Low Myocardial Protein Kinase G Activity in Heart Failure With Preserved Ejection Fraction

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Background—Prominent features of myocardial remodeling in heart failure with preserved ejection fraction (HFPEF) are high cardiomyocyte resting tension ($F_{\text{passive}}$) and cardiomyocyte diameter. In experimental models, both reacted favorably to raised protein kinase G (PKG) activity. The present study assessed myocardial PKG activity, its downstream effects on cardiomyocyte $F_{\text{passive}}$ and cardiomyocyte diameter, and its upstream control by cyclic guanosine monophosphate (cGMP), nitrosative/oxidative stress, and brain natriuretic peptide (BNP). To discern altered control of myocardial remodeling by PKG, HFPEF was compared with aortic stenosis and HF with reduced EF (HFREF).

Methods and Results—Patients with HFPEF ($n=36$), AS ($n=43$), and HFREF ($n=43$) were free of coronary artery disease. More HFPEF patients were obese ($P<0.05$) or had diabetes mellitus ($P<0.05$). Left ventricular myocardial biopsies were procured transvascularly in HFPEF and HFREF and perioperatively in aortic stenosis. $F_{\text{passive}}$ was measured in cardiomyocytes before and after PKG administration. Myocardial homogenates were used for assessment of PKG activity, cGMP concentration, proBNP-108 expression, and nitrotyrosine expression, a measure of nitrosative/oxidative stress. Additional quantitative immunohistochemical analysis was performed for PKG activity and nitrotyrosine expression. Lower PKG activity in HFPEF than in aortic stenosis ($P<0.01$) or HFREF ($P<0.001$) was associated with higher cardiomyocyte $F_{\text{passive}}$ ($P<0.001$) and related to lower cGMP concentration ($P<0.001$) and higher nitrosative/oxidative stress ($P<0.05$). Higher $F_{\text{passive}}$ in HFPEF was corrected by in vitro PKG administration.

Conclusions—Low myocardial PKG activity in HFPEF was associated with raised cardiomyocyte $F_{\text{passive}}$ and was related to increased myocardial nitrosative/oxidative stress. The latter was probably induced by the high prevalence in HFPEF of metabolic comorbidities. Correction of myocardial PKG activity could be a target for specific HFPEF treatment. (Circulation. 2012;126:830-839.)

Key Words: diastole ■ heart failure ■ muscle cells ■ natriuretic peptides ■ nitric oxide

Despite modern heart failure (HF) therapy, prognosis of heart failure with preserved ejection fraction (HFPEF) has not improved over the last decades. This epidemiological finding confirmed the neutral result of numerous large outcome trials testing modern pharmacotherapy in HFPEF. The neutral outcome of these trials has been variably ascribed to trial design and to failure to address features of myocardial dysfunction and remodeling prominently present in HFPEF.

Prominent clinical features of myocardial dysfunction in HFPEF are slow relaxation and high diastolic stiffness. High diastolic stiffness is also present in muscle strips isolated from left ventricular (LV) myocardium of HFPEF patients. Slow relaxation and high diastolic stiffness have both been shown to react favorably to raised protein kinase G (PKG) activity after in vivo administration of sildenafil, which raises myocardial PKG activity through inhibited breakdown of cyclic guanosine monophosphate (cGMP) by phosphodiesterase 5A (PDE5A). Sildenafil restored LV relaxation kinetics in mice exposed to transverse aortic constriction (TAC) and reduced diastolic LV stiffness.

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in an old hypertensive dog model,9 in patients with HF and reduced EF (HFREF)10 and in HFPEF patients with pulmonary hypertension.11 Administration of sildenafil to old hypertensive dogs lowered diastolic LV stiffness through re-stored phosphorylation of the N2B segment of titin,9 which was recently shown to be hypophosphorylated in HF patients12 and to have PKG phosphorylation sites.13 Prominent was recently shown to be hypophosphorylated in HF patients and to have PKG phosphorylation sites.13 Prominent features of myocardial remodeling in HFPEF are cardiomyocyte hypertrophy and interstitial fibrosis.7 Modulation of the soluble guanylate cyclase (sGC)-PKG-PDE5A axis also affected myocardial remodeling with less cardiomyocyte hypertrophy and interstitial fibrosis in TAC mice exposed to sildenafil.8

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Because of these promising experimental and clinical effects of sildenafil on myocardial dysfunction and remodeling in HFPEF and because of the ongoing large outcome trial testing sildenafil in HFPEF (RELAX study; http://www.clinicaltrials.gov; unique identifier: NCT00763867), the presence study measured PKG activity, upstream control of PKG activity, and downstream effects of PKG activity in LV myocardial biopsies of HFPEF patients. To discern altered control by PKG of the myocardial remodeling process in HFPEF, measurements were compared with measurements obtained from LV myocardium remodeled concentrically by severe aorticstenosis (AS) or eccentrically by nonischemic HFREF.

Methods

Patients

HF patients had been hospitalized for worsening HF and referred for cardiac catheterization and transvascular LV endomyocardial biopsy procurement because of suspicion of infiltrative or inflammatory myocardial disease. Patients were included if coronary angiography ruled out significant epicardial coronary artery stenosis (>50%) and if histological examination of the biopsy showed no evidence of infiltrative or inflammatory myocardial disease. The HF patient study cohort consisted of 79 patients, of whom 36 were classified as HFPEF and 43 as HFREF. Patients were diagnosed as HFPEF if they had LV ejection fraction (LVEF) <50%, LV end-diastolic volume index <97 mL/m2, and LV end-diastolic pressure >16 mm Hg.14 Patients were classified as HFREF if LVEF was <45%. No HF patient had cardiac resynchronization therapy or a LV assist device. The AS group consisted of 67 patients with symptomatic AS (mean aortic valve area 0.53±0.04 cm²) and without significant coronary disease. In this group, LV biopsy material consisted of LV endomyocardial tissue resected from the LV outflow tract (Morrow procedure) because of concomitant LV outflow tract narrowing. Hemodynamic data of HFREF, HFPEF, and AS patients are listed in the Table. LV peak-systolic pressure, LVEDP, LV end-diastolic volume index, and LVEF were derived from biplane LV angiograms. Diastolic posterior wall thickness and LV mass index were derived from 2-dimensional echocardiograms. The local ethics committee approved the study protocol. Written informed consent was obtained from all patients, and there were no complications related to procedure or biopsy procurement.

Histomorphometric Myocardial Analysis

Histomorphometric analysis of biopsy samples was performed on hematoxylin-and-eosin-stained 4-μm-thick sections of tissue placed in 5% formalin. Images of these sections were acquired with a projection microscope (×200) and subsequently analyzed with Slidebook 4.0 software (3I, Denver, CO) to determine cardiomyocyte diameter (μm, 17.1±1.5 cardiomyocytes measured per patient) and reactive interstitial fibrosis, expressed as collagen volume fraction (%, 4.1±0.3 representative fields per patient).7,15

Force Measurements in Isolated Cardiomyocytes

Force measurements were performed in single, mechanically isolated cardiomyocytes as described previously (2.6±0.1 cardiomyocytes per patient).5,7,13 Biopsy samples (5 mg wet weight) were defrosted in relaxing solution, mechanically disrupted, and incubated for 5 minutes in relaxing solution supplemented with 0.2% Triton X-100 to remove all membrane structures. Single cardiomyocytes were stretched to a sarcomere length of 2.2 μm to measure passive force (Fpassive). Fpassive measurements were repeated after 40 minutes of incubation in relaxing solution containing PKG1α (0.1 U/mL; Sigma, St. Louis, MO, batchh34K1336) and cGMP (10 μmol/L, Sigma).

Myocardial PKG Activity

Myocardial PKG activity was assessed in homogenized tissue samples. Tissues samples were homogenized in 25 mL/L Tris (pH 7.4), 1 mL/L EDTA, 2 mL/L EGTA, 5 mL/L dithiothreitol, 0.05% Triton X-100, and protease inhibitor cocktail (Sigma-Aldrich) and centrifuged for 5 minutes. Supernatants containing equal amounts of total protein were analyzed for PKG activity. The reaction mixture contained (at final concentration): 40 mL/L Tris-HCl (pH 7.4), 20 mL/L magnesium acetate, 0.2 mL/L [32P]ATP (500–1000 cpm pmol−1; Amersham Pharmacia Biotech, Amersham, UK), 113 mg/mL heptapeptide (RKRSRAE), 3 μmol/L cGMP (Promega, Madison, WI), and a highly specific inhibitor of cyclic adenosine monophosphate-dependent protein kinase (5–24, Calbiochem, Schiphol-Rijk, the Netherlands). The reaction mixtures were incubated at 30°C for 10 min, followed by termination of the reaction by spotting 70 μL of the reaction mix onto Whatman P-81 filters, which were then soaked with 75 mL/L H₃PO₄ for 5 min and washed 3 times with 75 mL/L H₃PO₄ to remove any unbound [32P]ATP. Filters were rinsed with 100% ethanol and air dried before quantification. For quantification of PKG activity, counts were taken in a Wallac 1409 Liquid Scintillation Counter using universal scintillation cocktail (ICN). Specific activity of PKG was expressed as pmol of [32P] incorporated into the substrate (pmol/min/mg protein).

Immunohistochemical analysis was performed on formalin-fixed tissue sections. Myocardial PKG activity was measured immunohistochemically by ratio of vasodilatory-stimulated phosphoprotein (VASP) phosphorylated at Ser149 (pVASP) to total VASP (pVASP/VASP ratio).16 Biopsy samples were stained with mouse monoclonal anti Ser149 pVASP antibody (16C2; #676602, Calbiochem) for detection of pVASP, and with mouse monoclonal anti-VASP antibody (A-11; 1:50; sc-46668, Santa Cruz Biotechnology, Inc, Santa Cruz, CA) for detection of VASP. All incubations were done for 1 hour at room temperature. Samples were then incubated with antimouse/ rabbit Envision (undiluted; K5007; Dako Cytomation) for 30 minutes at room temperature. Samples were then incubated with antimouse/ rabbit Envision (undiluted; K5007; Dako Cytomation) for 30 minutes at room temperature. Staining was visualized using 3,3'-diaminobenzidine (DAB, 0.1 mg/mL, 0.02% H₂O₂). Sections were then counterstained with hematoxylin, dehydrated, and covered. With each staining a PBS control or an irrelevant antibody was used. All these controls yielded negative results. pVASP and VASP levels were calculated, using Slidebook 4.0 software, as representative myocellular positive staining areas relative to total myocardial area (4.8±0.4 representative fields per patient).

Myocardial cGMP Concentration

Myocardial cGMP was determined in homogenates by use of parameter cGMP assay immunoassay kit (R&D Systems, Minneapolis, MN), in which cGMP present in the homogenate competes with fixed amount of horseradish peroxidase–labeled cGMP for sites on a rabbit polyclonal antibody. The homogenates were diluted in cell lysis buffer, and 100 μL of 0.025 μg/μL protein aliquots were
Myocardial Nitrotyrosine, proBNP-108, sGC, and PDE5A Expression

Gel electrophoresis and Western blot were performed to analyze expression of nitrotyrosine protein (dilution 1:1000, Upstate, Billerica, MA) and proBNP-108 (dilution 1:500, NOVUS Biologicals, Littleton, CO). Biopsies were resuspended in 1 mL cold 10% trichloroacetic acid solution–treated tissue pellets were homogenized in sample buffer containing 15% glycerol, 62.5 mmol/L Tris (pH 6.8), 1% (wt/vol) dithiothreitol). Trichloroacetic acid solution–treated tissue samples were stained with rabbit polyclonal guanylate cyclase beta1 antibody (1:10, ab14672, Abcam), rat polyclonal PDE5A antibody (1:100, ab24824, Abcam), and rabbit polyclonal nitrotyrosine antibody (HM.11, 1:50, ab7048, Abcam). Myocardial sGC and PDE5A levels were calculated as respective myocardial positive staining areas relative to total myocardial area (representative fields per patient: sGC 6.9 ± 0.7; PDE5A 22.7 ± 1.2). For detection of myocardial sGC and PDE5A levels, biopsy samples were stained with rabbit polyclonal guanylate cyclase beta1 antibody (1:100; ab24824, Abcam) and rabbit polyclonal PDE5A antibody (1:10, ab14672, Abcam), respectively. Myocardial sGC and PDE5A levels were calculated as respective myocardial staining areas relative to total myocardial area (representative fields per patient: sGC 6.9 ± 0.7; PDE5A 22.7 ± 3.5). For immunohistochemical analysis of nitrotyrosine content and sGC or PDE5A expression, incubation procedures, secondary antibodies, and visualization techniques were similar to the aforementioned immunohistochemical assessment of pVASP or VASP.

### Table. Clinical, Hemodynamic, and Echocardiographic Characteristics

<table>
<thead>
<tr>
<th></th>
<th>HFREF (n=43)</th>
<th>HFPEF (n=36)</th>
<th>AS (n=67)</th>
<th>P Value (HFPEF vs HFREF)</th>
<th>P Value (HFPEF vs AS)</th>
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</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>60.0±2.1</td>
<td>63.8±2.0</td>
<td>65.3±1.6</td>
<td>0.21</td>
<td>0.57</td>
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<tr>
<td>Male, %</td>
<td>70</td>
<td>56</td>
<td>47</td>
<td>0.24</td>
<td>0.53</td>
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<tr>
<td>Hypertension, %</td>
<td>16</td>
<td>78</td>
<td>58</td>
<td>&lt;0.0001</td>
<td>0.07</td>
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<tr>
<td>DM %</td>
<td>30</td>
<td>47</td>
<td>26</td>
<td>0.16</td>
<td>0.047</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>27.5±0.8</td>
<td>30.4±1.0</td>
<td>28.1±0.6</td>
<td>0.023</td>
<td>0.031</td>
</tr>
<tr>
<td>Body surface area, (BSA, m²)</td>
<td>1.91±0.03</td>
<td>1.94±0.04</td>
<td>1.77±0.03</td>
<td>0.57</td>
<td>0.002</td>
</tr>
<tr>
<td>Atrial fibrillation, %</td>
<td>33</td>
<td>14</td>
<td>2</td>
<td>0.067</td>
<td>0.028</td>
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<tr>
<td>Medication</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>ACE-I/ARB, %</td>
<td>81</td>
<td>81</td>
<td>43</td>
<td>1.00</td>
<td>&lt;0.0001</td>
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<tr>
<td>β-blocker, %</td>
<td>63</td>
<td>53</td>
<td>61</td>
<td>0.49</td>
<td>0.52</td>
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<tr>
<td>Diuretic, %</td>
<td>72</td>
<td>78</td>
<td>54</td>
<td>0.61</td>
<td>0.028</td>
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<tr>
<td>Aldosterone receptor antagonist, %</td>
<td>74</td>
<td>47</td>
<td>7</td>
<td>0.020</td>
<td>&lt;0.0001</td>
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<tr>
<td>Digoxin, %</td>
<td>33</td>
<td>14</td>
<td>2</td>
<td>0.067</td>
<td>0.028</td>
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<td>Statin, %</td>
<td>21</td>
<td>42</td>
<td>46</td>
<td>0.054</td>
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<tr>
<td>Metformin, %</td>
<td>14</td>
<td>17</td>
<td>3</td>
<td>0.76</td>
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<tr>
<td>Bronchodilators, %</td>
<td>19</td>
<td>17</td>
<td>9</td>
<td>1.00</td>
<td>0.34</td>
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<tr>
<td>Hemodynamics</td>
<td></td>
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</tr>
<tr>
<td>HR, beats/min</td>
<td>82±4</td>
<td>75±2.0</td>
<td>74±2.0</td>
<td>0.073</td>
<td>0.71</td>
</tr>
<tr>
<td>LVSP, mm Hg</td>
<td>120±3</td>
<td>166±6</td>
<td>223±4</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>LVEDP, mm Hg</td>
<td>22.3±1.4</td>
<td>25.1±1.1</td>
<td>22.8±1.4</td>
<td>0.12</td>
<td>0.21</td>
</tr>
<tr>
<td>LVEDVI, ml/m²</td>
<td>127±5</td>
<td>80±3</td>
<td>55±2</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>LVEF, %</td>
<td>29.4±1.5</td>
<td>58.4±2.1</td>
<td>64.0±1.2</td>
<td>&lt;0.0001</td>
<td>0.016</td>
</tr>
<tr>
<td>Echocardiography</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>PWTd, mm</td>
<td>9.56±0.32</td>
<td>11.0±0.26</td>
<td>11.0±0.23</td>
<td>0.0011</td>
<td>0.93</td>
</tr>
<tr>
<td>LVM/LVEDVI</td>
<td>1.03±0.06</td>
<td>1.74±0.07</td>
<td>2.55±0.17</td>
<td>&lt;0.0001</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

HFREF indicates heart failure with reduced ejection fraction; HFPEF, heart failure with preserved ejection fraction; AS, aortic stenosis; DM, diabetes mellitus; BMI, body mass index; ACE-I, angiotensin-converting enzyme inhibitor; ARB, angiotensin II receptor blocker; HR, heart rate; LVSP, left ventricular peak-systolic pressure; LVEDP, left ventricular end-diastolic pressure; LVEDVI, left ventricular end-diastolic volume index; LVEF, left ventricular ejection fraction; PWTd, diastolic posterior wall thickness; and LVM, left ventricular mass index.

Results of duplicate determinations were averaged and expressed as pmol/mL.

assayed according to manufacturer’s instructions. Results of duplicate determinations were averaged and expressed as pmol/mL.

**Myocardial Nitrotyrosine, proBNP-108, sGC, and PDE5A Expression**

Gel electrophoresis and Western blot were performed to analyze expression of nitrotyrosine protein (dilution 1:1000, Upstate, Billerica, MA) and proBNP-108 (dilution 1:500, NOVUS Biologicals, Littleton, CO). Biopsies were resuspended in 1 mL cold 10% trichloroacetic acid solution (dissolved in acetone containing 0.1% [wt/vol] dithiothreitol). Trichloroacetic acid solution–treated tissue pellets were homogenized in sample buffer containing 15% glycerol, 62.5 mmol/L Tris (pH 6.8), 1% (wt/vol) SDS, and 2% (wt/vol) dithiothreitol. Samples (dry weight) were applied in a concentration that was within the linear range of detection. After one-dimensional separation, proteins were transferred to Hybond ECL nitrocellulose membranes. Blots were preincubated with 0.5% milk powder or 3% bovine serum albumin in TTBS (TWEEN Tris-buffered saline; 10 mmol/L Tris-HCl pH 7.6, 75 mmol/L NaCl, 0.1% [wt/vol] TWEEN) for 1 hour at room temperature. The blots were incubated overnight at 4°C with primary rabbit polyclonal antibodies against nitrotyrosine (dilution 1:500, NOVUS Biologicals) and proBNP-108. After washing with TTBS, primary antibody binding was visualized using a secondary horseradish peroxidase-labeled, goat-antirabbit or goat-antimouse (dilution 1:1000; DakoCytomation), and enhanced chemiluminescence (ECL Western blotting detection, Amersham Biosciences). All signals were normalized to actin (dilution 1:1000; clone KJ43A; Sigma) stained on the same blots. Staining was visualized using the LAS-3000 Image Reader (460 nm/605 nm Ex/Em: 2-s illumination), and signals were analyzed with AIDA software.

Myocardial nitrotyrosine content was also assessed immunohistochemically with biopsy samples stained with mouse monoclonal anti-nitrotyrosine antibody (HM.11:1.50,ab7048, Abcam). Myocardial nitrotyrosine levels were calculated as respective myocardial staining areas relative to total myocardial area (representative fields per patient: 9.7±1.2). For detection of myocardial sGC and PDE5A levels, biopsy samples were stained with rabbit polyclonal guanylate cyclase beta1 antibody (1:100, ab24824, Abcam) and rabbit polyclonal PDE5A antibody (1:10, ab14672, Abcam), respectively. Myocardial sGC and PDE5A levels were calculated as respective myocardial positive staining areas relative to total myocardial area (representative fields per patient: sGC 6.9±1.0; PDE5A 22.7±3.5). For immunohistochemical analysis of nitrotyrosine content and sGC or PDE5A expression, incubation procedures, secondary antibodies, and visualization techniques were similar to the aforementioned immunohistochemical assessment of pVASP or VASP.
Results

Clinical, Hemodynamic, and Echocardiographic Characteristics

Hypertension was more prevalent in HFPEF than in HFREF, and diabetes mellitus (DM) was more prevalent in HFPEF than in AS (Table). HFPEF patients had a higher body mass index than both HFREF and AS patients. In both HF groups, there was more frequent use of angiotensin converting enzyme inhibitor or angiotensin II receptor blockers, diuretics, and digoxin. Aldosterone receptor antagonists were used less frequently in HFREF than in HFPEF and AS, whereas statins were used less frequently in HFPEF than in HFREF and AS. There was a progressive rise of LV peak-systolic pressure, LVEF, and LV mass index/LV end-diastolic volume index from HFREF to HFPEF and to AS, and a progressive fall of LV end-diastolic volume index from HFREF to HFPEF and to AS. LVEDP was similar in all 3 conditions. Diastolic posterior wall thickness was equally elevated in HFPEF and AS compared with HFREF.

Myocardial PKG Activity and cGMP Concentration

PKG activity in myocardial tissue homogenates was significantly lower in HFPEF (5.1±0.2 kN/m²; n=22; P<0.001; Figure 1A and 1B). When patients were subdivided in accordance to absence or presence of DM (Table) (DM⁻, DM⁺), Fpassive was higher in HFPEF-DM⁻ than in AS-DM⁻ or HFREF-DM⁻ and also higher in HFPEF-DM⁺ than in AS-DM⁺ or HFREF-DM⁺ (Figure I in the online-only Data Supplement). After administration of PKG, Fpassive fell significantly in HFREF, HFPEF, and AS (Figure 1B). The fall in Fpassive was significantly larger in HFPEF than in AS or HFREF (Figure 1C). Fpassive of normal human cardiomyocytes is 2.8±0.1 kN/m² and remains unchanged after PKG administration (2.6±0.1 kN/m²).6,12

Figure 1A, Single cardiomyocyte mounted between force transducer and piezoelectric motor. B, Higher cardiomyocyte Fpassive in HFPEF than in HFREF or AS with a significant fall in Fpassive after PKG administration in all 3 conditions. C, Larger fall in cardiomyocyte Fpassive after PKG administration in HFPEF than in HFREF or AS. HFPEF indicates heart failure with preserved ejection fraction; HFREF, heart failure with reduced ejection fraction; AS, aortic stenosis; and PKG, protein kinase G.

Statistical Analysis

Values are given as mean±SEM. A 2-tailed test with a P value <0.05 was considered significant. Single comparisons were assessed by an unpaired Student t test. Bonferroni-adjusted t tests were used subsequent for multiple comparisons after ANOVA. If Levene test revealed unequal variances, unequal-variance t test or Welch’s ANOVA was used. The significance for categorical variables was determined by the Fisher’s exact test. Statistical analysis was performed with SPSS (Version 15.0; SPSS Inc, Chicago, IL).

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Force Measurements in Isolated Cardiomyocytes

Cardiomyocyte Fpassive was higher in HFPEF (7.6±0.4 kN/m²; n=18) than in AS (3.4±0.2 kN/m²; n=39; P<0.001) and in HFREF (5.1±0.2 kN/m²; n=22; P<0.001; Figure 1A and 1B). When patients were subdivided in accordance to absence or presence of DM (Table) (DM⁻, DM⁺), Fpassive was higher in HFPEF-DM⁻ than in AS-DM⁻ or HFREF-DM⁻ and also higher in HFPEF-DM⁺ than in AS-DM⁺ or HFREF-DM⁺ (Figure I in the online-only Data Supplement). After administration of PKG, Fpassive fell significantly in HFREF, HFPEF, and AS (Figure 1B). The fall in Fpassive was significantly larger in HFPEF than in AS or HFREF (Figure 1C). Fpassive of normal human cardiomyocytes is 2.8±0.1 kN/m² and remains unchanged after PKG administration (2.6±0.1 kN/m²).6,12
and also lower in HFPEF-DM$^+$ than in AS-DM$^+$ or HFREF-DM$^+$ (Figure I in the online-only data supplement).

**Myocardial Nitrotyrosine, proBNP-108, sGC, and PDE5A Expression**

Unequal nitrotyrosylation of proteins in HFPEF ($n=12$), AS ($n=16$), and HFREF ($n=18$) myocardium was investigated for proteins ranging from 240 kDa to 10 kDa. Strong differential nitrotyrosylation (HFPEF: $0.34\pm0.08$ a.u.; AS: $0.05\pm0.02$ a.u., $P<0.01$; HFREF: $0.10\pm0.03$ a.u., $P<0.01$) was observed in a 66-kDa protein (Figure 4A) and weak differential nitrotyrosylation in 210-kDa and 20-kDa proteins. None of these proteins was identified. Immunohistochemical determination of myocardial nitrotyrosine content was significantly higher in HFPEF ($3.94\pm0.24\%$) than in both AS ($3.26\pm0.13\%$, $P=0.05$) and HFREF ($2.64\pm0.24\%$, $P<0.001$; Figure 4B and 4C). When HFPEF, AS, and HFREF patients were pooled, myocardial nitrotyrosine content related to cardiomyocyte $F_{\text{passive}}$ ($r=0.68$, $P=0.016$).

Myocardial proBNP-108 expression was significantly lower in HFPEF ($0.22\pm0.07$ a.u.; $n=12$) than in HFREF ($0.76\pm0.26$ a.u.; $n=16$, $P<0.05$) and similar in HFPEF and AS ($0.18\pm0.05$ a.u.; $n=16$; Figure 5). Immunohistochemical analysis demonstrated similar myocardial expression levels of sGC (HFPEF: $3.67\pm0.38\%$, $n=4$; AS: $3.37\pm0.14\%$, $n=6$; HFREF: $3.93\pm0.34\%$, $n=4$) and PDE5A (HFPEF: $20.4\pm1.0\%$, $n=6$; AS: $21.4\pm0.8\%$, $n=7$; HFREF: $23.3\pm1.3\%$, $n=5$) in all groups.

**Histomorphometric Myocardial Analysis**

Cardiomyocyte diameter was similarly elevated in HFPEF ($28.1\pm0.2\ \mu m$, $n=12$) and AS ($28.7\pm0.3\ \mu m$, $n=18$), but...
lower in HFREF (24.7±0.3 μm; n=21; P<0.001 versus HFPEF). Collagen volume fraction was comparable in HFPEF (11.3±0.3%; n=31) and HFREF (12.2±0.4%; n=36) but higher in AS (14.1±1.1%; n=33; P=0.002 versus HFPEF).

Discussion

The present study reveals reduced PKG activity and lower cGMP concentration in concentrically remodeled LV myocardium of HFPEF patients than in concentrically remodeled myocardium of AS patients and than in eccentrically remodeled LV myocardium of HFREF patients. Reduced PKG activity and lower myocardial cGMP concentration in HFPEF did not result from altered myocardial sGC or PDE5A expression, which was similar in all groups nor from unequal natriuretic peptide (NP) expression, which was comparable in HFPEF and AS. The downregulated cGMP-PKG signaling in HFPEF was more likely related to low myocardial nitric oxide (NO) bioavailability because of high nitrosative/oxidative stress. High nitrosative/oxidative stress in HFPEF was inferred in the present study from the higher myocardial nitrotyrosine content in HFPEF than in both HFREF and AS. Lower PKG activity affected myocardial dysfunction in HFPEF as evident from the higher cardiomyocyte Fpassive in HFPEF and the correction of this higher Fpassive by in vitro administration of PKG.

Myocardial PKG Signaling in HFPEF

The present study is the first to comprehensively compare myocardial PKG activity, its downstream effects on cardiomyocyte stiffness and hypertrophy, and its upstream control by cGMP, sGC, PDE5A, nitrosative/oxidative stress, and proBNP-108 in different forms of human cardiac remodeling, consisting respectively of HFPEF- and AS-associated concentric remodeling and of HFREF-associated eccentric remodeling. Upreregulated PDE5A expression was previously observed in remodeled human myocardial tissue procured at cardiac surgery from hypertrophied right ventricles of congenital cardiac malformations or at cardiac transplantation from explanted dilated or ischemic cardiomyopathic hearts. Although PDE5A and sGC enzyme activities were not directly measured in the present study, the lower PKG activity and cGMP concentration in HFPEF than in AS or HFREF cannot be ascribed to higher expression of PDE5A nor to lower expression of sGC or proBNP-108. They therefore probably derived from lower myocardial NO bioavailability because of higher nitrosative/oxidative stress, which is known to directly impair NO-cGMP-PKG signaling. In the present study, higher nitrosative/oxidative stress was inferred from higher myocardial nitrotyrosine content in HFPEF than in both HFREF and AS. The higher myocardial nitrotyrosine content in HFPEF than in HFREF or AS, assessed by Western blot analysis and normalized for actin. B, Representative immunohistochemical images stained for nitrotyrosine in HFREF, HFPEF, and AS. C, Higher myocardial nitrotyrosine content assessed by quantitative immunohistochemistry in HFPEF than in HFREF or in AS. HFPEF indicates heart failure with preserved ejection fraction; HFREF, heart failure with reduced ejection fraction; and AS, aortic stenosis.
PKG Activity and Myocardial Diastolic Dysfunction in HFPEF

Initial clinical studies on the myocardial contractile effects of NO already observed NO to acutely improve LV relaxation and diastolic stiffness.26 This beneficial action of NO on diastolic LV function was explained by PKG-dependent phosphorylation of troponin-I or titin and PKG-independent phosphorylation of phospholamban. PKG-dependent phosphorylation of the giant cardiomyocyte cytoskeletal protein titin is of special interest as PKG-induced phosphorylation of its N2B segment lowers cardiomyocyte cytoskeletal protein titin of special interest as PKG-induced phosphorylation of its N2B segment lowers F_{passive} in human HF cardiomyocytes12 or in cardiomyopathic myocardial strips.13 The present study establishes lack of cardiomyocyte PKG activity as an important contributor to myocardial diastolic dysfunction in HFPEF as it observed lower myocardial PKG activity in HFPEF to correspond with higher cardiomyocyte F_{passive} and in vitro administration of PKG to acutely lower the high F_{passive} of HFPEF cardiomyocytes. Apart from PKG-dependent control of diastolic LV dysfunction in HFPEF, the present study also supports involvement of nitrosative/oxidative stress. Such involvement has also been suggested in recent experimental and clinical studies. Oxidative stress leads to formation of disulfide bridges within the titin molecule, which raises its overall stiffness.27 In a mouse HFPEF model, oxidative stress uncouples cardiac NOS and induces diastolic LV dysfunction.28 Finally, high plasma levels of methylated L-arginine metabolites were strongly related to diastolic LV dysfunction in patients with HFREF. Accumulation in circulating blood of methylated L-arginine metabolites mainly results from oxidative stress inhibiting their breakdown by dimethylarginine dimethylaminohydrolase.30

PKG Activity and Myocardial Remodeling in HFPEF

In LV cardiomyocytes cultured from neonatal rat hearts, NO or a cGMP analog attenuated the norepinephrine-induced hypertrophic response through modulation of Ca^{2+} influx.31 NO or a cGMP analog exerted these effects by raising PKG activity, which suppressed prohypertrophic calcineurin-NFAT signaling.32 These in vitro findings were in line with subsequent in vivo experiments in mice subjected to TAC.8 In these mice, sildenafil raised myocardial PKG activity and prevented or reversed cardiomyocyte hypertrophy and interstitial fibrosis by deactivating multiple prohypertrophic pathways.8 Furthermore, in a transgenic mouse model, cardiomyocyte-specific PDE5 overexpression induced an exaggerated hypertrophic and fibrotic response to LV pressure-overload, which was reversed by enhancing cGMP-PKG activity through suppressed PDE5 gene expression.33 The ability of cGMP-PKG activity to dampen maladaptive hypertrophy during LV pressure overload was, however, seriously challenged by recent experiments showing deletion of PKG to have no effect on maladaptive myocardial hypertrophy in mice subjected to TAC.34 The present study failed to observe relations between myocardial PKG activity and cardiomyocyte diameter or collagen volume fraction, when HFPEF patients were compared with HFREF and AS. This failure could relate to different prohypertrophic signaling pathways prevailing in the different conditions83 and to more frequent use in the present study of angiotensin converting enzyme inhibitors, angiotensin II receptor blockers, and aldosterone receptor antagonists in HF than in AS. The finding in AS of PKG activity corresponding with F_{passive} but not with cardiomyocyte diameter suggests cardiomyocyte diastolic stiffness to evolve independently from cardiomyocyte hypertrophy. This concept also emerged from the VALIIDD trial, in which only 3% of hypertensives had significant LV hypertrophy despite all having diastolic LV dysfunction.36

Therapeutic Implications

Treatment of HFPEF remains uncertain as many large outcome trials yielded neutral results. These neutral results were variably explained by flawed trial design and testing of treatment paradigms extrapolated from HFREF.3 By observing lower PKG activity and higher nitrotyrosine level in HFPEF myocardium, the present study supports use in HFPEF of NO-donors, PDE5 inhibitors, and drugs with antioxidative properties such as statins. Acute administration of NO donors is known to ameliorate diastolic LV function,26 whereas chronic use of isosorbide dinitrate combined with the antioxidant hydralazine improved outcome in V-HeFT I and A-HeFT trials.37,38 The clinical characteristics of the A-HeFT HFREF patients revealed a high prevalence of obesity and diabetes mellitus.39 HFPEF patients have a similar clinical profile, and the present study provides direct evidence for high myocardial oxidative stress in HFPEF. Combined use of isosorbide dinitrate and the antioxidant hydralazine could therefore be potentially favorable in HFPEF. In HFREF, acute administration of sildenafil ameliorated cardiac performance by lowering LV load,40 and in both HFREF and HFPEF chronic administration ameliorated diastolic LV dysfunction.10,11 The effect on diastolic LV dysfunction contributed, respectively, to improved clinical status in HFREF and reduced pulmonary hypertension in HFPEF. Acute administration of sildenafil was recently also reported to lower diastolic LV stiffness and to increase myocardial titin phosphorylation in an old hypertensive HFPEF dog model,9 and chronic administration of sildenafil was previously demonstrated to improve TAC-induced concentric LV remodeling in mice.5 Because of these encouraging clinical and experimental results, sildenafil has been proposed as a potential therapy for HFPEF.2,35 The present study, however, suggests no superior effectiveness of sildenafil in HFPEF as myocardial expression of PDE5A was similar in all patient groups. In the present study, myocardial proBNP-108 expression was comparable between HFPEF and AS and higher in HFREF than in both HFPEF and AS. These observations are
consistent with proBNP-108 expression being regulated by diastolic LV wall stress, which is higher in HFREF because of eccentric LV remodeling and similar in HFPEF and AS because of concentric LV remodeling. Because of comparable proBNP-108 expression in HFPEF and AS, BNP is unlikely to account for the widely different PKG activities and cGMP concentrations observed in both conditions, which were therefore attributed to higher nitrosative/oxidative stress in HFPEF than in AS. Lower proBNP-108 expression in HFPEF than in HFREF also explains the low positive predictive value of BNP for the diagnosis of HFPEF. Acute BNP administration was recently reported to lower diastolic LV stiffness and to increase myocardial titin phosphorylation in an old hypertensive HFPEF dog model but failed to improve clinical end points in acutely decompenated HF patients with LVEF ≥40%.43

Finally, the present study supports use of statins in HFPEF because of their antioxidative properties. In experimental hypertensive HF, statins were known to exert a variety of favorable actions on concentric myocardial remodeling such as regression of LV hypertrophy and prevention of myocardial fibrosis. Moreover, usefulness of statins in HFPEF was already demonstrated in a preliminary study that showed statins to lower mortality with a relative risk reduction of 22%.45

Limitations
The in vivo relevance of low myocardial PKG activity for diastolic LV dysfunction in HFPEF is currently only supported by the lower diastolic LV stiffness observed in an old hypertensive HFPEF dog model after administration of sildenafil or BNP and still needs to be clinically confirmed by mechanistic studies and large outcome trials.

Myocardial ischemia was excluded only by absence of significant (>50%) epicardial coronary artery stenosis. In a concentrically remodeled left ventricle, which was present in both HFPEF and AS patients, absence of significant coronary artery stenosis does not exclude subendocardial ischemia at the site of biopsy procurement. Subendocardial ischemia resulting from concentric LV remodeling is, however, an unlikely cause for the higher F\textsubscript{passive} in HFPEF because AS patients had a higher LV mass index/LV end-diastolic volume index ratio but a lower F\textsubscript{passive} than HFPEF patients.

Because of the higher prevalence of DM in the HFPEF patients, higher F\textsubscript{passive} and lower myocardial PKG activity or cGMP concentration could have resulted from DM-induced upregulation of oxidative stress. In a subset analysis of DM\textsuperscript{−} and DM\textsuperscript{+} patients, the findings in the DM\textsuperscript{−} patients, however, resembled the overall results. This confirms the higher F\textsubscript{passive} and lower PKG activity or cGMP concentration in HFPEF to be associated with the HFPEF phenotype and not with a higher prevalence of DM in the HFPEF group. Furthermore, the findings in the DM\textsuperscript{+} patients also resembled the overall results apart from low PKG activity in the AS-DM\textsuperscript{+} patients. The latter finding was, however, consistent with a recent report on DM worsening diastolic LV dysfunction and raising F\textsubscript{passive} in aortic stenosis.15

Finally, immunohistochemical quantification of PKG activity using pVASP phosphorylated at Ser\textsuperscript{239} lacks specificity as Ser\textsuperscript{239} can also be phosphorylated by PKA. It therefore only provides confirmatory evidence of the PKG enzyme activity measurements performed on tissue homogenates.

Conclusions
HFPEF myocardium is characterized by downregulated NO-cGMP-PKG signaling probably resulting from high nitrosative/oxidative stress. Low PKG activity raises cardiomyocyte stiffness and is a potential target for a specific HFPEF treatment strategy.

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Disclosures
None.

References


CLINICAL PERSPECTIVE

Large trials testing modern heart failure pharmacotherapy in heart failure with preserved ejection fraction (HFPEF) had a neutral outcome, probably because of failure to address features of myocardial dysfunction specific for heart failure with preserved ejection fraction such as high-diastolic left ventricular stiffness. The latter results not only from myocardial fibrosis, but also from high cardiomyocyte stiffness, which is linked to hypophosphorylation of the cytoskeletal protein titin and corrected by in vitro administration of protein kinase G (PKG). In vivo administration of sildenafil, which raises myocardial PKG activity by inhibiting breakdown of cyclic guanosine monophosphate, similarly improves diastolic left ventricular stiffness in a HFPEF dog model and HFPEF patients with pulmonary hypertension. Because of these findings and the ongoing RELAX trial testing sildenafil in HFPEF, the present study compared PKG activity, its downstream effects, and its upstream controls in left ventricular endomyocardial biopsies of patients with HFPEF, HF with reduced ejection fraction, and aortic stenosis. Patients were free of coronary artery disease, but more HFPEF patients were obese or had diabetes mellitus. The lowest myocardial PKG activity was observed in HFPEF and associated with the highest cardiomyocyte stiffness, the lowest myocardial cyclic guanosine monophosphate concentration, and the highest myocardial nitrosative/oxidative stress. The high prevalence among HFPEF patients of metabolic disorders probably accounted for their high myocardial nitrosative/oxidative stress, which led to high cardiomyocyte stiffness through a cascade of derangements consisting of low cyclic guanosine monophosphate concentration, low PKG activity, and reduced phosphorylation of titin by PKG. Metabolic derangements therefore underlie diastolic left ventricular dysfunction in HFPEF and should be targeted in future HFPEF treatment strategies.
Low Myocardial Protein Kinase G Activity in Heart Failure With Preserved Ejection Fraction


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SUPPLEMENTAL MATERIAL

Low Myocardial Protein Kinase G Activity in Heart Failure with Preserved Ejection Fraction

by Van Heerebeek L. et al.

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Suppl. Figure

A

DM-

F_{\text{Passive}} (kN/m^2)

p<0.05  p<0.001

HFREF HFPEF AS

B

DM+

F_{Passive} (kN/m^2)

p<0.05  p<0.01

HFREF HFPEF AS

C

PKG enzyme activity (pmol/min/mg protein)

p<0.01  p<0.001

HFREF HFPEF AS

D

PKG enzyme activity (pmol/min/mg protein)

p<0.01  p<0.001

HFREF HFPEF AS

E

cGMP (pmol/ml)

p<0.001  p<0.001

HFREF HFPEF AS

F

cGMP (pmol/ml)

p<0.01  p<0.001

HFREF HFPEF AS
Supplemental Figure: Patients are subdivided in accordance to absence (DM−) or presence (DM+) of diabetes mellitus (HFPEF-DM−: n=19; HFPEF-DM+: n=17; HFREF-DM−: n=30; HFREF-DM+: n=13; AS-DM−: n=50; AS-DM+: n=17). HFPEF-DM− patients had higher cardiomyocyte $F_{\text{passive}}$ and lower myocardial PKG enzyme activity or cGMP concentration than AS-DM− or HFREF-DM− patients. HFPEF-DM+ patients had higher cardiomyocyte $F_{\text{passive}}$ and lower cGMP concentration than AS-DM+ or HFREF-DM+ patients. HFPEF-DM+ patients also had lower PKG enzyme activity than HFREF-DM+ patients.