Endothelial Mineralocorticoid Receptor Deletion Prevents Diet-Induced Cardiac Diastolic Dysfunction in Females


Abstract—Overnutrition and insulin resistance are especially prominent risk factors for the development of cardiac diastolic dysfunction in females. We recently reported that consumption of a Western diet (WD) containing excess fat (46%), sucrose (17.5%), and high fructose corn syrup (17.5%) for 16 weeks resulted in cardiac diastolic dysfunction and aortic stiffening in young female mice and that these abnormalities were prevented by mineralocorticoid receptor blockade. Herein, we extend those studies by testing whether WD-induced diastolic dysfunction and factors contributing to diastolic impairment, such as cardiac fibrosis, hypertrophy, inflammation, and impaired insulin signaling, are modulated by excess endothelial cell mineralocorticoid receptor signaling. Four-week-old female endothelial cell mineralocorticoid receptor knockout and wild-type mice were fed mouse chow or WD for 4 months. WD feeding resulted in prolonged relaxation time, impaired diastolic septal wall motion, and increased left ventricular filling pressure indicative of diastolic dysfunction. This occurred in concert with myocardial interstitial fibrosis and cardiomyocyte hypertrophy that were associated with enhanced profibrotic (transforming growth factor β1/Smad) and progrowth (S6 kinase-1) signaling, as well as myocardial oxidative stress and a proinflammatory immune response. WD also induced cardiomyocyte stiffening, assessed ex vivo using atomic force microscopy. Conversely, endothelial cell mineralocorticoid receptor deficiency prevented WD-induced diastolic dysfunction, profibrotic, and progrowth signaling, in conjunction with reductions in macrophage profibrotic polarization and improvements in insulin metabolic signaling. Therefore, our findings indicate that increased endothelial cell mineralocorticoid receptor signaling associated with consumption of a WD plays a key role in the activation of cardiac profibrotic, inflammatory, and growth pathways that lead to diastolic dysfunction in female mice. (Hypertension. 2015;66:1159-1167. DOI: 10.1161/HYPERTENSIONAHA.115.06015.)

Key Words: cardiovascular diseases ■ diet, Western ■ insulin resistance ■ obesity ■ receptors, mineralocorticoid

Cardiovascular disease (CVD) is strikingly increased in women with obesity, the metabolic syndrome, and diabetes mellitus,1,2 all of which are promoted by increased consumption of a Western diet (WD) high in both fat and sugar. For example, the Framingham Heart Study showed that left ventricular (LV) mass and wall thickness is proportionately greater in women than in men with worsening glucose intolerance.1 Emerging data also indicate that aldosterone levels are higher in overweight and hypertensive women and that the elevated plasma aldosterone is positively associated with markers of concentric cardiac remodeling like LV wall thickness in females but not in males.3,4 Mineralocorticoid excess and enhanced macrophage, vascular, and cardiac mineralocorticoid receptor (MR) signaling have been associated with increased fibrosis, inflammation, oxidative stress, and maladaptive cardiac remodeling.7-14 An early functional manifestation of this maladaptive remodeling and fibrosis is diastolic dysfunction characterized by impaired diastolic relaxation with preserved systolic function.3,4 In this regard, there is increasing evidence that obese and diabetic women are more likely to develop diastolic dysfunction than their male counterparts.3,14-18 Furthermore, diastolic dysfunction is often associated with systemic and cardiovascular fibrosis, LV hypertrophy, and insulin resistance, suggesting that there is an imbalance between

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Endothelial dysfunction often occurs in conditions of obesity and insulin resistance.22,23–25 In insulin-resistant states, there is decreased cardiovascular insulin metabolic signaling, resulting in attenuated endothelial nitric oxide synthase (eNOS) activation, and an increased profibrotic environment.19,22 Furthermore, increased generation of reactive oxygen species plays a role in impaired vascular insulin metabolic signaling by increasing redox sensitive serine (Ser) kinase activation/phosphorylation (p), such as S6K-1.18,21,24–26 These activated Ser kinases decrease insulin receptor-1 (IRS-1) tyrosine (p) and engagement with phosphoinositol 3-kinase (PI3-K), with a resultant decrease in protein kinase B (Akt)-mediated (p)/activation of eNOS.19,22,25–27 There is also mounting evidence that elevated plasma aldosterone and systemic and cardiovascular MR signaling contributes to systemic and cardiovascular fibrosis, insulin resistance,4,19,22 and associated endothelial dysfunction.21,28 Endothelial cells (ECs) express MRs, as well as the 11β-hydroxysteroid dehydrogenase enzyme, which limit glucocorticoid signaling through these receptors.29,31 Increased MR expression and signaling in ECs of rodent models has been shown to increase generation of vascular reactive oxygen species and reduce bioavailable nitric oxide (NO) and thus impair endothelial-mediated vascular relaxation.29,30

Utilization of cell-specific MR null mice has identified a role for cardiomyocyte, macrophage, and endothelial cell mineralocorticoid receptors (ECMRs) in aldosterone/deoxycorticosterone acetate (DOCA) and salt-induced oxidative stress and inflammation-mediated cardiac tissue fibrosis, remodeling, and dysfunction in male mice.3–13 In each of these cell-specific null models, there was protection afforded by the cell-specific MR deletion. However, those EC studies were conducted with Tie2-promoter driving expression of Cre-recombinase in ECs and other myeloid lineage cells. Given the importance of macrophage MRs in promoting cardiac remodeling,4,11 the protection in this MR null model may have reflected, in part, the deletion of MRs from macrophages and other myeloid cells. The choice to study females was predicted on the fact that all previous studies were done in males and our previous observations that females are more prone than males to develop elevated plasma aldosterone levels, cardiac fibrosis, and diastolic dysfunction in the setting of WD consumption and the development of insulin resistance.4,19,32

Materials and Methods

Animals and Treatments

ECMR knockout (KO; ECMR−/−) mice were kindly provided by Drs Jaffe and Mueller from Tufts University Medical Center who also contributed to the design of this investigation.33 In these mice, exons 5 and 6 of the MR gene are flanked by loxP sites via homologous recombination (MRf/f), as previously described.33 ECMR KO was generated by crossing MRf/f mice with Cad-Cre+ mice.33,34 MRf/f Cad-Cre+ littermates were used as controls.35 All animal procedures were performed in accordance with the Animal Use and Care Committee at the University of Missouri, Columbia, the Subcommittee for Animal Safety at the Harry S. Truman Memorial Veterans’ Hospital and National Institutes of Health Guide for the Care and Use of Laboratory Animals. Groups of 4-week-old female mice were fed a WD36 consisting of high fat (46%) and a high carbohydrate component as constituted with sucrose (17.5%) and high fructose corn syrup (17.5%) for 16 weeks. Parallel groups of age-matched female controls (ECMR+/+) were fed regular control diet for the same period of time. Detailed description of procedures is available in Methods in the online-only Data Supplement.

Results

Effects of ECMR KO on Body Composition and Insulin Sensitivity of Mice Fed a WD

Twenty-week-old ECMR+/+ and ECMR−/− female mice fed control diet had similar whole body fat mass, body weight, and insulin sensitivity as assessed by homeostasis model assessment–estimated insulin resistance (Table). As expected, consumption of a WD for 16 weeks by ECMR+/+ mice induced an 83% increase in whole body fat mass, a 22% increase in body perireproductive fat mass and insulin resistance compared with control diet–fed mice (P<0.05; Table). There was no significant difference in WD-induced changes in body composition or insulin sensitivity in ECMR−/− versus ECMR+/+ mice. Of note, there were also no significant differences in lean body weight or mean arterial pressures between any of the groups (Table).

ECMR−/− Prevents Development of Diastolic Dysfunction

Consistent with our previous report,4 WD induced LV diastolic dysfunction and stiffness in ECMR+/+ female mice (Figure 1).
After 16 weeks of WD feeding, ECMR\textsuperscript{+/+} mice exhibited prolonged diastolic relaxation time in concert with slower initial and peak filling rates as assessed by cine-magnetic resonance imaging (Figure 1A–1D). These impairments were consistent with abnormal echocardiographic-derived diastolic parameters, including impaired septal annular wall motion, which was indicated by a reduction in the tissue Doppler-derived E'/A' ratio, an abnormal myocardial performance index and a prolonged period of isovolumic relaxation relative to control (Figure 1E–1G). Furthermore, ECMR\textsuperscript{−/−} mice also exhibited an increase in cardiomyocyte stiffness assessed in vitro as determined by atomic force microscopy (Figure 1H). These abnormalities were not observed in WD-fed ECMR\textsuperscript{−/−} mice, indicating that the absence of ECMR affords protection from the deleterious effects of WD that promote diastolic dysfunction and stiffness (Figure 1).

**Figure 1.** Western diet (WD)–induced cardiac diastolic dysfunction is prevented in ECMR\textsuperscript{−/−} female mice. **A**, Representative midventricle short-axis cine-magnetic resonance imaging (MRI) images that correspond to end-diastole, end-systole, and early diastole phases of cardiac cycle from control diet (CD)–fed ECMR KO mouse (CD ECMR\textsuperscript{−/−}, the second row), WD-fed wild-type mouse (WD ECMR\textsuperscript{+/+}, the third row), and WD-fed ECMR KO mouse (WD ECMR\textsuperscript{−/−}, lower row) compared with the CD-fed wild-type mouse (CD ECMR\textsuperscript{+/+}). **B**, Left ventricular (LV) diastolic relaxation time, **C** peak filling rate, and **D** LV initial filling rate were derived from in vivo cine-MRI. **E**, Representative tissue Doppler is shown of the early and late (E' and A') motion of the septal annulus during diastole and during early systole (E), myocardial performance index (MPI; **F**), and isovolumic relaxation time (IVRT; **G**). **H**, Atomic force microscopy (AFM) indentation measurements of the elastic modulus (stiffness) of acutely isolated ventricular cardiomyocytes. WD feeding caused an increase in cardiomyocyte stiffness in wild-type mice but not in ECMR KO mice. \(n=4\) to 6 per group. ECMR indicates endothelial cell mineralocorticoid receptor; and KO, knockout. *\(P<0.01\) compared with CD ECMR\textsuperscript{+/+}; #\(P<0.05\) compared with WD ECMR\textsuperscript{+/+}.
ECMR−/− prevented WD-induced LV interstitial fibrosis and tissue remodeling in concert with reduced transforming growth factor β1 (TGF-β1)/Smad signaling cascades.

To determine the role of ECMR in structural parameters involved in impairments in cardiac diastolic relaxation, we evaluated myocardial fibrosis by Verhoeff–van Gieson staining for total collagen and immunostaining for collagen 1, connective TGF, and fibronectin in ECMR+/+ and ECMR−/− mice fed a WD. WD induced an increase in cardiac fibrosis that was accompanied by increases in expression of TGF-β1 and phosphorylated-Smad2/3, whereas (p) of Smad7 was decreased (Figure 2; Figure S1 in the online-only Data Supplement). Importantly, ECMR−/− also attenuated the WD-induced increases in cardiac immunostaining for collagen 1, connective TGF, and fibronectin, as well as TGF-β1/Smad signaling, in conjunction with prevention of interstitial fibrosis (Figure 2; Figure S1).

**ECMR−/− Prevented WD-Induced Myocardial Oxidative Stress, Adaptive Proinflammatory Cytokines, and Macrophage M1/M2 Polarization**

In wild-type mice, WD promoted cardiac oxidant stress as indicated by increases in 3-nitrotyrosine immunostaining. This was associated with increases in cardiac tissue expression of the proinflammatory cytokines monocyte chemotactant protein-1, interleukin (IL) 1, and IL17, as well as expression of the M1 macrophage markers, CD86 and CD11b (Figure 3). ECMR−/− mice significantly prevented WD-induced increases in proinflammatory cytokines, such as IL17 and CD11b. Furthermore, WD-fed ECMR+/+ mice also displayed increased

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**Figure 2.** Western diet (WD)-induced cardiac fibrosis is prevented in ECMR−/− mice. A, Representative images of left ventricular immunostaining for interstitial fibrosis using picrosirius red with quantification of interstitial collagen deposition by average gray scale intensities. Representative images immunostaining for collagen 1 (B), connective tissue growth factor (CTGF; C), and fibronectin (D) with corresponding measures of average gray scale intensities. Scale bar=50 μm. E, Representative blots of TGF-β (F), phosphorylation of Smad2/3 (G), and Smad7 (H) in left ventricle tissues using Western blot with corresponding quantitative analysis. Control diet wild-type (CD ECMR+/+), ECMR KO control diet (CD ECMR−/−), WD wild-type (WD ECMR+/+), and WD ECMR KO (WD ECMR−/−) mice. n=3 to 5 per group. ECMR indicates endothelial cell mineralocorticoid receptor; and KO, knockout. *P<0.05 compared with CD ECMR+/+; #P<0.05 compared with WD ECMR+/+. Downloaded from http://hyper.ahajournals.org/ by guest on October 30, 2017
M2 marker expression (CD206 and IL10) and the ratio of M2/M1 marker gene expression (Figure 3).

ECMR−/− Prevents Abnormal Cardiac Insulin Metabolic Signaling

To investigate the roles of S6 kinase (S6K) in the phosphorylation of pSer-IRS-1 and p85-PI3K, S6K and IRS-1 were immunoprecipitated and then pSer-IRS-1 and p85-PI3K were immunoblotted, respectively. We have previously shown that WD feeding leads to increased serine/reduced tyrosine pIRS-1, less downstream PI-3K/Akt/eNOS activation in cardiovascular tissues.32 WD feeding induced abnormal upregulation of S6K signaling (Figure 4A and 4B) and this occurred in concert with upregulation of phosphorylated IRS-1 (Figure 4C) and downregulation of PI-3K/Phosphorylated Akt/Phosphorylated eNOS signaling (Figure 4D–4F). The absence of ECMR prevented the development of WD-induced impairments in upstream and downstream pathways associated with insulin signaling (Figure 4).

ECMR−/− Prevents WD-Induced LV Hypertrophy in Concert With Reduced Cardiac Growth Pathway Signaling

WD was associated with increases in cardiomyocyte size in conjunction with upregulation (p) of the growth promoting S6K-1 and extracellular signal–regulated kinase (ERK1/2; Table; Figures 4 and 5). Although ECMR−/− had no impact on blood pressure, body weight, or HOMA-IR, it was associated with substantial reductions in WD-induced increases in cardiomyocyte size and (p) of S6K-1 and ERK 1/2 (Figures 4 and 5).

Discussion

In this study, we observed that consumption of a WD for 16 weeks in female mice resulted in cardiac diastolic relaxation impairment. This diastolic dysfunction was associated with the presence of LV fibrosis, cardiomyocyte stiffness, and increased inflammation, oxidative stress, and fibrosis promoting signaling pathways. Concomitantly, cardiac insulin metabolic signaling was reduced and associated with diminution of eNOS phosphorylation/activation. The diastolic dysfunction, as well as the structural and biochemical changes associated with WD consumption, was absent in a specific ECMR−/− female mouse model. We had previously observed that a WD was associated with impaired diastolic relaxation at an earlier age in female mice (8 weeks) than in male mice.4 Low-dose spironolactone, a global MR inhibitor, prevents the development of myocardial and vascular stiffness and diastolic dysfunction in females consuming a WD.37,38 Here, we uniquely demonstrate...
that EC-specific deletion of the MR prevents the development of WD-associated impairment in diastolic relaxation in conjunction with reductions in profibrotic signaling pathways, inflammation, and improvement in cardiac insulin metabolic signaling female mice. In particular, WD promotion of cardiac interstitial fibrosis, oxidative stress, M1/2 macrophage polarization, and hypertrophy was also largely absent in ECMR−/− female mice. The failure to develop abnormalities in cardiac structure and function in ECMR−/− female mice occurred without abrogation of WD-induced alterations in body weight, fat mass and systemic insulin resistance, or changes in mean arterial pressure. This suggests that WD-induced structural and functional changes in cardiovascular tissues occur before overt development of hypertension as has been previously reported for conduit vascular stiffness.39 To the best of our knowledge, this is the first study examining the impact of cell-specific deletion of the MR in females and the first endeavor to investigate the impact of endothelial-specific MR deletion on

**Figure 4.** ECMR−/− prevents Western diet (WD)-impaired insulin metabolic signals resulting in downregulation of phosphorylation of Akt/eNOS. A, The expression and activation of S6K, PI3/K, Akt, and endothelial nitric oxide synthase (eNOS) were performed with immunoprecipitation (IP) and immunoblotting (IB). B–F, Representative blots of S6K, PI3/K, Akt, and eNOS in left ventricle tissues using IB and IP with corresponding quantitative analysis. Control diet wild-type (CD ECMR+/+), ECMR KO control diet (CD ECMR−/−), WD wild-type (WD ECMR+/+), and WD ECMR KO (WD ECMR−/−) mice. n=3 per group. ECMR indicates endothelial cell mineralocorticoid receptor; and KO, knockout. *P<0.05 compared with CD ECMR+/+; #P<0.05 compared with WD ECMR+/+.

**Figure 5.** Western diet (WD)-induced cardiac hypertrophy is prevented in ECMR−/− mice. A, Representative images of myocardial immunostaining for hypertrophy with quantitative analysis of the cardiomyocyte sizes. B, Phosphorylation (p) of extracellular signal-regulated kinase (ERK1/2) in left ventricular tissues using Western blot with representative analysis below or the ratio of (p) ERK1/2 to total. Control diet wild-type (CD ECMR+/+), ECMR KO control diet (CD ECMR−/−), WD wild-type (WD ECMR+/+), and WD ECMR KO (WD ECMR−/−) mice. n=3 to 5 per group. ECMR indicates endothelial cell mineralocorticoid receptor; and KO, knockout. *P<0.05 compared with CD ECMR+/+; #P<0.05 compared with WD ECMR−/−.
cardiac structure and function. Taken together, these findings support the notion that increased ECMR signaling associated with consumption of a WD plays a key role in the development of cardiac fibrosis and diastolic dysfunction.

We previously reported that young female C57BL6 mice exhibit higher serum levels of aldosterone compared with males (+50% higher in females versus males), but WD had no effect on aldosterone levels in males or females. Therefore, we speculate that the interaction of higher aldosterone levels in females and consumption of a WD high in fat, fructose, and sucrose may act synergistically to promote the observed increases in oxidative stress, inflammation, fibrosis, and diastolic dysfunction. Indeed, data from our and other laboratories have suggested that consumption of a WD by excess intake of fat and carbohydrates can activate the renin-angiotensin–aldosterone system and result in cardiovascular dysfunction and impaired cardiovascular tissue insulin metabolic signaling.

Activation of the profibrotic TGF-β1/Smad signaling pathway in association with the observed increase in myocardial interstitial fibrosis in WD-fed mice are likely the major factors contributing to diastolic dysfunction in our study, and this was largely prevented in the ECMR−/− mice. To this point, TGF-β1 is a powerful initiator for the synthesis of collagen and other major extracellular matrix components in cardiovascular tissue. TGF-β1 regulates fibroblast proliferation and extracellular matrix production, particularly of collagen and fibronectin, while reducing degradation of these components through Smad2/3. Smad7 is also a ligand of TGF-β1 but it is a TGF-β1 antagonist and interacts with activated TGF-β1, thereby blocking the association, phosphorylation, and activation of Smad2/3. Connective TGF and collagen 1 are profibrotic factors that are involved in promoting cardiac stiffness and are increased through TGF-β1/Smad signaling. The WD-induced increase in expression of these matrix/fibrotic proteins was prevented/abrogated by ECMR deletion. To our knowledge, our report is the first to link ECMR to cardiac stiffness.

The results of this study are consistent with data from a recent study showing a role of the ECMR in modulating myocardial profibrotic and proinflammatory responses to DOCA/salt. In that study, we also observed reduced macrophage polarization in ECMR null mice. To this point, monocyte chemoattractant protein-1-directed macrophage recruitment plays a direct role in MR-dependent DOCA/salt-induced cardiac fibrosis and hypertension. However, in the previous study, the precise role of ECMR−/− was confounded by the use of a Tie2/Tek promoter to drive the expression of Cre recombinase. In this regard, the Tie2 promoter has been shown to affect all myeloid cells, including macrophages. This is an important distinction as the macrophage MR has been reported to promote inflammatory macrophage polarization and associated fibrosis, hypertrophy, and remodeling. However, the beneficial cardiac effects observed with ECMR KO in this study were similar to that observed previously using the Tie2 promoter in male mice subjected to mineralocorticoid/salt treatment. Thus, the current study is the first to demonstrate that selective ECMR KO prevents fibrosis and remodeling and associated diastolic dysfunction in the translational setting of a WD.

The results of this investigation further suggest that consumption of a WD plays a role in the generation of oxygen-free radicals and ECMR−/− prevents oxidative stress (myocardial 3-nitrotyrosine levels) and related fibrosis. This observation highlights the potential role of ECMR in promotion of reactive oxygen species–mediated fibrogenesis. Furthermore, oxygen-free radicals likely induced and increased proinflammatory cytokines, including monocyte chemoattractant protein-1, IL1, and IL17 in female mice fed a WD. These data are consistent with a model in which EC injury because of active MR signaling promotes immune cell activation, including T lymphocytes migration to secondary lymphoid organs, as well as recruitment and activation of macrophages into the cardiac tissues. Indeed, MR activation in immune cells has been reported to promote differentiation of naïve CD4 T cells into Th1 and Th17 proinflammatory phenotypes and downregulation of regulatory T lymphocytes, which are involved in the pathogenesis of cardiac fibrosis and diastolic dysfunction. Moreover, consumption of a WD promoted a proinflammatory macrophage M1/M2 polarization that was prevented in ECMR−/−. This finding is consistent with previous results showing that the loss of MR in macrophage-MR KO mice resulted in reduction in M1 phenotype and mRNA levels for markers of cardiovascular inflammation. Macrophage polarization favoring an enhanced M1 proinflammatory response and suppressing an M2 anti-inflammatory response occurs in diet-induced cardiac disease. The proinflammatory M1 macrophages secrete inflammatory cytokines to cause insulin resistance and cardiac dysfunction. In contrast, M2 macrophages secrete IL10, which attenuates cardiomyocyte hypertrophy and cardiac fibrosis. Thus, activation of ECMR resulting in an increased adaptive inflammation response and macrophage M1/M2 polarization could contribute to cardiac fibrosis, stiffness, and diastolic dysfunction that is characteristic of longstanding heart failure in females with obesity, the metabolic syndrome, and type 2 diabetes mellitus.

The results of this investigation further suggest that a WD promotes cardiac hypertrophy, driven by ECMR-mediated reductions in insulin metabolic signaling and associated activation of growth promoting S6K-1 and ERK signaling. Indeed, both S6K-1 and ERK have been implicated as mediators of cardiac hypertrophy in both cell culture and in genetically modified mouse models. These kinases become activated in cardiac cardiomyocytes in response to many stimuli, such as inflammation, oxidative stress, reductions in bioavailable NO, and aldosterone. The role of ECMR signaling in modulating these growth promoting kinases is underscored by our findings that ECMR−/− prevented WD-induced S6K-1 and ERK activation and cardiac hypertrophy. Reductions in eNOS activity and increased reactive oxygen species destruction of NO leading to reduced bioavailable NO may also have contributed to cardiomyocyte hypertrophy in WD-fed female mice.

Perspectives

The results of this investigation suggest that signaling through ECMR plays a critical role in development of WD-induced cardiac diastolic dysfunction in females. Indeed, ECMR deletion prevented the cardiac inflammation, oxidative stress,
maladaptive immune responses, and fibrosis associated with consumption of a high fat/sucrose/high fructose corn syrup diet. One limitation of this study is that we did not compare these pathophysiological changes in male and female ECMR KO mice, although the cardiac impact of ECMR KO is similar to that seen previously in males subjected to a different treatment paradigm. Thus, future more long-term studies need to done in male ECMR KO mice subjected to WD feeding. Nevertheless, this investigation has uncovered potentially important mechanism by which overweight and insulin-resistant females are at especially high risk of developing diastolic heart failure. Finally, targeting the ECMR may be an effective strategy to limit heart failure development and progression in overweight/obesity females that are at substantial risk of cardiovascular events.

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Disclosures
None.

References
Novelty and Significance

What Is New?

- These data indicate that consumption of a Western diet, high in fat and refined sugars promotes fibrosis, stiffness, and impaired cardiac diastolic relaxation in female mice. Development of this cardiac phenotype can be prevented by selective deletion of the endothelial cell mineralocorticoid receptor.

What Is Relevant?

- Metabolic cardiomyopathy characterized by selective impairment of diastolic relaxation often occurs in young overweight and insulin-resistant females. Using a very translational model of insulin resistance, female mice fed a Western diet, this research show that diet-induced activation of the endothelial cell mineralocorticoid receptor is critical for development of impaired diastolic relaxation in females.

Summary

The results of this investigation suggest that enhanced endothelial cell mineralocorticoid receptor activation plays an integral role in development of cardiac fibrosis and diastolic dysfunction that is promoted by consumption of a Western diet in females.

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Endothelial mineralocorticoid receptor deletion prevents diet induced cardiac diastolic dysfunction in females

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Materials and methods:

Structural, biochemical parameters, and blood pressure measurements

Mice were placed into a thin-walled plastic container while awake and under no anesthesia or distress. All measurements were performed during the same time of day. Mice were weighed and euthanized via exsanguination under isoflurane anesthesia (above). Heart weights and visceral fat weights were obtained after harvesting along with tibial lengths measured to normalize weights and eliminate confounding effects of differences in size. After 16 weeks of feeding, mice underwent body composition analysis for whole body fat mass, lean mass and total body water utilizing an EchoMRI-500 for quantitative magnetic resonance analysis (Echo Medical Systems, Houston, TX, USA) by a method established in our laboratory. Venous blood samples were collected from a subset of fasting mice in each treatment group and plasma was stored at −80°C for glucose and insulin assay and homeostatic model assessment of insulin resistance (HOMA-IR) as previously described.1 Average systolic, diastolic and mean arterial pressures (MAP) were determined by catheterization of the right carotid artery under isoflurane anesthesia as previously described.1 At the end of the 16 weeks feeding trial and immediately prior to being euthanized for tissue collections, mice were anesthetized with isoflurane (1.75% isoflurane in 100% O2). The right carotid artery was isolated and a high fidelity 1.2 French mouse pressure catheter (Transonic) was inserted and advanced to a position proximate to the aortic arch. After a brief acclimation period and when blood pressures were stable, average systolic (SBP), diastolic (DBP) and mean arterial pressures (MAP) were determined utilizing the Avantage Data Acquisition System (Scisense, Ontario Canada).

In vivo high-resolution cine-MRI and echocardiography

Non-invasive cine-MRI scans were performed on mice using a horizontal-bore 7 Tesla Bruker AVANCE III BioSpec MRI system (Bruker Corp, Billerica, MA) equipped with a 35 mm quadrupole detection radiofrequency coil. Left ventricular (LV) morphology and functions were measured and analyzed as established previously.2 Animals were weighed and anesthetized using 1.8–2.7% isoflurane on a nose cone nonrebreathing system supplying continuous oxygen. ECG and respiratory monitoring and gating were performed with a small animal monitoring system (SA Instruments, Stony Brook, NY). Warm air was circulated through the MRI bore to maintain body temperature. ECG/respiratory gated gradient echo sequences were acquired with 1-mm slice thickness and 65 × 45- and 45 × 45-mm² field of views for the LV in long- and short-axis images, respectively. LV functional parameters were determined using a series of cine images of the LV in long-axis view acquired at 20 equally spaced time points throughout the entire cardiac cycle with a frame rate of 8–12 ms/frame. At each time point, the endocardial borders were traced to measure the LV chamber area using VnmrJ software (Agilent) by two experienced MRI readers. LV volumes (LVVs) at each phase were calculated with the following modified ellipsoid equation: $LVV = \frac{8A^2}{3\pi L}$, where $A$ is the endocardial area and $L$ is the length of the LV long-axis chamber. The LVV
The curve was plotted as LVV versus time throughout a cardiac cycle. For the LV diastolic function measurements, the first derivatives of LVV against time were calculated to extract the diastolic filling rates and relaxation time. Diastolic IFR was defined as the slope of the first four time points on the early diastolic curve. Diastolic peak filling rate (PFR) was defined as the maximum derivative of the LVV curve. Diastolic relaxation time (DRT) was defined as the time duration from the end of systolic phase to the peak filling phase. Normalized DRT, which is the ratio of DRT to the R-R interval, was used to compare LV diastolic relaxation among groups, where normalized DRT = [DRT × (HR /6,000)].

Two-dimensional echocardiograms were performed in the apical 4-chamber view. The myocardial performance index (MPI), also known as the Tei index, was calculated as the sum of isovolumic contraction and relaxation times divided by ejection time. The time from cessation of the mitral valve A wave to the onset of the mitral valve E wave of the next cardiac cycle (a) is equal to the total isovolumic time plus the ejection time (b). The MPI was calculated therefore by the formula (a − b)/b. This was obtained from the apical 4-chamber view, with pulsed-wave Doppler by placing a small sample volume at the level of the LV outflow tract. Early transmirtal peak diastolic flow velocity (E wave) was obtained from the Doppler pulse-wave with the sample volume placed in the mitral inflow adjacent to the mitral leaflets. All Doppler spectra were recorded for 5–8 cardiac cycles at a sweep speed of 200 mm/sec. Parameters were assessed using an average of 3 beats from 3 different spectra, and calculations were made in accordance with the American Society of Echocardiography guidelines as well as specific guidelines for rodent echocardiography. All data were acquired and analyzed offline by a single blinded observer.

**Cardiomyocyte isolation and atomic force microscopy (AFM) nanoindentation**

Hearts were excised and cannulated via the aorta for retrograde coronary perfusion of the myocardium. Calcium-free Physiological Saline Solution (containing 135 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 10 mM D-glucose, 10 mM Heps, and 2 U/ml heparin, pH 7.4 with NaOH) was perfused through the heart for 10 minutes, and was followed by perfusion of a MEM-based enzymatic isolation solution supplemented with 7 mM NaHCO₃, 2 mM Na-Pyruvate, 7 mM NaHEPES, 7 mM HEPES, 5 mM Taurine, 10 mM Creatine, 5 mM 2,3-Butanedione monoxime in addition to 20 µM CaCl₂, 4,000 U/L insulin, 50,000 U/L Penicillin-Streptomycin, and 45 µg ml⁻¹ Liberase Blendzyme TH (Roche Applied Science, Indianapolis, IN, USA) for ~10 minutes. All perfusion solutions were oxygenated with 95% O₂ / 5% CO₂ and maintained at 37 °C. After coronary perfusion the left ventricle and septum were then isolated, minced, agitated, and filtered, all at 22-25 °C. Atomic force microscopy nanoindentation was used to estimate the elastic modulus (stiffness) of single myocytes. A cardiomyocyte suspension was applied to laminin-coated dishes and cardiomyocytes were allowed to adhere for 40 minutes. Cardiomyocytes were washed 3 times in nominally calcium-free Physiological Saline Solution at 22-25 °C. Cardiomyocyte elasticity was monitored in contact mode using silicon nitride microlevers (model MLCT with spring constants 10-30 pN/nm; Veeco Mertrology Inc, Santa Barbara, CA) and a Bioscope AFM System (Veeco
Metryrology) mounted on an Olympus IX81 Microscope (Olympus Inc, NY). Probes repeatedly approached and retracted the cardiomyocyte surface at 0.5 Hz (tip speed of 800 nm/s) with an indentation depth of approximately 150-250 nm. Cell elasticity (Young’s modulus = $E$) was calculated from approach curves according to the equation: $F = \tan (\alpha) \times \left[ (2 \times E \times \delta^2) / \pi (1 - \nu^2) \right]$, where $\alpha$ represents probe shape (conical with an approximate half-angle of 21.25 degrees), $\delta$ is indentation depth calculated as the difference in the AFM piezo $z$-movement and the measured deflection of the probe, and $\nu$ is the Poisson ratio (0.5 for the cell). Indentation force ($F$) was calculated using Hooke’s law ($F = k \Delta x$), where $k$ is the AFM probe spring constant and $\Delta x$ is the measured deflection of the probe.

**Quantification of myocardial interstitial fibrosis and cardiomyocyte hypertrophy**

Immunohistochemistry was performed according to previously published protocols in our group using antibodies collagen I (Col 1) (Abcam, Cambridge, MA), connective tissue growth factor (CTGF) (Abcam, Cambridge, MA), and fibronectin (Epitomies, Cambridge, MA). Five µm sections of LV samples from mice of treatment groups were stained with picro-sirus-red for evaluation of interstitial fibrosis. Slides were viewed with a Nikon50i microscope and five images were randomly captured from each LV with a cool snap cf camera and auto leveled with Photoshop. Morphometric analysis was performed using MetaVue software. In each image, the areas of hot pink color and their intensities, which are representative of interstitial fibrosis, were quantified. Col 1 and CTGF were evaluated by immunostaining specific antibodies. For cardiomyocyte hypertrophy quantification, 5 µm paraffin embedded heart sections were dewaxed, rehydrated with ethanol series and HEPES wash buffer and stained with wheat Germ Agglutinin (WGA). Slides were blindly analyzed by one or two observers with a Nikon50i (Nikon, Tokyo, Japan) microscope. To keep uniformity and avoid error, each section was thoroughly checked. Five representative areas were captured with ×40 images from each section with a CoolSNAP cf camera (Roper Scientific Germany, Trenton, NJ). The areas and intensities of pink regions, which are indicative of interstitial fibrosis, were quantified on both transverse and longitudinal sections of the left ventricle using MetaVue software (Molecular Devices, Sunnyvale, CA). The average grayscale intensity due to collagen was recorded. An average value of these intensities was determined for each animal. Average cardiomyocyte cross-sectional dimensions from different treatments were measured and compared.

**3-Nitrotyrosine (3-NT)**

LV content of 3-NT was quantified as previously described. Sections were washed and incubated with secondary antibodies (biotinylated linked and streptavidin-HRP conjugated) for 30 min each. After several rinses with Tris-buffered saline-Tween 20, diaminobenzidine was applied for 8 min, and sections were then rinsed several times with distilled water, stained with hematoxylin for 80 s, dehydrated, and mounted with a permanent media. Slides were inspected under a bright-field (50i, Nikon) microscope, and ×40 images from each section were captured with a CoolSNAP cf camera. Signal
intensities of brownish color, which is indicative of the 3-NT level, were quantified by MetaVue software.

**Immunoprecipitation and western-blots**

Protein concentrations of cardiac tissue homogenates were measured as previously described.\(^7\) 200 μl tissue lysates were added with antibody S6K or IRS-1 (Cell Signaling Technology, Danvers, MA). Antibody-protein complexes were incubated with gentle rocking overnight at 4°C and collected with protein G-coupled magnetic beads. Isolated protein complexes or sample proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. Blots were incubated overnight at 4°C with primary antibodies against T\(^{389}\) phosphorylated (p)-S6K/S6K (Cell Signaling Technology, Danvers, MA), pSer-IRS-1 (Cell Signaling Technology, Danvers, MA), p85-PI3-K (Cell Signaling Technology, Danvers, MA), pSer-Akt/Akt (Cell Signaling Technology, Danvers, MA), pSer-eNOS/eNOS (BD Biosciences, San Jose, CA), p-Erk/Erk (Cell Signaling Technology, Danvers, MA), TGF-β1 (Abcam, Cambridge, MA), p-Smad2/3/Smad2/3 (Santa Cruz Biotec, Dallas, Texas), Smad7 (Santa Cruz Biotec, Dallas, Texas), and Pan-actin (Cell Signaling Technology, Danvers, MA). After a rinse, blots were incubated with secondary antibodies (1:5,000 dilution of each antibody) for 1 hour at room temperature. Bands were visualized by chemiluminescence, and images were recorded using a Bio-Rad ChemiDoc XRS image-analysis system. Quantitation of (p) protein band density, normalized to the density of total protein for each sample, was performed using Image Lab (Bio-Rad).

**RNA isolation and quantitative PCR**

Total RNA was isolated with the TRIzol reagent (Sigma) method as previously described.\(^7\) Real-time PCR was done using 8 μl cDNA, 10 μl SYBR green PCR master mix (Bio-Rad Laboratories) and forward and reverse primers (10 pM/μl) (Integrated DNA Technologies, San Diego, CA) using a real-time PCR system (CFX96; Bio-Rad Laboratories). The yield of RNA was quantified using a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE). First-strand cDNA synthesis was done using 1 μg total RNA with oligo dT (1 μg), 5× reaction buffer, MgCl\(_2\), dNTP mix, RNAse inhibitor, and Improm II reverse transcriptase as per Improm II reverse transcription kit (Promega, Madison, WI). After the first strand synthesis, real-time PCR was done using 8 μl cDNA, 10 μl SYBR green PCR master mix (Bio-Rad Laboratories) and forward and reverse primers (10 pM/μl) (Integrated DNA Technologies, San Diego, CA) using a real-time PCR system (CFX96; Bio-Rad Laboratories). The primer sequences used were: MCP-1, Forward: 5’-GATGCAGTTAATGCCCCACT-3’; Reverse: 5’-TTCCCTATGCGGCTCAGC-3’; IL1, Forward: 5’-CTCCATGAGCTTGTACAGAAGG-3’; Reverse: 5’-TGCTGATGATCCAGTTGCGG-3’; IL17, Forward: 5’-CTTTCCTTTTGACTCTGCAGAC-3’; Reverse: 5’-GGGCGGACATAGAGGAAAC-3’; CD86, Forward: 5’-GACCGTTGTGTTGTCAGC-3’; Reverse: 5’-GATGAGCACTCACAAGGA-3’; CD11b, Forward: 5’-TTCCTTTGAGCTCAGC-3’; Reverse: 5’-AAGGACGTGACAGGCAGAAGG-3’.
CCAAGACGATCTCAGCATCA-3’, Reverse: 5’- TTCTGGCTTGCTGAATCTTT-3’; IL-10, Forward: 5’- CCAAGCTTTATCGAAAATGA-3’, Reverse: 5’- TTTCACAGGGGAGAAATCG-3’; CD206, Forward: 5’- CAAGGAAAGGTTGGCATTGT-3’, Reverse: 5’- CTTTTTCAGTCTTTGCAAGC-3’; GAPDH, Forward: 5’-GGGAAACCTGCCAAGTATGA-3’, Reverse: 5’- TCCTCAGTGTAGCCCAAGA-3’. The specificity of the primers was analyzed by running a melting curve. The PCR cycling conditions used were 5 min at 95°C for initial denaturation, 40 cycles of 30 s at 95°C, 30 s at 58°C and 30 s at 72°C. Each real-time PCR was carried out using three individual samples in triplicates, and the threshold cycle values were averaged. Calculations of relative normalized gene expression were done using the Bio-Rad CFX manager software based on the ΔCt method. The results were normalized against housekeeping gene GAPDH.

**Statistical analysis**

Histologic data were collected by genotype- and treatment-blinded investigators. Data are reported as means ± SEM. Differences in outcomes were determined using one- or two-way ANOVA and paired t tests and were considered significant when \( P < 0.05 \). All statistical analyses were performed using Sigma Plot (version 12) software (Systat Software).


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**Fig.S1:** Western diet induced collagen I, CTGF, and fibronectin is prevented in ECMR KO mice. Representative images immunostaining for Collagen-I, connective tissue
growth factor (CTGF), and fibronectin with corresponding measures of average gray scale intensities below. Scale bar = 50 μm.