CLINICAL RESEARCH

Myocardial Microvascular Inflammatory Endothelial Activation in Heart Failure With Preserved Ejection Fraction

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ABSTRACT

OBJECTIVES The present study investigated whether systemic, low-grade inflammation of metabolic risk contributed to diastolic left ventricular (LV) dysfunction and heart failure with preserved ejection fraction (HFpEF) through coronary microvascular endothelial activation, which alters paracrine signalling to cardiomyocytes and predisposes them to hypertrophy and high diastolic stiffness.

BACKGROUND Metabolic risk is associated with diastolic LV dysfunction and HFpEF.

METHODS We explored inflammatory endothelial activation and its effects on oxidative stress, nitric oxide (NO) bioavailability, and cyclic guanosine monophosphate (cGMP)-protein kinase G (PKG) signalling in myocardial biopsies of HFpEF patients and validated our findings by comparing obese Zucker diabetic fatty/Spontaneously hypertensive heart failure F1 hybrid (ZSF1)-HFpEF rats to ZSF1-Control (Ctrl) rats.

RESULTS In myocardium of HFpEF patients and ZSF1-HFpEF rats, we observed the following: 1) E-selectin and intercellular adhesion molecule-1 expression levels were upregulated; 2) NADPH oxidase 2 expression was raised in macrophages and endothelial cells but not in cardiomyocytes; and 3) uncoupling of endothelial nitric oxide synthase, which was associated with reduced myocardial nitrite/nitrate concentration, cGMP content, and PKG activity.

CONCLUSIONS HFpEF is associated with coronary microvascular endothelial activation and oxidative stress. These lead to a reduction of NO-dependent signalling from endothelial cells to cardiomyocytes, which can contribute to the high cardiomyocyte stiffness and hypertrophy observed in HFpEF. (J Am Coll Cardiol HF 2016;4:312–24) © 2016 by the American College of Cardiology Foundation.
Metabolic risk is increasingly recognized as an important contributor to diastolic left ventricular (LV) dysfunction and to heart failure with preserved ejection fraction (HFpEF). Recent longitudinal noninvasive studies over a 4-year time interval revealed close correlations between diastolic LV stiffness and body mass index (BMI) (1,2). These studies concluded that weight loss and reduction of central adiposity could prevent diastolic LV dysfunction and eventual development of HFpEF. Similar evidence was provided by the ALLHAT trial (The Antihypertensive and Lipid-Lowering Treatment to Prevent Heart Attack Trial), which enrolled patients with arterial hypertension and 1 additional cardiovascular risk factor and observed that a high BMI at enrollment was the strongest predictor for development of HFpEF (3). This finding was consistent with the high prevalence of overweight and obesity in large trials and registries of HFpEF outcome registries, which almost uniformly reported a median BMI value in HFpEF patients in excess of 30 kg/m².

In primates developing diet-induced obesity, endothelial inflammatory activation, evident from adhesion molecule expression, appears to be the earliest manifestation of vascular damage (4). Endothelial inflammatory activation is associated with microalbuminuria, which was recently shown to be associated with diastolic LV dysfunction (5) and to predict development of HFpEF (6). When endothelial inflammatory activation evolves to endothelial dysfunction, vasomotor responses become blunted as shown by a lower reactive hyperemic response (7), which provides both diagnostic and prognostic information in HFpEF (8,9). In HFpEF, endothelial dysfunction also closely relates to worsening of symptoms (10), functional capacity (10), and precapillary pulmonary hypertension (11).

This prominent involvement of metabolic risk and endothelial inflammatory activation recently led to a new paradigm for development of HFpEF (12). In accordance with this paradigm, metabolic comorbidities drive LV remodeling and dysfunction in HFpEF through coronary microvascular endothelial inflammation, which alters paracrine signalling from endothelial cells to surrounding cardiomyocytes. It is especially the fall in nitric oxide (NO)-cyclic guanosine monophosphate (cGMP)-protein kinase G (PKG) signalling that predisposes cardiomyocytes to develop hypertrophy and high diastolic resting tension. Microvascular endothelial dysfunction as a mechanism of LV remodeling in HFpEF differs from heart failure with reduced ejection fraction (HFrEF), where eccentric LV remodeling results from cardiomyocyte cell death pathways such as accelerated autophagy, apoptosis, or necrosis (13). It also differs from aortic stenosis (AS), where concentric LV remodeling is induced by excessive systolic wall stress (14). To establish the validity of endothelial dysfunction controlling LV remodeling in HFpEF, the current study compared microvascular endothelial inflammatory activation and its effects on myocardial oxidative stress, NO bioavailability, and cGMP content in biopsies of myocardial tissue from HFpEF, HFrEF, and AS patients. Furthermore, we studied the ability of metabolic risk to induce HFpEF through myocardial microvascular endothelial inflammatory activation in leptin-resistant, obese, hypertensive Zucker diabetic fatty/Spontaneously hypertensive heart failure F1 hybrid (ZSF1) rats. These rats develop HFpEF phenotype after 20 weeks, which was evident from elevated LV filling pressures with preserved LV systolic function, increased lung weight because of pulmonary congestion, and increased stiffness of isolated myocardial strips (15). At that time, a similar assessment of myocardial microvascular endothelial inflammatory activation and its effects on oxidative stress, NO bioavailability, cGMP content, and PKG signalling was performed, and results were compared to those in age-matched, lean hypertensive ZSF1 rats with normal diastolic LV function and no lung congestion.

**METHODS**

A detailed Methods section can be found in the Online Appendix.

**HUMAN SAMPLES.** Human HFrEF and HFpEF samples were procured from LV biopsies (HFrEF [n = 43] and HFpEF [n = 36]). HFrEF and HFpEF patients were hospitalized for HF and underwent transvascular LV endomyocardial biopsy because of suspected infiltrative or inflammatory cardiomyopathy. Patients were included if significant coronary artery disease (stenosis >50%) was ruled out by angiography and if histological analysis of the biopsy sample showed no evidence of infiltrative or inflammatory myocardial disease. Patients were classified as HFpEF if LVEF was >50%, LV end diastolic volume index was <97 ml/m², and LV end-diastolic pressure was >16 mm Hg (16). If LVEF was <45%, a patient was classified as HFrEF. AS patients (n = 67) had severe AS (mean aortic valve area = 0.53 ± 0.04 cm²) without
concomitant coronary artery disease. Biopsy specimens from this group were procured from endomyocardial tissue resected from the septum (using the Morrow procedure) during aortic valve replacement. The local ethics committee approved the study protocol. Written informed consent was obtained from all patients. Control human samples (n = 4) were procured from patients with life-threatening arrhythmia, suspected infiltrative heart disease or myocarditis, and a preserved LVEF without coronary artery disease who had histology rule out myocarditis or infiltrative pathology. Because of limited availability of human myocardial tissue, histological and biochemical determinations could only be performed in subgroups of patients, randomly selected by blinded investigators.

**RAT SAMPLES.** Obese ZSF1 rats were previously shown to develop HFpEF phenotype over a 20-week lifespan (15) and are referred to as ZSF1-HFpEF (n = 8) in the present study. These rats are hypertensive and develop obesity and diabetes mellitus (DM) because of leptin resistance. ZSF1-lean rats are hypertensive but do not develop obesity or DM and have no HFpEF phenotype (15). The ZSF1-lean rats are referred to as ZSF1-Ctrls (n = 8) in the present study. All rats were sacrificed at 20 weeks of age.

**WESTERN BLOTTING.** Expression of total and phospho-proteins was measured in homogenized samples, as follows: expression of intercellular adhesion molecule (ICAM)-1 was measured in 16 ZSF1-Ctrls, 16 ZSF1-HFpEF, 4 AS, 7 HFrEF, and 4 HFpEF samples; E-selectin in 6 AS, 8 HFrEF, and 8 HFpEF samples; endothelial nitric oxide synthase (eNOS) in 8 ZSF1-Ctrls, 8 ZSF1-HFpEF, 5 AS, 7 HFrEF, and 7 HFpEF samples; and kinases in 8 to 10 ZSF1-Ctrls and 8 to 10 ZSF1-HFpEF samples.

**IMMUNOFLOURESCENCE.** Myocardial ICAM-1 expression was measured by immunofluorescence in frozen sections of 10-μm-thick rat myocardium samples from 12 ZSF1-HFpEF and 12 ZSF1-Ctrls.

**IMMUNOHISTOCHEMISTRY.** For immunohistochemical staining of myeloperoxidase (MPO), CD68, and NADPH oxidase-2 (NOX2), paraffin-embedded myocardial samples from 8 ZSF1-Ctrls and 8 ZSF1-HFpEF were used for MPO and CD68 analysis; and 8 ZSF1-Ctrls, 8 ZSF1-HFpEF, 4 HFrEF, and 4 Ctrl patient samples were used for NOX2 analysis.

**MYOCARDIAL HYDROGEN PEROXIDE QUANTIFICATION.** Hydrogen peroxide (H₂O₂) was assessed in human and rat myocardial tissue homogenates from 10 ZSF1-Ctrls, 10 ZSF1-HFpEF, 16 AS, 16 HFrEF, and 16 HFpEF samples.

**NITRATE/NITRITE CONCENTRATION.** Concentrations of nitrite/nitrate were measured in tissue homogenates from 10 ZSF1-Ctrls, 10 ZSF1-HFpEF, 16 AS, 16 HFrEF, and 16 HFpEF samples by using a colorimetric assay kit (BioVision, Milpitas, California).

**IMMUNOELECTRON MICROSCOPIC QUANTIFICATION OF 3-NITROTYROSINE.** A standard pre-embedding immunogold electron microscopy protocol was used to quantify myocardial 3-nitrotyrosine in 6 ZSF1-HFpEF and 6 ZSF1-Ctrl rat samples.

**MYOCARDIAL PKA, PKC, PKG, AND CaMKII ACTIVITY.** Kinase activities were assessed in myocardial homogenates. Activity levels of 10 ZSF1-HFpEF and 10 ZSF1-Ctrls were analyzed as described previously for protein kinase A (PKA) and PKC (17) and for PKG and cGMP (18). Ca²⁺/calmodulin-dependent protein kinase II (CaMKII) activity was determined using a CaMKII assay kit (CycLex; MBL Corp., Woburn, MA), using 10 ZSF1-HFpEF and ZSF1-Ctrl samples.

**STATISTICAL ANALYSIS.** Differences among groups were analyzed by 1-way ANOVA, followed by Bonferroni-adjusted t test. Single comparisons were assessed by an unpaired Student t test. All analyses were performed using Prism version 6.0 software (GraphPad Inc., Lo Jolla, CA).

**RESULTS**

**PATIENT CHARACTERISTICS.** More HFpEF than HFrEF patients were hypertensive, and DM was more prevalent in HFpEF than AS patients (Table 1). BMI was higher in HFpEF than in AS patients. Angiotensin-converting enzyme inhibitors or angiotensin II receptor blockers, diuretic agents, and digoxin were more frequently used by HFpEF patients than by HFrEF patients, compared to AS patients. Aldosterone receptor antagonists were used more by HFpEF patients than by AS patients, but were used even more frequently by HFrEF patients. LV peak systolic pressure and LVEF were higher in HFpEF patients than in HFrEF patients but were highest in AS patients. LV end-diastolic volume index was lower in HFpEF patients than in HFrEF patients but were lowest in AS patients. LV end-diastolic pressures were equally elevated in all patient groups.

**MICROVASCULAR INFLAMMATION AND MACROPHAGE ACTIVATION IN HFpEF.** Microvascular inflammation and macrophage activation were assessed by analysis of expression of the vascular adhesion molecules ICAM-1 and E-selectin. Expression levels of both of the markers were increased in HFpEF compared to those in AS or HFrEF patients (Figures 1A and 1B),
consistent with the comorbidity-induced proinflammatory status of HFpEF patients.

Similarly, immunofluorescence showed increased endothelial ICAM-1 and E-selectin expression levels in ZSF1-HFpEF rats compared to those in ZSF1-Ctrls (Figures 1C and 1D). ZSF1-HFpEF rats also had higher myocardial CD68 and MPO expression levels than ZSF1-Ctrls, indicating monocyte/macrophage recruitment and neutrophil activation (Figures 1E and 1F).

**INCREASED OXIDATIVE STRESS IN HFpEF.** To quantify myocardial oxidative stress, H$_2$O$_2$ concentrations were measured and shown to be higher in HFpEF patients than in HFrEF and AS patients (Figure 2A), again consistent with the comorbidity-induced pro-inflammatory status of HFpEF patients. Findings were reproduced in ZSF1-HFpEF rats, which also had increased myocardial H$_2$O$_2$ levels compared to those in ZSF1-Ctrls (Figure 2B).

To account for myocardial oxidative stress, we compared NOX2 expression levels in HFpEF patients with those in control subjects. Human HFpEF myocardium contained more NOX2-expressing macrophages than control patients (Figure 2C). Expression in cardiomyocytes, however, was comparable those in both HFpEF and control patients (Figure 2C). Findings were confirmed in ZSF1-HFpEF myocardium by the presence of more NOX2-expressing macrophages than in ZSF1-Ctrl. Similar to the human findings, NOX2 expression levels were equal to those in cardiomyocytes of ZSF1-HFpEF and ZSF1-Ctrl rats (Figure 2D). Furthermore, NOX2 expression was also manifest in microvascular endothelial cells of ZSF1-HFpEF rats (Figure 2D), indicative of a systemic proinflammatory status.

**DECREASED NO BIOAVAILABILITY IN HFpEF.** Because of the high oxidative stress, bioavailability of NO becomes jeopardized. NO bioavailability was therefore assessed by measurement of myocardial nitrite/nitrate concentrations in human biopsy samples. Nitrite/nitrate concentration was indeed lower in HFpEF than in AS and HFrEF samples (Figure 3A). These findings were also confirmed in the rat model (Figure 3B).

Furthermore, immunogold-labeled electron microscopy allowed for quantification of 3-nitrotyrosine in different myocardial cellular compartments (Figure 3C). 3-Nitrotyrosine formation reflects concentration of peroxynitrite, which is generated from

**TABLE 1** Baseline Characteristics and Hemodynamics of Patients

<table>
<thead>
<tr>
<th></th>
<th>HFpEF (n = 36)</th>
<th>HFrEF (n = 43)</th>
<th>AS (n = 67)</th>
<th>Control (n = 4)</th>
<th>p Value (HFpEF vs. HFrEF)</th>
<th>p Value (HFpEF vs. AS)</th>
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<tr>
<td>Age, yrs</td>
<td>63.8 ± 2.0</td>
<td>60.0 ± 2.1</td>
<td>65.3 ± 1.6</td>
<td>51 ± 4</td>
<td>0.21</td>
<td>0.57</td>
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<td>% of males</td>
<td>56</td>
<td>70</td>
<td>47</td>
<td>25</td>
<td>0.24</td>
<td>0.53</td>
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<tr>
<td>% of hypertension</td>
<td>78</td>
<td>16</td>
<td>58</td>
<td>–</td>
<td>&lt;0.0001</td>
<td>0.07</td>
</tr>
<tr>
<td>% of DM</td>
<td>47</td>
<td>30</td>
<td>26</td>
<td>–</td>
<td>0.16</td>
<td>0.047</td>
</tr>
<tr>
<td>BMI, kg/m$^2$</td>
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<td>27.5 ± 0.8</td>
<td>28.1 ± 0.6</td>
<td>–</td>
<td>0.023</td>
<td>0.031</td>
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<tr>
<td>GFR, ml/min/1.73 m$^2$</td>
<td>72.9 ± 2.3</td>
<td>73.3 ± 2.7</td>
<td>68.9 ± 18.5</td>
<td>–</td>
<td>0.49</td>
<td>0.2</td>
</tr>
<tr>
<td>% of atrial fibrillation</td>
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<td>33</td>
<td>2</td>
<td>–</td>
<td>0.067</td>
<td>0.028</td>
</tr>
<tr>
<td>% taking medications</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>ACEI/ARB</td>
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<td>81</td>
<td>43</td>
<td>–</td>
<td>1.00</td>
<td>&lt;0.0001</td>
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<td>Beta-blocker</td>
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<td>63</td>
<td>61</td>
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<td>0.49</td>
<td>0.52</td>
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<tr>
<td>Diuretic agent</td>
<td>78</td>
<td>72</td>
<td>54</td>
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<td>0.61</td>
<td>0.028</td>
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<tr>
<td>Aldosterone receptor antagonist</td>
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<td>7</td>
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<td>0.020</td>
<td>&lt;0.0001</td>
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<tr>
<td>Digoxin</td>
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<td>33</td>
<td>2</td>
<td>–</td>
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<td>0.028</td>
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<td>19</td>
<td>9</td>
<td>–</td>
<td>1.00</td>
<td>0.34</td>
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</table>

Hemodynamics

|                          |               |               |            |                |                          |                        |
| HR, beats/min            | 75 ± 2        | 82 ± 4        | 74 ± 2     | 80 ± 16       | 0.073                    | 0.71                   |
| LVSPS, mm Hg             | 166 ± 6       | 120 ± 3       | 223 ± 4    | 135 ± 15      | <0.0001                  | <0.0001                |
| LVEDP, mm Hg             | 25.1 ± 1.1    | 22.3 ± 1.4    | 22.8 ± 1.4 | 13 ± 4        | 0.12                     | 0.21                   |
| LVEDVI, ml/m$^2$         | 80 ± 3        | 127 ± 5       | 55 ± 2     | 78 ± 23       | <0.0001                  | <0.0001                |
| % of LVEF                | 58.4 ± 2.1    | 29.4 ± 1.5    | 64.0 ± 1.2 | 73 ± 3        | <0.0001                  | <0.0001                |

Values are mean ± SD or %.

ACEI = angiotensin-converting enzyme inhibitor; ARB = angiotensin II receptor blocker; AS = aortic stenosis; BMI = body mass index; DM = diabetes mellitus; HFpEF = heart failure with preserved ejection fraction; HFrEF indicates heart failure with reduced ejection fraction; HR = heart rate; LVEDP = left ventricular end-diastolic pressure; LVEDVI = left ventricular end-diastolic volume index; LVEF = left ventricular ejection fraction; LVSP = left ventricular peak-systolic pressure.
FIGURE 1 Microvascular Inflammation in HFpEF Patients and ZSF1-HFpEF Rats

(A) ICAM-1 expression was higher in HFpEF than in AS (#p < 0.05 vs. AS; *p < 0.05 vs. HFrEF).
(B) E-selectin levels were higher in HFpEF patients than in those with HFrEF and AS (#p < 0.05 vs. AS; *p < 0.05 vs. HFrEF). Expression levels of ICAM-1 (C) and E-selectin (D) were higher in ZSF1-HFpEF myocardium than in that of ZSF1-Ctrls (*p < 0.05).
(E and F) ZSF1-HFpEF rats had higher levels of myocardial CD68 and MPO than ZSF1-Ctrls (*p < 0.05). AS = aortic stenosis; Ctrls = controls; HFpEF = heart failure with preserved ejection fraction; HFrEF = heart failure with reduced ejection fraction; ICAM = intercellular adhesion molecule; MPO = myeloperoxidase.

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Superoxide anion and NO. 3-Nitrotyrosine expression was higher in endothelial cells than in cardiomyocytes (Figures 3D and 3E). Its endothelial expression tended to decrease in ZSF1-HFpEF (Figure 3D), probably as a result of reduced NO bioavailability, whereas cardiomyocyte expression remained unaltered (Figure 3E).

**Uncoupling of Nitric Oxide Synthase in HFpEF.** Endothelial nitric oxide synthase (eNOS) produces NO as a dimer, but “uncouples” into monomers in the presence of inflammation/oxidative stress, producing superoxide anion. HFpEF patients had significantly higher expression of the eNOS monomer than HFrEF or AS patients (Figure 4A). In ZSF1-HFpEF rats, there was a similar trend toward higher expression of the eNOS monomer than in ZSF1-Ctrl rats (Figure 4B). Levels of eNOS dimers were equal among human groups and between the 2 groups of rats (Figures 4C and 4D). Phosphorylated, and hence activated, eNOS monomers tended to be higher in HFpEF than in HFrEF and AS patients (p = 0.06) and in ZSF1-HFpEF than in ZSF1-Ctrl rats (Figures 4E and 4F). Concentrations of active, phosphorylated NO-producing dimer were lower in HFpEF patients than in AS (p = 0.06) and HFrEF (p = 0.07) patients (Figure 4G). In rats, phosphorylation of the eNOS dimer was similar (Figure 4H).
DECREASED cGMP CONCENTRATION AND PKG ACTIVITY IN HFpEF. Because of decreased NO bioavailability, soluble guanylate cyclase (sGC) activity falls. This leads to reduced production of cGMP, which regulates PKG activity. PKG activity and cGMP concentration were indeed lower in myocardium of ZSF1-HFpEF rats than in ZSF1-Ctrl rats (Figures 5A and 5B). PKG lowers cardiomyocyte...
Expression of eNOS monomer was higher in HFpEF than in AS and HFrEF and higher in HFrEF than in AS (\#p < 0.05 vs. AS; *p < 0.05 vs. HFrEF). A similar trend was observed in a comparison between ZSF1-HFpEF and ZSF1-Ctrl rats. E N O S dimer concentrations were comparable in AS, HFrEF, and HFpEF patients as well as in ZSF1-HFpEF and ZSF1-Ctrl rats. E N O S m o n o m e r phosphorylation tended to be higher in HFpEF patients and in ZSF1-HFpEF rats. E N O S dimer phosphorylation tended to be lower in HFpEF patients but was comparable in rats. eNOS = endothelial nitric oxide synthase; other abbreviations as in Figure 1.
stiffness through phosphorylation of titin, the giant intracellular protein responsible for cardiomyocyte-based stiffness (15,17–19). Activity and expression of other protein kinases and phosphatases, reported to modulate titin phosphorylation (19), were measured as well. Activity levels of PKC and PKA in ZSF1-HFpEF rats were not significantly different from those in ZSF1-Ctrl rats (Figures 5C and 5D). There were no significant differences between the expression levels of the active, phosphorylated state of extracellular signal-regulated kinase (ERK) and those of ZSF1-HFpEF rats and Ctrl rats (Figure 5E). CaMKII also lowers cardiomyocyte stiffness through titin phosphorylation (20). CaMKII activity was increased in ZSF1-HFpEF rats compared to that in ZSF1-Ctrl rats (Figure 5F), and altered CaMKII activity, therefore, fails to explain the increased cardiomyocyte stiffness observed in previous experiments in ZSF1-HFpEF rats (15). Finally, titin can also be affected by dephosphorylating protein phosphatases (PP) such as PP1 and PP2a (19). Both of the proteins were increased in ZSF1-HFpEF compared to those in ZSF1-Ctrl rats (Figures 5G and 5H).

**DISCUSSION**

The present study provides comprehensive evidence for microvascular endothelial activation, high oxidative stress, eNOS uncoupling, and low NO levels in LV myocardium of HFpEF patients. These findings were reproduced in leptin-resistant, obese, hypertensive ZSF1 rats, which develop HFpEF after 20 weeks, in contrast to lean hypertensive ZSF1 rats, which maintain normal LV function after a similar time period. The present study also demonstrated that low myocardial NO level was associated with reduced myocardial cGMP/PKG signalling in ZSF1-HFpEF rats. A similar reduction was previously demonstrated in LV myocardium of HFpEF patients and shown to be associated with titin hypophosphorylation which contributes to high myocardial diastolic stiffness (18).

**MICROVASCULAR INFLAMMATION.** In the present study, myocardial expression levels of E-selectin and ICAM-1 were upregulated in HFpEF patients and ZSF1-HFpEF rats (Figure 6). Upregulated myocardial expression of vascular cell adhesion molecule (VCAM) and myocardial microvascular rarefaction compatible with microvascular inflammation was previously reported in HFpEF (21,22). Because findings in HFpEF patients were similar to those in ZSF1-HFpEF rats, we attribute the endothelial inflammatory activation in HFpEF patients to their metabolic risk profile. HFpEF patients had significantly higher BMI than AS and HFrEF patients, and the prevalence of DM was also higher in HFpEF patients than in AS patients. The high prevalence of a metabolic risk profile in HFpEF patients in the present study was consistent with findings of the recent MEDIA (The METabolic Road to DIAstolic Heart Failure) European HFpEF registry (23). This registry was the first to report the prevalence of metabolic syndrome in HFpEF and observed that 85% of HFpEF patients satisfied National Cholesterol Education Program III criteria of metabolic syndrome.

Expression of adhesion molecules favors myocardial infiltration of inflammatory cells, which was evident in the current study in HFpEF patients and in ZSF1-HFpEF rats, respectively, by the presence of NOX2-producing macrophages and by the high expression levels of CD68 and MPO. In contrast to viral myocarditis, the myocardial presence of macrophages in HFpEF is not accompanied by evidence of cardiomyocyte cell death (24,25). A recent study provided an explanation for this intriguing finding, observing that macrophages activated by obesity had a distinct proinflammatory phenotype (26). Hitherto, macrophage activation was conceived to proceed either to an M1 phenotype with potent proinflammatory properties or to an M2 phenotype with anti-inflammatory properties. However, when macrophages are activated by obesity, a distinct phenotype is induced with low production of proinflammatory cytokines because of peroxisome proliferator-activated receptor γ (PPAR γ) partially inhibiting induction of nuclear factor kappa-light chain-enhancer of activated B cells (NFκB). This last effect results from the abundance in obesity of free fatty acids such as palmitate, which stimulate PPAR γ activity. A recent study also demonstrated that development of HFpEF was associated with monocytosis and monocyte differentiation into M2 macrophages (27).

**OXIDATIVE STRESS.** Myocardial H₂O₂ concentration was significantly higher in HFpEF patients than in both HFrEF and AS patients. Similarly, ZSF1-HFpEF rats had higher myocardial H₂O₂ concentrations than ZSF1-Ctrl animals. H₂O₂ results from conversion of superoxide anion by superoxide dismutase, and the high H₂O₂ concentrations therefore suggested increased superoxide anion production in HFpEF. Possible sources of superoxide anion production are NADPH oxidases (NOX2, NOX4), uncoupled NO synthases (eNOS, iNOS), xanthine oxidase, and mitochondria (Figure 6). Cellular localization of NOX2 expression was immunohistochemically visualized in myocardial tissue from HFpEF patients and ZSF1-HFpEF rats. Upregulation
Decreased PKG Activity and cGMP Concentration in HFpEF

(A and B) Myocardial PKG activity and cGMP concentration were lower in ZSF1-HFpEF than in ZSF1-Ctrl rats. 
(C and D) Comparable activity levels of PKC and PKA were seen in ZSF1-HFpEF and ZSF1-Ctrl rats. 
(E) Comparable expression levels of phosphorylated ERK were seen in ZSF1-HFpEF and ZSF1-Ctrl rats. 
(F) Expression of CaMKII was higher in ZSF1-HFpEF than in ZSF1-Ctrl rats. 
(G and H) Higher expression of PP1 and PP2a was seen in ZSF1-HFpEF than in ZSF1-Ctrl rats (*p < 0.05). 
CaMKII = Ca²⁺/calmodulin-dependent protein kinase II; cGMP = cyclic guanosine monophosphate; 
ERK = extracellular signal-regulated kinase; PKA = protein kinase A; PKC = protein kinase C; other abbreviations as in Figure 1.
of NOX2 expression was observed in macrophages and microvascular endothelium but not in cardiomyocytes. The current findings of upregulated endothelial NOX2 expression and unaltered NOX2 expression in cardiomyocytes in HFrpEF myocardium support the assertion that myocardial remodeling in HFrpEF is driven by endothelial activation, in contrast to HFrEF, where myocardial remodeling is driven by cardiomyocyte cell death triggered by upregulated NOX2 activity within cardiomyocytes (28).

**NO-cGMP-PKG SIGNALLING.** Similar to NOX2 expression, immunoelectron microscopy revealed myocardial 3-nitrotyrosine expression in ZSF1-Ctrl and ZSF1-HFrpEF rats was localized mainly in endothelial cells, with less expression in cardiomyocytes. Development of HFrpEF failed to affect 3-nitrotyrosine expression in cardiomyocytes but reduced 3-nitrotyrosine expression in endothelial cells. 3-Nitrotyrosine formation reflects peroxynitrite concentration, which is generated from superoxide anion and NO. Because of increased H2O2 concentration, the trend for reduced 3-nitrotyrosine in endothelial cells of HFrpEF myocardium probably resulted from low NO production. The latter was consistent with the low nitrite/nitrate concentrations observed in myocardium of both HFrpEF patients and ZSF1-HFrpEF rats. Reduced NO production can be explained by eNOS uncoupling, which was confirmed in both HFrpEF patients and ZSF1-HFrpEF rats. eNOS uncoupling switches eNOS from the NO producing dimer to the superoxide anion generating monomer (29). Apart from eNOS uncoupling, the present study also observed modified eNOS phosphorylation in HFrpEF patients, with a higher phosphorylation of monomeric eNOS increasing superoxide production.
and a lower phosphorylation of dimeric eNOS decreasing NO production. Low NO production affects sGC activity and results in decreased levels of cGMP and low PKG activity (Figure 6). This was previously observed in human HFpEF myocardium (18) and was currently confirmed in ZSF1-HFpEF myocardium. Low PKG activity increases diastolic stiffness through reduced phosphorylation of the giant cytoskeletal protein titin (Figure 6) (30,31).

The phosphorylation and distensibility of titin are also affected by phosphatases and other kinases, such as PKCz, PKA, ERK and CaMKII (20,30,31). In the present study, ZSF1-HFpEF rats showed higher expression levels of PP1a and PP2a. PKC, PKA, and ERK activity levels were comparable, but CaMKII activity was increased. Phosphorylation of titin by CaMKII augments titin distensibility (32) and the higher CaMKII activity therefore cannot explain the high cardiomyocyte resting tension previously observed in ZSF1-HFpEF rats (15). The latter more likely results from imbalanced activities of PKG and phosphatases.

STUDY LIMITATIONS. Except for immunohistochemical assessment of NOX2 expression, the present study compared myocardial tissue of HFpEF patients to tissue of HFrEF and AS patients because of limited availability of myocardial tissue from control subjects. This limitation was partially accounted for by inclusion of an animal model consisting of obese and lean ZSF1 rats (15). Both HFpEF and HFrEF patients were investigated following admission for acute heart failure. An inflammatory component related to the acute heart failure episode could have contributed to the microvascular inflammation.

CONCLUSIONS

Microvascular inflammatory endothelial activation, high oxidative stress, eNOS uncoupling, and impaired cGMP-PKG signalling were observed in LV myocardium of HFpEF patients. Similar changes were reproduced in obese, leptin-resistant, hypertensive ZSF1 rats, which developed a HFpEF phenotype after 20 weeks, but not in lean, hypertensive ZSF1 rats. Because of these findings, myocardial microvascular inflammation induced by metabolic comorbidities could be an important contributor to development of HFpEF.

REFERENCES

13. González A, Ravassa S, Beaumont J, López B, Diez J. New targets to treat the structural...


KEY WORDS endothelium, heart failure, inflammation, nitric oxide, oxidative stress

APPENDIX For a detailed Methods section, please see the online version of this article.