>
<td>Visceral fat weight/tibial length (mg/mm)</td>
<td>396.691</td>
<td>&lt;0.0001</td>
<td>20.528</td>
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<td>Subcutaneous fat weight/tibial length (mg/mm)</td>
<td>290.052</td>
<td>&lt;0.0001</td>
<td>17.903</td>
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<tr>
<td>IVST (mm)</td>
<td>223.405</td>
<td>&lt;0.0001</td>
<td>0.518</td>
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<tr>
<td>LVPWT (mm)</td>
<td>364.338</td>
<td>&lt;0.0001</td>
<td>0.031</td>
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<tr>
<td>LVDd (mm)</td>
<td>2.776</td>
<td>0.1054</td>
<td>5.214</td>
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<tr>
<td>LVFS (%)</td>
<td>9.319</td>
<td>0.0045</td>
<td>40.768</td>
</tr>
<tr>
<td>LV mass (mg)</td>
<td>179.378</td>
<td>&lt;0.0001</td>
<td>2.610</td>
</tr>
<tr>
<td>RWT</td>
<td>57.669</td>
<td>&lt;0.0001</td>
<td>1.721</td>
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<tr>
<td>DcT (ms)</td>
<td>119.848</td>
<td>&lt;0.0001</td>
<td>55.754</td>
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<tr>
<td>IRT (ms)</td>
<td>66.554</td>
<td>&lt;0.0001</td>
<td>40.171</td>
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<tr>
<td>Tei index</td>
<td>112.785</td>
<td>&lt;0.0001</td>
<td>84.109</td>
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<tr>
<td>LVEDP (mm)</td>
<td>92.802</td>
<td>&lt;0.0001</td>
<td>61.167</td>
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<tr>
<td>Tau (ms)</td>
<td>28.332</td>
<td>&lt;0.0001</td>
<td>26.314</td>
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<tr>
<td>LVEDP/LVDd (mmHg/mm)</td>
<td>37.029</td>
<td>&lt;0.0001</td>
<td>38.092</td>
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<tr>
<td>Myocyte cross-sectional area</td>
<td>180.078</td>
<td>&lt;0.0001</td>
<td>29.060</td>
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<tr>
<td></td>
<td>Mean</td>
<td>P-Value</td>
<td>Mean</td>
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<td>------------------</td>
<td>------</td>
<td>---------</td>
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<tr>
<td>ANP</td>
<td>90.202</td>
<td>&lt;0.0001</td>
<td>0.185</td>
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<td>BNP</td>
<td>39.422</td>
<td>&lt;0.0001</td>
<td>0.748</td>
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<tr>
<td>β-MHC</td>
<td>42.521</td>
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<td>1.237</td>
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<td>Perivascular fibrosis</td>
<td>61.471</td>
<td>&lt;0.0001</td>
<td>12.430</td>
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<td>Interstitial fibrosis</td>
<td>41.009</td>
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<td>10.734</td>
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<td>Collagen type I</td>
<td>39.456</td>
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<td>21.182</td>
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<td>TGF-β1</td>
<td>17.478</td>
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<td>CTGF</td>
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<td>Relative DHE fluoroescence</td>
<td>54.719</td>
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<td>23.548</td>
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<td>NADPH oxidase activity</td>
<td>42.922</td>
<td>0.0003</td>
<td>15.211</td>
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<tr>
<td>p22&lt;sub&gt;p&lt;/sub&gt;</td>
<td>30.940</td>
<td>&lt;0.0001</td>
<td>14.222</td>
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<tr>
<td>gp91&lt;sub&gt;p&lt;/sub&gt;</td>
<td>47.327</td>
<td>&lt;0.0001</td>
<td>21.128</td>
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<tr>
<td>Rac1</td>
<td>42.582</td>
<td>&lt;0.0001</td>
<td>20.835</td>
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<tr>
<td>CD68-positive cells/mm&lt;sup&gt;2&lt;/sup&gt;</td>
<td>55.474</td>
<td>&lt;0.0001</td>
<td>22.497</td>
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<tr>
<td>MCP-1</td>
<td>22.455</td>
<td>&lt;0.0001</td>
<td>17.639</td>
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<td>Osteopontin</td>
<td>56.128</td>
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<td>44.285</td>
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<td>COX-2</td>
<td>27.277</td>
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<td>14.046</td>
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<td>ACE</td>
<td>41.126</td>
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<td>9.281</td>
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<td>11.291</td>
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<td>MR</td>
<td>57.081</td>
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<td>28.203</td>
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<td>Sgk1</td>
<td>52.143</td>
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<td>AT&lt;sub&gt;1&lt;/sub&gt; receptor</td>
<td>20.487</td>
<td>0.0002</td>
<td>8.676</td>
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</table>

Abbreviations: SBP, systolic blood pressure; RPP, rate-pressure product; IVST, interventricular septum thickness; LVPWT, left ventricular posterior wall thickness; LVDd, left ventricular end-diastolic dimension; LVFS, left ventricular fractional shortening; RWT, relative wall thickness; DcT, deceleration time; IRT, isovolumic relaxation time; LVEDP, left ventricular end-diastolic pressure; ANP, cardiac mRNA expression of atrial natriuretic factor; BNP, brain natriuretic peptide; TGF-β1, transforming growth factor-β1; CTGF, connective tissue growth factor; DHE, dihydroethidium; NADPH, nicotinamide adenine dinucleotide phosphate; p22<sub>phox</sub>, p22-phox; gp91<sub>phox</sub>, gp91-phox; Rac1, Rho GTPase-activating protein 1; CD68, cluster of differentiation 68; MCP-1, monocyte chemoattractant protein-1; COX-2, cyclooxygenase-2; ACE, angiotensin-converting enzyme; AT<sub>1A</sub> receptor, angiotensin II type 1A receptor; MR, mineralocorticoid receptor; Sgk1, serum/glucocorticoidregulated kinase 1; AT<sub>1</sub> receptor, angiotensin II type 1 receptor.
peptide; BNP, cardiac mRNA expression of brain natriuretic peptide; \(\beta\)-MHC, cardiac mRNA expression of \(\beta\)-myosin heavy chain; TGF-\(\beta\)1, cardiac mRNA expression of transforming growth factor-\(\beta\)1; CTGF, cardiac mRNA expression of connective tissue growth factor; DHE, Dihydroethidium; p22\(^{\text{phox}}\), gp91\(^{\text{phox}}\) and Rac1, cardiac mRNA expression of NADPH oxidase components; MCP-1, cardiac mRNA expression of monocyte chemoattractant protein-1; COX-2, cardiac mRNA expression of cyclooxygenase-2; ACE, cardiac mRNA expression of angiotensin-converting enzyme; AT\(_{1A}\) receptor, cardiac mRNA expression of angiotensin II type 1A receptor; MR, cardiac mRNA expression of mineralocorticoid receptor; Sgk1, cardiac mRNA expression of serum/glucocorticoid-regulated kinase 1; AT\(_{1}\) receptor, the abundance of angiotensin II type 1 receptor protein in the left ventricle.