Heart Failure With Preserved Ejection Fraction Induces Beiging in Adipose Tissue

María Valero-Muñoz, PhD; Shanpeng Li, BS; Richard M. Wilson, BS; Maarten Hulsmans, PhD; Tamar Aprahamian, PhD; José J. Fuster, PhD; Matthias Nahrendorf, MD, PhD; Philipp E. Scherer, PhD; Flora Sam, MD

Background—Despite the increasing prevalence of heart failure with preserved ejection fraction (HFpEF) in humans, there are no evidence-based therapies for HFpEF. Clinical studies suggest a relationship between obesity-associated dysfunctional adipose tissue (AT) and HFpEF. However, an apparent obesity paradox exists in some HF populations with a higher body mass index. We sought to determine whether HFpEF exerted effects on AT and investigated the involved mechanisms.

Methods and Results—Mice underwent d-aldosterone infusion, uninephrectomy, and were given 1% saline for 4 weeks. HFpEF mice developed hypertension, left ventricular hypertrophy, and diastolic dysfunction and had higher myocardial natriuretic peptide expression. Although body weights were similar in HFpEF and sham-operated mice, white AT was significantly smaller in HFpEF than in sham (epididymal AT, 7.59 versus 10.67 mg/g; inguinal AT, 6.34 versus 8.38 mg/g). These changes were associated with smaller adipocyte size and increased beiging markers (ucp-1, cidea, and eva) in white AT. Similar findings were seen in HFpEF induced by transverse aortic constriction. Increased activation of natriuretic peptide signaling was seen in white AT of HFpEF mice. The ratio of the signaling receptor, natriuretic peptide receptor type A, to the clearance receptor, nprc, was increased as was p38 mitogen-activated protein kinase activation. However, HFpEF mice failed to regulate body temperature during cold temperature exposure. In HFpEF, despite a larger brown AT mass (5.96 versus 4.50 mg/g), brown AT showed reduced activity with decreased uncoupling protein 1 (ucp-1), cell death-inducing DFFA-like effector a (cidea), and epithelial V-like antigen (eva) expression and decreased expression of lipolytic enzymes (hormone-sensitive lipase, lipoprotein lipase, and fatty acid binding protein 4) versus sham.

Conclusions—These findings show that HFpEF is associated with beiging in white AT and with dysfunctional brown AT.

© 2015 American Heart Association, Inc.

Key Words: brown adipose tissue ■ diastolic heart failure ■ natriuretic peptides ■ white adipose tissue

Heart failure (HF) with preserved ejection fraction (HFpEF), also known as diastolic HF, is a clinical syndrome characterized by lung congestion, increased left ventricular (LV) filling pressure and diastolic dysfunction (DD), with preservation of LV ejection fraction (LVEF). In contrast to HF with reduced EF, there are no evidence-based therapies for HFpEF. Randomized clinical trials, to date, in patients with HFpEF, failed to show any improvement in morbidity or mortality. Management is limited to the treatment of symptoms and comorbidities. Uncertainties surround the mechanism and pathophysiology of HFpEF; with HFpEF patients having more comorbidities than those with HF with reduced EF, eg, arterial hypertension, metabolic abnormalities such as diabetes mellitus, hyperlipidemia, and obesity.

Obesity is significantly associated with the future development of HF despite adjusting for established risk factors. In addition to other known risk factors, such as hypertension and coronary artery disease, obesity is highly prevalent in population-based studies and registries of HFpEF. Similarly, dysfunction of the vasculature, kidney, skeletal muscle, and adipose tissue (AT) are important contributors to the HFpEF phenotype. The role of obesity and adipose-derived adipokines has provided some insights into the onset and evolution of HFpEF. Recently, we showed that factors released by AT, such as adiponectin, may regulate the pathological heart or interact with neurohormones in HFpEF. Similarly, cardiac cells release regulatory peptides, called cardiokines, in response to changes in the cardiac environment. These cardiokines have physiological and pathological roles in organs distal from the heart, such as muscle, fat, and liver, where...
cell death, growth, fibrosis, and remodeling are regulated.18,19 However, much less is known about their role in mediating metabolic cross talk between the heart and the peripheral tissues.19,20 In addition to natriuresis, diuresis, and vasodilation, natriuretic peptides released from the heart enhance lipolysis and lipid mobilization.21–23 Moreover, it has also been described that these natriuretic peptides induce changes in white AT (WAT) depots promoting the development of brown AT (BAT) characteristics (such as increased thermogenesis and increase energy expenditure), a phenomenon termed browning or beiging.23 These findings provide further evidence of communication between the heart and the peripheral tissues. However, it is unknown whether cardiokines and the heart may regulate the AT phenotype and biology in nonobese HFpEF. Thus, we sought to investigate whether AT function is regulated in HFpEF in nonobese mice because this may provide mechanistic insight into the relationship between HFpEF and obesity and similarly set the stage for the development of novel treatments for HFpEF.

Methods
A detailed Methods is available in the Data Supplement.

Experimental Model
The Institutional Animal Care and Use Committee at Boston University School of Medicine approved all study procedures related to the handling and surgery of the mice. There were 2 groups of mice experiments.

Eight-week C57BL/6J old mice were anesthetized with 80 to 100 mg/kg ketamine and 5 to 10 mg/kg xylazine intraperitoneally. Mice (20–25 g) underwent uninephrectomy and then received either a continuous infusion of saline (sham) or d-aldosterone (0.30 μg/h; Sigma-Aldrich, St. Louis, MO; HFpEF) for 4 weeks via osmotic minipumps (Alzet; Durect, Cupertino, CA). The animals were euthanized after 4 weeks at which time there was a HFpEF phenotype.16,24 Another group of C57BL/6J mice underwent transverse aortic constriction (TAC). Briefly, mice were anesthetized with sodium pentobarbital (50 mg/kg intraperitoneally). The chest was opened, and after blunt dissection through the intercostal muscles, the thoracic aorta was identified. Silk suture (7-0) was placed around the transverse aorta and tied around a 26-gauge blunt needle, which was subsequently removed. Sham-operated mice underwent a similar surgical procedure without aortic constriction. After 14 days, cardiac structure and function were determined by echocardiography. Mice were then euthanized, the hearts were weighed and white adipose depots were collected.

Physiological Measurement
Heart rate and blood pressure (BP) were measured weekly using a noninvasive tail-cuff BP analyzer, BP-2000 Blood Pressure Analysis System (Visitech Systems, Inc., Apex, NC).16,24 Transthoracic echocardiography was performed at the end of 4 weeks in chronic aldosterone and after 14 days in TAC using the Vevo 770 High-Resolution In Vivo Micro-Imaging System and a Real-Time Micro Visualization 707B Scanhead (VisualSonics Inc., Toronto, Ontario, Canada), as previously described (further details are available in the Data Supplement).16 The ratio of wet:dedry lung weight ratio was used as an indicator of pulmonary congestion.

Metabolic Measurements and Assessment of Body Composition
Briefly, body composition, including fat mass and lean tissue mass, was measured by noninvasive quantitative magnetic resonance imaging (EchoMRI-700; Echo Medical System, Houston, TX). During the last week of the experiment, mice were subjected to a cold tolerance test (CTT). Glucose tolerance tests and nonfasting blood glucose levels were measured using tail vein blood samples using an Accu-Chek glucometer (Roche Diagnostics Corp, Indianapolis, IN). Details of CTT and glucose tolerance test are available in the Methods in the Data Supplement.

Cell Preparation and Flow Cytometry
Stromal vascular fraction from epididymal WAT was isolated according to Orr’s protocol.25 Cell staining was performed as previously described (Methods in the Data Supplement).26

Gene Expression Analysis by Quantitative Reverse Transcriptase Polymerase Chain Reaction
Details are available in the Methods and Table I in the Data Supplement.

Western Blot Analysis
Protein extraction and Western blot were performed as previously described (Details are available in the Data Supplement).26

Statistical Analysis
Data are shown as mean±SEM unless otherwise stated. Statistical significance of differences between 2 groups was assessed using the Student t tests (2 sided). In those cases when data were not sampled as a normal distribution, nonparametric Mann–Whitney U test was used. Results of glucose tolerance test and CTT experiments were evaluated by 2-way repeated measures ANOVA. P<0.05 values were considered significant. All statistical tests were performed using GraphPad Prism software (GraphPad Software Inc., La Jolla, CA).

Results
Murine Model of HFpEF
Characteristics and echocardiography parameters are presented in Tables 1 and 2. Weekly BP and heart rate are shown in Figure 1A in the Data Supplement. HFpEF mice demonstrated significantly increased systolic and diastolic BP by the end of 4 weeks of aldosterone infusion. Heart rate was comparable between sham and HFpEF (Table 1). LV structure and systolic and diastolic functions are shown in Table 2. LV EF was preserved, and there was evidence of lung congestion in HFpEF mice (Tables 1 and 2). HFpEF mice demonstrated increased LV mass and total wall thickness compared with sham (P<0.001). Similarly, the heartbeat:body weight ratio was also increased in HFpEF (P<0.001 versus sham). Although there was a trend to smaller LV cavity size in HFpEF mice, it was not significantly different to sham. Relative wall thickness was increased in HFpEF mice (P<0.01 versus sham). At similar heart rate, HFpEF mice showed severe DD (Table 2). Both mitral E and A velocities were elevated in HFpEF mice (P<0.01 versus sham; Figure II in the Data Supplement). The increased A velocities indicate that LV filling was increasingly dependent on an increase in atrial pressure to drive blood into the LV, during diastole. As early diastolic transmural pressure gradient increased, early diastolic flow velocities also rose, and the E wave increased, causing the E/A ratio to normalize. Similar to humans, where isovolumic relaxation time initially increases with impaired relaxation but then decreases with progressive worsening of diastolic function, isovolumic relaxation time was significantly shorter (P<0.05 versus Sham),
Diastolic blood pressure, mm Hg
failure preserved ejection fraction; and iWAT, inguinal white adipose tissue.
brown adipose tissue; eWAT, epididymal white adipose tissue; HFpEF, heart
Liver weight/body weight, mg/g 44.81±1.68 47.45±1.40
Gastrocnemius weight/body weight, mg/g 5.66±0.16 4.90±0.22‡
BAT weight/body weight, mg/g 4.50±0.34 5.96±0.42‡
Gastrocnemius weight/body weight, mg/g 5.66±0.16 4.90±0.22‡
Liver weight/body weight, mg/g 44.81±1.68 47.45±1.40
Nonfasting glucose levels, mg/dL 203.0±11.2 211.0±13.7

Data are expressed as means±SEM (n=6–12 mice per group). BAT indicates brown adipose tissue; eWAT, epididymal white adipose tissue; HFpEF, heart failure preserved ejection fraction; and iWAT, inguinal white adipose tissue.
*P<0.01 vs sham, †P<0.01 vs sham, and ‡P<0.05 vs sham.

and deceleration time was significantly decreased in HFpEF mice (P<0.001 versus sham). In addition to DD, there was evidence of HF with the preservation of LVEF after 4 weeks of d-aldosterone infusion.

**Body Composition, Characteristics, and Glucose Levels**

Body weights were comparable between both groups during the 4 weeks of HFpEF induction (Table 1; Figure IB in the Data Supplement). Magnetic resonance imaging analysis, performed to assess total lean and fat mass, showed no difference between HFpEF and sham mice (Figure 1A). Measurements of WAT weights, however, showed that both epididymal (eWAT) and inguinal (iWAT) adipose depots were significantly smaller in HFpEF mice versus sham (eWAT, 7.59 versus 10.67 mg/g; iWAT, 6.34 versus 8.38 mg/g; Table 1; Figure 1B, top and middle). Importantly, there was a relative increase in BAT weight in HFpEF mice versus sham (5.96 versus 4.50 mg/g; P<0.05; Table 1; Figure 1B, bottom).

Although there was no evidence of ascites in HFpEF mice, spleen weight was significantly increased in HFpEF versus sham (P<0.001). Similarly, there was a trend toward increased liver mass (P=NS versus sham). Skeletal muscle weight measurement, specifically gastrocnemius muscle, was smaller in HFpEF mice (P<0.05 versus sham). Glucose levels were comparable in both experimental groups under similar nonfasting and fasting conditions (Table 1; Figure IC in the Data Supplement). Similarly, glucose metabolism, as determined by the glucose tolerance test, was similar in HFpEF and sham mice (Figure IC in the Data Supplement).

**AT Morphology**

To examine whether weight changes in adipose depots were because of morphological alterations, eWAT, iWAT, and BAT histological analysis were performed (Figure IB). HFpEF mice demonstrated significant alterations in WAT morphology. There was a shift to smaller adipocyte size in HFpEF mice in both eWAT (867.91 vs 986.71 μm²; P<0.01) and iWAT (858.43 vs 1006.22 μm²; P<0.05) compared to sham. The smaller adipocytes had multilocular lipid droplets, a typical feature of WAT undergoing beiging or brown-like AT (Figure IC).

**Phenotype Switch of AT in HFpEF**

We sought to investigate molecular changes that may accompany the morphological changes observed in WAT. There was a marked phenotype switch in eWAT and iWAT in HFpEF mice, with a significant increase in beiging transcripts such as uncoupling protein 1 (ucp-1), cell death-inducing DFFA-like effector a (cidea), and epithelial V-like antigen (eva) by 5-, 2-, and 3-fold, respectively, in eWAT and 9-, 2-, and 3-fold in iWAT (Figure 2A and 2B). Conversely, there was a significant decrease in mRNAs of typical white adipose markers, such as leptin, resistin, and even adiponectin in the case of eWAT in HFpEF mice (Figure 2A and 2B).

We next evaluated the molecular phenotype of BAT. Despite the significant increase in BAT weight in HFpEF (Table 1), there was a marked decrease in expression levels of ucp-1, cidea, and eva (P<0.05 versus sham; Figure 2C). Furthermore, transcript levels of lipolytic proteins hormone-sensitive lipase, lipoprotein lipase, and fatty acid binding protein 4 were all significantly decreased in BAT of HFpEF mice (P<0.05 versus sham; Figure 2C). Importantly, these changes were also associated with an impaired thermogenic response in HFpEF mice during the CTT (Figure 2D). Body temperature was initially the same between groups (37.3°C), but core body temperature dramatically decreased in HFpEF mice after 4 hours of cold (27.8±1.8 versus 34.1±0.8°C; P<0.05 versus sham). Similarly, after only 4 hours of CTT, HFpEF mice had a significant decrease in body weight (~2.1±0.2 versus ~1.2±0.2 g; P<0.05 versus sham). These data suggest that BAT function may be decreased or impaired because normal BAT function is expected to improve thermoregulation.

<p>| Table 1. Characteristics of Sham and HFpEF Mice After 4 Weeks of Saline or d-Aldosterone Infusion |
|-------------------------------------------------|-------------------------------------------------|</p>
<table>
<thead>
<tr>
<th>Sham (n=6)</th>
<th>HFpEF (n=12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Systolic blood pressure, mm Hg</td>
<td>129±3</td>
</tr>
<tr>
<td>Diastolic blood pressure, mm Hg</td>
<td>89±6</td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>666±29</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>26.6±1.0</td>
</tr>
<tr>
<td>Heart weight/body weight, mg/g</td>
<td>3.45±0.07</td>
</tr>
<tr>
<td>Wet lung/dry lung weight ratio</td>
<td>4.01±0.10</td>
</tr>
<tr>
<td>Spleen weight/body weight, mg/g</td>
<td>3.29±0.09</td>
</tr>
<tr>
<td>eWAT weight/body weight, mg/g</td>
<td>10.67±0.95</td>
</tr>
<tr>
<td>BAT weight/body weight, mg/g</td>
<td>4.50±0.34</td>
</tr>
<tr>
<td>Gastrocnemius weight/body weight, mg/g</td>
<td>5.66±0.16</td>
</tr>
<tr>
<td>Liver weight/body weight, mg/g</td>
<td>44.81±1.68</td>
</tr>
<tr>
<td>Nonfasting glucose levels, mg/dL</td>
<td>203.0±11.2</td>
</tr>
</tbody>
</table>

Data are expressed as means±SEM (n=6–12 mice per group). BAT indicates brown adipose tissue; eWAT, epididymal white adipose tissue; HFpEF, heart failure preserved ejection fraction; and iWAT, inguinal white adipose tissue.
*P<0.01 vs sham, †P<0.01 vs sham, and ‡P<0.05 vs sham.

<p>| Table 2. Echocardiographic Parameters of Sham and HFpEF Mice After 4 Weeks of Saline or d-Aldosterone Infusion |
|-------------------------------------------------|-------------------------------------------------|</p>
<table>
<thead>
<tr>
<th>Sham (n=6)</th>
<th>HFpEF (n=12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LV structure and systolic function</td>
<td></td>
</tr>
<tr>
<td>LV mass, mg</td>
<td>89.43±3.35</td>
</tr>
<tr>
<td>Total wall thickness, mm</td>
<td>0.83±0.03</td>
</tr>
<tr>
<td>LV end-systolic diameter, mm</td>
<td>1.66±0.16</td>
</tr>
<tr>
<td>LV end-diastolic diameter, mm</td>
<td>3.34±0.13</td>
</tr>
<tr>
<td>LV ejection fraction, %</td>
<td>82.18±3.05</td>
</tr>
<tr>
<td>Relative wall thickness</td>
<td>0.50±0.04</td>
</tr>
<tr>
<td>Diastolic function</td>
<td></td>
</tr>
<tr>
<td>Mitral E velocity, mm/s</td>
<td>664±45</td>
</tr>
<tr>
<td>Mitral A velocity, mm/s</td>
<td>498±70</td>
</tr>
<tr>
<td>E/A</td>
<td>1.28±0.09</td>
</tr>
<tr>
<td>Deceleration time, ms</td>
<td>22.67±1.65</td>
</tr>
<tr>
<td>Isovolumic relaxation time, ms</td>
<td>20.36±1.10</td>
</tr>
</tbody>
</table>

Data are expressed as mean±SEM (n=6–12 mice per group). HFpEF indicates heart failure with preserved ejection fraction; and LV, left ventricle.
*P<0.001 vs sham, †P<0.01 vs sham, and ‡P<0.05 vs sham.
Valero-Muñoz et al
Adipose Tissue Regulation in HFpEF

Inflammatory, Fibrotic, and Oxidative Status in WAT of HFpEF

We further characterized WAT in HFpEF mice by assessing the inflammatory, fibrotic, and oxidative status in WAT. Recent studies showed changes in the inflammatory response in WAT during beiging and adaptive thermogenesis.27,28 Fluorescence-activated cell sorter analysis of stromal vascular fraction from eWAT revealed significantly increased neutrophil content in HFpEF mice versus sham (6.25×10²±1.2×10² versus 1.43×10²±0.36×10²; *P*<0.05; Figure 3A and 3B). Ly6C<sup>high</sup> monocytes (proinflammatory function), Ly6C<sup>low</sup> macrophages (patrolling behavior), and Ki67<sup>+</sup> Ly6C<sup>low</sup> macrophages (macrophage proliferation) were comparable between HFpEF and sham mice. We also analyzed the inflammatory state in iWAT by quantitative reverse transcriptase polymerase chain reaction. Similar to the results obtained in eWAT, there was greater ly6g gene expression (indicating an increase in neutrophil infiltration) in this adipose depot in HFpEF mice (*P*<0.05 versus sham; Figure 3C). There were no differences in f4/80, tnfα, and mcp1 transcript expression in iWAT between HFpEF and sham mice (Figure 3C). There were also no differences in fibrotic (collagen 1, 3, and 6) and oxidative markers (NAD(P)H subunits p22phox and p47 phox) in eWAT or iWAT in HFpEF versus sham mice (Figure III in the Data Supplement). Similar results were found in BAT (Figure 3D; Figure III in the Data Supplement).

Mechanisms Implicated in AT Phenotype Changes

To further investigate beiging activation in WAT during HFpEF, we measured p38 mitogen-activated protein kinase (MAPK) activation in eWAT because it was previously shown that phosphorylation of p38 MAPK can induce UCP-1 protein expression.23,29 In eWAT, p38 MAPK phosphorylation was increased by 137±27% in HFpEF when compared with sham mice (*P*<0.01; Figure 4A). Furthermore, this increase in p38 MAPK expression was accompanied by an increase in hormone-sensitive lipase phosphorylation in eWAT of HFpEF mice (*P*<0.05 versus sham; Figure 4B). With the increase in p38 MAPK activation seen in eWAT, we sought to address the upstream pathway leading to p38 MAPK activation. We first evaluated β3-adrenergic expression, which has been shown to be related to beiging activation.27,30 In eWAT of HFpEF mice, β3-receptor protein expression was diminished by −35±7% when compared with sham (*P*<0.05; Figure 4C). However, protein kinase A phosphorylation, an important downstream target of the β3-adrenergic pathway, was not altered in eWAT of either HFpEF or sham mice (Figure 4D). We sought to determine natriuretic peptide receptors expression in eWAT and BAT. In eWAT, the ratio of gene expression of the natriuretic peptide receptor type A (npra: active form) to natriuretic peptide receptor type C (nrpc: clearance receptor) was increased in HFpEF when compared with sham mice (*P*=0.001; Figure 4E), but not different between these groups in BAT (Figure 4F). These results were also accompanied by an increase in myocardial, LV atrial natriuretic peptide and brain natriuretic peptide expressions by 2.7- and ≈2-fold, respectively, in HFpEF mice, as previously described16 (*P*<0.001 and *P*<0.05, respectively, versus sham; Figure 4G).

To ensure that these findings were not a model-specific finding, ie, an effect of chronic aldosterone, a group of wild-type mice was subjected to TAC or sham procedure. Two weeks after TAC, mice developed HFpEF and showed echocardiographic features consistent with DD (Table II in the Data Supplement). TAC mice demonstrated higher E velocity and higher E/A ratio consistent with impaired DD. TAC mice had
increased LV mass, total wall thickness, and relative wall thickness \((P<0.05\) versus sham). LVEF was preserved. TAC mice had significantly increased lung congestion (data not shown).

Body weight was comparable in TAC and sham-operated mice \((28.16\pm0.47\) versus \(27.61\pm1.09\) g; Figure 5A). WAT weight from TAC was similar to that observed in aldosterone-infused HFpEF mice (Figure 5B). WAT depot had smaller adipocytes (Figure 5C and 5D). Moreover, WAT from TAC mice showed elevated transcript levels of \(ucp-1\) \((P<0.05)\) and trends to increase \(cidea\) and \(eva\) expressions \((P=0.07\) and \(P=0.07,\) respectively). Conversely leptin and resistin expressions were downregulated \((P<0.05\) and \(P=0.06,\) respectively; Figure 5E). Similarly, the ratio of \(npra/nprc\) in WAT was increased in TAC \((P<0.05\) versus sham; Figure 5F). This was because of increases in \(npra\) expression in TAC (data not shown).

**Discussion**

In this study, we show that HFpEF is associated with changes in AT in nonoverweight mice. Aldosterone-infused mice developed hypertension, LV hypertrophy, DD, and lung congestion, while maintaining a preserved LVEF, thus resulting in HFpEF.\(^{15,16,24}\) Similar changes were seen in TAC-induced HFpEF. There were no changes in total body weight or modifications in total lean and fat mass. However, there was a shift in WAT and BAT weight, with a marked decrease in white adipose depots, whereas the brown depot was significantly increased. The macroscopic decrease of WAT mass was associated with a reduction in adipocyte size in both visceral (eWAT) and subcutaneous (iWAT) AT. The smaller adipocyte size in WAT was associated with the presence of brown-like structures, islets composed of small adipocytes with large...
Figure 3. Inflammatory markers in adipose depots of sham and heart failure with preserved ejection fraction (HFpEF) mice. A, Number of neutrophils, Ly6C\textsuperscript{low} macrophages, and Ly6C\textsuperscript{high} monocytes per milligram of epididymal white adipose tissue (eWAT) and Ki67+ Ly6C\textsuperscript{low} macrophages proliferation. B, Representative flow cytometry staining of stromal vascular fraction (SVF) cells from eWAT. C, Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) analysis of inflammatory markers in inguinal WAT (iWAT). D, qRT-PCR analysis of inflammatory markers in brown AT (BAT); n=6–12 animals per group.
nuclei and multilocular cytoplasm. Clusters of UCP-expressing adipocytes have been shown to develop in WAT in response to various stimuli such as sympathetic activation.30 These adipocytes have been named beige or brite (brown-in-white). Similar to adipocytes in BAT, beige cells are defined by their multilocular lipid droplet morphology, high mitochondrial content, and the expression of a core set of brown fat-specific genes (such as ucp-1, cidea, or eva).31 We showed an increase in these brown-specific markers, in both eW AT and iW AT in HFpEF mice. Furthermore, there was a reduction in classical murine white adipose markers such as leptin, adiponectin, and resistin.32 Together, these indicate that HFpEF is associated with the activation of beiging in WAT. Similar findings were seen in TAC-induced HFpEF mice.

When HFpEF mice were challenged in a cold tolerance experiment, they showed an ineffective response of shivering thermogenesis. This failure could indicate an inability to maintain shivering, but may also indicate an inadequate function of BAT.30 BAT, in contrast to bona fide WAT, dissipates energy through uncoupled respiration and heat production (thermogenesis). This is mediated by the major thermogenic factor UCP-1 and can be activated by stimuli, such as cold exposure and adrenergic factors.33–35 Although WAT depots possess the capacity to acquire brown fat characteristics in response to thermogenic stimuli, it is more likely that the beiging of WAT observed here is because of increased natriuretic peptides because circulating levels of brain natriuretic peptide and atrial natriuretic peptide are elevated in HF.2

Analysis of BAT molecular markers also showed a decrease in browning markers that were also accompanied by decreased expression of lipolytic enzymes, such as hormone-sensitive lipase, lipoprotein lipase, and fatp4, which are responsible for AT regulation of lipid breakdown and the generation of free fatty acids.32 Thus, the effects of the natriuretic peptides on WAT were also associated with alterations in BAT machinery. It seems that functional and molecular changes occurring in active BAT depots are also present in adult humans and can be readily activated when exposed to stimuli, such as mild cold.34–36 Beiging of WAT is usually associated with beneficial effects such as an improvement of insulin sensitivity, or amelioration of obesity.31,37,38 However, recent studies suggest that this process may not always be beneficial. Among several such settings, beiging of WAT is thought to be a driver for weight loss during cancer-associated cachexia.27 In the same way, our data suggest that the activation of beiging in WAT seems to be inadequate in HFpEF. Although we have not proven cause, notably there are parallel downregulation of the BAT transcripts that are crucial in fuel generation and thermal homeostasis and needs further study.

Interestingly, the changes observed in WAT were not accompanied by significant changes in fibrosis or oxidative stress status as we might expect in response to HFpEF.39,40 Notably, neutrophil content was higher in both eW AT and iW AT. Neutrophils are short-lived cells and are recognized as primary effector cells in acute inflammatory responses and participate in the initiation of immune responses and resolution

Figure 4. Mechanisms involved in white adipose tissue (AT) phenotypic change during heart failure with preserved ejection fraction (HFpEF). Quantitative analysis and representative blots of (A) p38 total protein and p38 phosphorylation, (B) hormone-sensitive lipase (HSL) total protein expression and HSL phosphorylation, (C) β3-receptor protein, and (D) protein kinase A (PKA) total protein and PKA phosphorylation in epididymal white AT (eWAT) from sham and HFpEF mice. E. Natriuretic peptide receptor type A (npra) to natriuretic peptide receptor type C (n.rpc) gene expression ratio in eWAT and F. brown AT (BAT) from sham and HFpEF mice. G. Atrial natriuretic peptide (anp) and brain natriuretic peptide (bnp) left ventricular gene expressions in sham and HFpEF mice (n=4–14 animals per group).
of inflammation. Thus, although neutrophils are implicated in the modulation of AT inflammation in the early stages of obesity, their presence in AT in response to a high-fat diet may last ≤90 days. Our results therefore suggest that the onset of immune activation in WAT may be related to ongoing changes, such as continued neutrophil infiltration in WAT, setting the stage for tissue infiltration by other immune cells, such as macrophages.

Several mechanisms are implicated in WAT beiging, including prolonged cold exposure and adrenergic activation, that both converge with p38 MAPK activation, as well as increases in vascular endothelial growth factor. In HFpEF mice, p38 MAPK phosphorylation was increased in WAT, and this increase was associated with an elevation in hormone-sensitive lipase phosphorylation, indicating increased AT lipolysis. Adrenergic signaling is classically thought to be the principal activator of thermogenic machinery. Elevated catecholamines increase intracellular cAMP levels via the β3-receptor, directly activating cAMP-dependent protein kinase A, which can also phosphorylate kinases in the p38 MAPK pathway. However, in HFpEF mice, there was downregulation of β3-receptor expression and protein kinase A phosphorylation indicating that the β3 pathway was not implicated in the changes observed in our experimental model. It is well known that BAT is densely innervated, and consequently, thermogenesis in this tissue is regulated primarily by adrenergic tone. In contrast, white adipose depots are less extensively innervated, suggesting that alternate pathways may control p38 MAPK activation and subsequent UCP-1 expression. We thus focused on other effectors that could be related to the observed changes. Bordicchia et al showed that cardiac natriuretic peptides mediate beiging in WAT via the p38 MAPK pathway. Similarly, we examined natriuretic peptide receptors in adipose depots and found that the ratio of npra/nrpc was increased in eWAT of HFpEF mice indicating increased signaling or decreased clearance, and demonstrating that natriuretic peptides may play a role in p38 MAPK activation. Furthermore, atrial natriuretic peptide and brain natriuretic peptide expression levels, which are primarily synthesized in the heart with increased expression in HFpEF, were increased in the LV from mice with HFpEF. We thus propose that the natriuretic peptides secreted from the heart play an important role in the changes observed in WAT, and this pathway may be a target for drug development in HFpEF.

Sources of Funding

This work was supported by the National Institutes of Health (HL117153) to Dr Sam.

Disclosures

None.

References

Valero-Muñoz et al  Adipose Tissue Regulation in HFpEF


**CLINICAL PERSPECTIVE**

The heart and adipose tissue (AT) are linked by the association of obesity and diseases of the heart, such as coronary artery disease and hypertension. Obesity is a comorbidity that is also highly associated with heart failure with preserved ejection fraction (HFpEF). Increasing evidence demonstrates that the heart can directly regulate AT via cardiokines, but the role of cardiokines in mediating cross talk between the heart and the AT is incompletely understood. The present study demonstrates that during HFpEF, there was an attempt of white AT to turn on a beiging program, which is presumed to be beneficial but appears ineffective. This novel role of white AT during HFpEF might provide further insight into how AT is regulated in HFpEF. A plausible model is that AT, as an endocrine organ, also responds and regulates the pathological cardiac phenotype. Others have described the effect of natriuretic peptides on white AT in relation to metabolism, but to our knowledge this has never been described in HFpEF. Similarly, changes seen in brown AT in HFpEF have not been described. These data have important implications for HFpEF and future drug therapies, given the many negative human HFpEF clinical trials to date. This study emphasizes the importance of cross talk between the heart and AT and raises new insights into the pathogenesis of HFpEF.
SUPPLEMENTAL MATERIAL

SUPPLEMENTAL METHODS

Mice:

The Institutional Animal Care and Use Committee at Boston University School of Medicine approved all study procedures related to the handling and surgery of the mice. Eight-weeks C57BL/6J old mice were purchased from the Jackson Laboratory and were housed in a 12:12-h light-dark cycle in a temperature-controlled (19–21°C) room. Mice were maintained on a standard rodent chow and 1.0% sodium chloride drinking water ad libitum for 4 weeks in the chronic aldosterone or saline experiment. The number of mice used in each experiment is indicated in the figure legends.

Transthoracic echocardiography to assess cardiac structure:

This was done using the Vevo 770 High-Resolution In Vivo Micro-Imaging System and a Real-Time Micro Visualization 707B Scanhead (VisualSonic Inc., Toronto, Ontario, Canada) as previously reported1. Interventricular septum wall thickness (IVST), LV posterior wall thickness (LVPWT), LV end-diastolic diameter (LVEDD), LV end-systolic diameter (LVESD), and LV ejection fraction (LVEF) were obtained. Total wall thickness (TWT) was derived from an average of the IVST and LVPWT. Relative wall thickness (RWT) was calculated as (2 X LVPWT)/LVEDD. LV mass was calculated using the formula LV mass= 1.05 x [(LVEDD + IVST + PWT)³ - (LVEDD)³] as described by Kiatchoosakun et al².

Doppler echocardiography for diastolic function:

Mitral Doppler flow studies were performed as previously described². Images were acquired using a 30-MHz transducer. HR was maintained around 350 beats/min by using isoflurane (0.5–1.5%) with minimal effects on diastolic function as previously described². Images were recorded for 30–40 cardiac cycles with measurements made from representative cycles. The mitral Doppler inflow spectrum was recorded from the apical four-chamber view. Peak early (E) and late (A) mitral inflow velocities, isovolumic relaxation time (IVRT) and deceleration time (DT) of early filling were measured.

Body temperature and acute cold tolerance test:

During the final week of the experiment, animals were subjected to a cold tolerance test (CTT), and body temperature was measured at baseline and then hourly up to 4 hours. During cold exposure, mice were kept in individual cages without access to food in a cold room (4-6°C). Body weight was measured before and after performing the test. Mice were implanted with programmable subcutaneous microchip transponders (IPTT-300 Extended Accuracy Calibration; Bio Medic Data Systems, Seaford, DE) as instructed by the manufacturer. Temperature measurements from the microchip transponders were obtained by using a compatible reader held at a distance of 5 to 6 cm from the implantation area.
**Glucose tolerance test:**

Mice were injected intraperitoneally with 1 g glucose/Kg body weight after fasting for 16 hours. Blood glucose levels were measured immediately before and 15, 30, 60, 90, and 120 minutes after glucose injection. AUC values were calculated with the GraphPad Prism software (GraphPad Software Inc., La Jolla, CA).

**Cell preparation and flow cytometry:**

Stromal vascular fraction (SVF) from epididymal WAT was isolated according to Orr’s protocol\(^2\). Single cell suspensions were obtained and the total number of cells per mg of tissue was determined using a hemocytometer and Trypan Blue staining method for cell viability (Cellgro, Mediatech, Inc., Manassas, VA). Cells were stained at 4°C in PBS supplemented with 0.5% bovine serum albumin. Fluorochrome-conjugated antibodies specific to mouse CD90.2 (clone 53-2.1), B220 (clone RA3-6B2), CD49b (clone DX5), NK1.1 (clone PK136), Ly6G (clone 1A8), Ter119 (clone TER-119), CD45.2 (clone 104), CD11b (clone M1/70), F4/80 (clone BM8), and Ly6C (clone AL-21) were used. Antibodies were purchased from either eBioscience (San Diego, CA), Biolegend (San Diego, CA), or BD Biosciences (Franklin Lakes, NJ). Ly6C\textsuperscript{high} monocytes were identified as Lineage (B220/CD49b/CD90.2/Ly6G/NK1.1/Ter119)\textsuperscript{low}, CD45\textsuperscript{+}, CD11b\textsuperscript{+}, F4/80\textsuperscript{low}, Ly6C\textsuperscript{high}. Ly6C\textsuperscript{low} macrophages were identified as Lineage (B220/CD49b/CD90.2/Ly6G/NK1.1/Ter119)\textsuperscript{int}, CD45\textsuperscript{+}, CD11b\textsuperscript{+}, F4/80\textsuperscript{high}, Ly6C\textsuperscript{low}. Neutrophils were identified as Lineage (B220/CD49b/CD90.2/Ly6G/NK1.1/Ter119)\textsuperscript{high}, CD45\textsuperscript{int}, CD11b\textsuperscript{+}. For cell proliferation analysis with Ki67 intracellular staining, cells were fixed and permeabilized using the transcription factor staining buffer set (eBioscience) and stained with FITC anti-Ki67 antibody (eBioscience, clone SolA15). Data were acquired on a LSRII (BD Biosciences) and analyzed with FlowJo software (Tree Star, Ashland, OR).

**Histology:**

Samples of adipose tissue were fixed in 4% paraformaldehyde, dehydrated and embedded in paraffin. 4μm-thick histological sections were stained with hematoxylin and eosin (H&E, Sigma-Aldrich, St. Louis, MO). Adipocyte size was evaluated by computer-assisted morphometric analysis using Adiposoft\(^4\).

**Western blot analysis:**

Protein extracts were obtained using ice-cold lysis buffer (Bio-Rad, Hercules, CA) supplemented with protease and phosphatase inhibitors (Roche Applied Science, Indianapolis, IN). Equal amounts of protein lysates were resolved by SDS-PAGE. The following antibodies were used for immunoblotting: β3 adrenergic receptor (1:500, ab94506, Abcam Inc., Cambridge, MA), HSL (1:1000, #4107, Cell Signaling Technologies, Danvers, MA), phosphor-HSL (1:1000, #4139, Cell Signaling Technologies, Danvers, MA), p38-MAP Kinase (1:1000, #9212, Cell Signaling Technologies, Danvers, MA), phospho-p38-MAP Kinase (Thr180/Tyr182) (1:1000, #9211, Cell Signaling Technologies, Danvers, MA), PKA C-α (1: 1000, #4782, Cell Signaling Technologies, Danvers, MA) and phospho-PKA C (Thr197) (1:1000,#4781s, Cell Signaling Technologies). Membranes were then probed with either anti-goat, anti-rabbit or anti-mouse horseradish peroxidase conjugated secondary antibodies (1:5000, Santa Cruz Biotechnology, Santa Cruz, CA). Blots were normalized to glyceraldehyde-3-phosphate dehydrogenase.
(GAPDH, 1:2500, ab9484, Abcam Inc., Cambridge, MA) as a loading control housekeeping protein. Blots were detected with ECL-TM Western Blotting Detection Reagent (Amersham, Buckinghamshire, England), and chemiluminescence was quantified by densitometry using ImageJ measuring software (National Institutes of Health, Bethesda, MD). Protein expression was normalized for equal protein loading, and data is expressed in arbitrary units relative to control.

**Gene expression analysis by qRT-PCR:**

RNA was obtained using Trizol reagent (Life Technologies) and RNeasy kit (QIAGEN, Valencia, CA). 2 μg of RNA were retro-transcribed using qScript XLT cDNA Supermix synthesis kit (Quanta Biosciences, Gaithersburg, MD). qRT-PCR was performed with PerfeCta SYBR® Green FastMix (Quanta Biosciences, Gaithersburg, MD) in a ViiA7 PCR system (Life Technologies, Carlsbad, CA). Primers sequences are shown in **Supplemental Table 1.** Results were analyzed with the ΔΔ Ct method using 36B4 expression as reference for normalization.
### Supplemental Table 1: Primer sequences for qRT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Sequence</th>
<th>Reverse Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>adiponectin</td>
<td>TCCTGGAGAGAAGGGAGAGAAAG</td>
<td>TCATTCAACATCTCCTGCTCTCA</td>
</tr>
<tr>
<td>anp</td>
<td>TTCCTCGTCTTGCCCTTTTG</td>
<td>CCTCATCTTCTACCGGGCATC</td>
</tr>
<tr>
<td>bnp</td>
<td>GTCCAGCAGAGACCTCAAAA</td>
<td>AGGCAGAGTCAGAAACTGGA</td>
</tr>
<tr>
<td>cidea</td>
<td>GCCGTGTAAAGGAATCTGCTG</td>
<td>TGCTCTTCTGTATCGCCAGTG</td>
</tr>
<tr>
<td>collagen 1</td>
<td>CAGAAGATGTAGGAGTCAGAG</td>
<td>GGACCCAAGGGAGACCTGG</td>
</tr>
<tr>
<td>collagen 3</td>
<td>GTGGACTGCGCTGGACCTCCA</td>
<td>GGTATCAAAGGCCAGCTG</td>
</tr>
<tr>
<td>collagen 6</td>
<td>CAGAACCATTGTCTTCTACT</td>
<td>AGGACTACATCATCTTTTCAC</td>
</tr>
<tr>
<td>eva</td>
<td>GCATTTTAACCGAACATCTGTCC</td>
<td>CCACCTCTCTGSGTTTACAGC</td>
</tr>
<tr>
<td>fabp4</td>
<td>AACACCGAGATTTCCTT</td>
<td>ACACATCCACCAG</td>
</tr>
<tr>
<td>f4/80</td>
<td>TTTTCTCGCTCTGTCTTTC</td>
<td>CCCCGTCTCTGTATTCACCA</td>
</tr>
<tr>
<td>hsl</td>
<td>GGATGGCAGGTGTGAACT</td>
<td>AGAGTATGTCAGCTACACAA</td>
</tr>
<tr>
<td>leptin</td>
<td>TTCACACACGCAGTGGGTATC</td>
<td>GGCTGGTGAGGACCTGG</td>
</tr>
<tr>
<td>ly6g</td>
<td>TGGACTCTCACAGAACAAAG</td>
<td>GCAGAGGTCTTCTCTCACAAC</td>
</tr>
<tr>
<td>lpl</td>
<td>AGGACCCCTGAAGACAC</td>
<td>GCACCAACACTCTATA</td>
</tr>
<tr>
<td>mcp1</td>
<td>TGGTGATCTCTCTGTAGCTCTCC</td>
<td>CCACTCACCTGCTGCTA</td>
</tr>
<tr>
<td>npra</td>
<td>CGAACAGATGTCATCTTACAG</td>
<td>TGGAACACAGTCACAACAGC</td>
</tr>
<tr>
<td>nprc</td>
<td>AGCTGGCTACAGCAAGAAAG</td>
<td>CGGCCATACCTCTAACAG</td>
</tr>
<tr>
<td>p22NAD(P)H ox</td>
<td>GTCCACCATGGAGACGATGTG</td>
<td>CAATGGCAGAGCAGACG</td>
</tr>
<tr>
<td>p47NAD(P)H ox</td>
<td>GATGTCCCCATTAGGGCCG</td>
<td>GTCGCCAGTCAGTGG</td>
</tr>
<tr>
<td>resistin</td>
<td>ACAAGACTTACACTCCTGTTC</td>
<td>TTTCTCAGGATGTCAGC</td>
</tr>
<tr>
<td>tnfα</td>
<td>CCCTCACAATCAGATCATCTCT</td>
<td>GCTACGACGTTGGG</td>
</tr>
<tr>
<td>ucp-1</td>
<td>GCCCTCTACGACTCAGTCCA</td>
<td>TAAAGGGCTCAGATCAG</td>
</tr>
<tr>
<td>36b4</td>
<td>CCCACTTACTGAAAAGG</td>
<td>GGCAGGATTAGTCGAA</td>
</tr>
</tbody>
</table>
**Supplemental Table 2: Echocardiographic Parameters of Sham and HFpEF mice after 2 weeks of TAC.**

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>HFpEF</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>LV Structure and Systolic Function</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LV mass (mg)</td>
<td>95±5.1</td>
<td>130.2±7.6*</td>
</tr>
<tr>
<td>Total wall thickness (mm)</td>
<td>0.80±0.04</td>
<td>1.05±0.05*</td>
</tr>
<tr>
<td>LV end-systolic diameter (mm)</td>
<td>1.78±0.06</td>
<td>1.31±0.10*</td>
</tr>
<tr>
<td>LV end-diastolic diameter (mm)</td>
<td>3.40±0.08</td>
<td>3.10±0.09*</td>
</tr>
<tr>
<td>LV ejection fraction (%)</td>
<td>75.0±5.0</td>
<td>71.0±3.0</td>
</tr>
<tr>
<td>Relative wall thickness (RWT)</td>
<td>0.50±0.03</td>
<td>0.76±0.05**</td>
</tr>
<tr>
<td><strong>Diastolic Function</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mitral E velocity, mm/s</td>
<td>667±55</td>
<td>1144±102*</td>
</tr>
<tr>
<td>Mitral A velocity, mm/s</td>
<td>567±80</td>
<td>625±106</td>
</tr>
<tr>
<td>E/A</td>
<td>1.27±0.05</td>
<td>1.79±0.06**</td>
</tr>
<tr>
<td>Deceleration time (ms)</td>
<td>20.15±0.6</td>
<td>19.40±1.1</td>
</tr>
<tr>
<td>Isovolumic relaxation time (ms)</td>
<td>19.39±0.6</td>
<td>18.8±1.2</td>
</tr>
</tbody>
</table>

Data are expressed as mean±SEM. Abbreviations: LV, left ventricle. (n=3-5 animals/group)

E; early, A; late. *P<0.05 vs. Sham; **P≤0.01 vs. Sham.
Supplemental Figure 1

A. SBP (mmHg)

B. Body weight (g)

C. Glucose tolerance test
Supplemental Figure 2

A.

B.
Supplemental Figure 3

A. eWAT

B. iWAT

C. BAT
**SUPPLEMENTAL FIGURE LEGENDS**

**Supplemental Figure 1:**

A) Systolic and diastolic blood pressure (SBP and DBP, respectively) and heart rate (HR) over 4 experimental weeks in Sham and HFpEF mice (n=6-10 animal/group). B) Weekly evolution of body weight in Sham and HFpEF mice (n=6-12 animals/group). C) Glucose tolerance test (*left panel*) and area under the curve (AUC) analysis (*right panel*) in Sham and HFpEF mice after 4 weeks of saline or *d*-aldosterone infusion (n=6-12 animals/group). *P<0.05 vs. Sham; **P<0.01 vs. Sham.

**Supplemental Figure 2:**

Representative pulse wave Doppler images in Sham (A) and HFpEF (B) mice after 4 weeks of saline or *d*-aldosterone infusion. B) Representative images of diastolic dysfunction demonstrating: Flow Reversal, Fused Peaks. Separation of E and A occurred at lower heart rates. HR indicates heart rate; IVRT, isovolumetric relaxation time; E, peak early transmitral flow velocity; A, peak late transmitral flow velocity; DT, early filling deceleration time.

**Supplemental Figure 3:**

qRT-PCR analysis of fibrotic (collagen 1, 3 and 6) and oxidative (*p22phox-NAD(P)H oxidase and p47phox-NAD(P)H oxidase*) markers in A) eWAT, B) iWAT and C) BAT of Sham and HFpEF mice. (n=5-12 animals/group). *P<0.05 vs. Sham.
Supplemental References


Heart Failure With Preserved Ejection Fraction Induces Beiging in Adipose Tissue
María Valero-Muñoz, Shanpeng Li, Richard M. Wilson, Maarten Hulsmans, Tamar Aprahamian, José J. Fuster, Matthias Nahrendorf, Philipp E. Scherer and Flora Sam

Circ Heart Fail. 2016;9:e002724
doi: 10.1161/CIRCHEARTFAILURE.115.002724
Circulation: Heart Failure is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2015 American Heart Association, Inc. All rights reserved.
Print ISSN: 1941-3289. Online ISSN: 1941-3297

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circheartfailure.ahajournals.org/content/9/1/e002724

Data Supplement (unedited) at:
http://circheartfailure.ahajournals.org/content/suppl/2015/12/31/CIRCHEARTFAILURE.115.002724.DC1.html

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

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

Subscriptions: Information about subscribing to Circulation: Heart Failure is online at:
http://circheartfailure.ahajournals.org/subscriptions/