Myocardial Titin Hypophosphorylation Importantly Contributes to Heart Failure with Preserved Ejection Fraction in a Rat Metabolic Risk Model

by

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ABSTRACT

**Background**—Obesity and diabetes are important metabolic risk factors and frequent comorbidities in heart failure with preserved ejection fraction (HFPEF). They contribute to myocardial diastolic dysfunction (DD) through collagen deposition or titin modification. The relative importance for myocardial DD of collagen deposition and titin modification was investigated in obese, diabetic ZSF1 rats after HFPEF development at 20 weeks of age.

**Methods and Results**—Four groups or rats (Wystar Kyoto, n=11; lean ZSF1, n=11; obese ZSF1, n=11 and obese ZSF1 with high fat diet, n=11) were followed over 20 weeks with repeat metabolic, renal and echocardiographic evaluations and hemodynamically assessed at sacrifice. Myocardial collagen, collagen crosslinking, titin isoforms and phosphorylation were also determined. Resting tension (F\textsubscript{passive})-sarcomere length relations were obtained in small muscle strips before and after KCl-KI treatment, which unanchors titin and allows contributions of titin and extracellular-matrix to F\textsubscript{passive} to be discerned. At 20 weeks of age, the lean ZSF1 group was hypertensive whereas both obese ZSF1 groups were hypertensive and diabetic. Only the obese ZSF1 groups had developed HFPEF, which was evident from increased lung weight, preserved LVEF and LV DD. The underlying myocardial DD was obvious from high muscle strip stiffness, which was largely (±80%) attributable to titin hypophosphorylation. The latter occurred specifically at the S3991 site of the elastic N2B segment and at the S12884 site of the PEVK segment.

**Conclusions**—Obese ZSF1 rats developed HFPEF over a 20 weeks time span. Titin hypophosphorylation importantly contributed to the underlying myocardial DD.

**Key Words**: diastole; heart failure; diabetes mellitus; obesity; myocardium
INTRODUCTION

Heart failure with preserved ejection fraction (HFPEF) is currently observed in 50% of all heart failure patients. The incidence of HFPEF relative to heart failure with reduced ejection fraction (HFREF) continues to rise and its prognosis fails to improve partly because of lack of a specific HFPEF therapy.

Prevalence of comorbidities is higher in HFPEF than in HFREF. Comorbidities such as obesity and diabetes mellitus (DM) are key constituents of metabolic risk and known to be associated with the progressive left ventricular (LV) remodeling and dysfunction characteristically observed in HFPEF. In HFPEF, body mass index has a U-shaped relation to mortality in contrast to HFREF where it displays an inverse relation with mortality. DM has long time been recognized to be associated with LV diastolic dysfunction. In HFPEF, HFREF and aortic stenosis (AS), DM worsens diastolic LV stiffness through a variety of mechanisms such as myocardial fibrosis, advanced glycation endproducts (AGEs) deposition and high cardiomyocyte stiffness. High cardiomyocyte stiffness was especially evident in HFPEF and AS patients with DM, was associated with hypophosphorylation of the giant cytoskeletal protein titin and corrected in-vitro by administration of protein kinase A (PKA) or G (PKG). Furthermore, patients with high metabolic risk frequently suffer from salt-sensitive hypertension, which is like obesity associated with systemic oxidative stress.

To elucidate the mechanisms underlying myocardial dysfunction in metabolic riskrelated HFPEF, the present study investigated: 1) LV hemodynamics; 2) myocardial histology; 3) in-vitro stiffness of small muscle strips; 4) cardiomyocyte stiffness and 5) myocardial titin phosphorylation in hypertensive ZSF1 rats, which became over a 20 weeks period morbidly obese and diabetic because of absence of satiation and unlimited access to a regular (ZSF1-obese) or high-fat diet (ZSF1-obese+HFD).

METHODS

An expanded Methods section is available in the Online Data Supplement.
Animal model
Nine-week old male ZSF1 lean (ZSF1-Lean, n=11), ZSF1 obese (ZSF1-Obese, n=22) and Wistar-Kyoto rats (WKY, n=11) were obtained from Charles River (Barcelona, Spain) and fed with Purina Diet (#5008). After a 1 week laboratory adaptation period, animals underwent phenotypic evaluation consisting of metabolic cage studies, blood sample collection and echocardiographic evaluation. To assess diastolic function, peak velocity of early (E) and late (A) mitral inflow signals and the ratio of E over E’ (peak velocity of early diastolic lateral mitral annular motion) were measured as an indication of LV pressure. From this point onward, a subgroup of ZSF1 obese rats (ZSF1-Obese+HFD, n=11) was randomly allocated to receive HFD (Research Diet Inc. #D12468). Weight gain and energy intake were recorded every third day. Phenotypic evaluation was repeated at the 14th and 18th week of life.

At 20 weeks of age, animals underwent hemodynamic evaluation under anesthesia and were subsequently sacrificed with procurement of myocardial tissue samples for histological, biochemical and biomechanical studies. Animals were kept in individually ventilated chambers in a controlled environment with a 12-h-light/-dark cycle at 20°C room temperature and had unlimited access to food. Investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the NIH (NIH Publication no. 85–23, revised 1996) and was approved by the ethics committee of the Faculty of Medicine of Porto.

Histology and Collagen Gene Expression
Collagen volume fraction (CVF) was determined by quantitative morphometry with an automated image analysis system in sections stained with collagen-specific picrosirius red.8,9 A sircol-based assay was performed to obtain and quantify total, soluble and insoluble collagen, which was calculated by subtracting the amount of soluble collagen from the amount of total collagen. The degree of cross-linking was calculated as the ratio between soluble and insoluble collagen. Gene expression of Collagen1A1 and Collagen3A1 was performed using real-time PCR.

Force measurements on small muscle strips and cardiomyocytes
Cardiomyocytes and muscle strips were incubated respectively for 5 and 30 minutes in relaxing solution supplemented with 0.2 % TritonX-100 to remove all membrane structures and subsequently attached between a force transducer and
length motor. Resting tension ($F_{\text{passive}}$) was recorded between 1.9 and 2.3 m sarcomere length (SL). $F_{\text{passive}}$ of cardiomyocytes was measured before and after PKG incubation. In muscle strips, thick and thin filaments were extracted by immersing the preparation in relaxing containing 0.6M KCl (45 min at 20°C) followed by relaxing solution containing 1.0 min at 20°C). Following the extraction procedure, the muscle bundles were stretched again and the passive force remaining after KCl/KI treatment was assumed to be extracellular matrix-based. Titin-based passive force was determined as total passive force minus extracellular matrix-based passive force.\(^{14}\)

**Titin analysis**

*Titin isoform separation*

Homogenized myocardial samples were analyzed by 1.8% sodium dodecyl-sulfate polyacrylamide-gel electrophoresis (SDS-PAGE). Protein bands were visualized by Coomassie blue or SYPRO Ruby, scanned, and analyzed densitometrically.\(^{15,16}\)

*Titin phosphorylation assays*

Following 1.8% SDS-PAGE, gels were stained with Pro-Q Diamond for 1 hour and subsequently with Sypro Ruby overnight. Phosphorylation signals on Pro-Q Diamond-stained gels were indexed to Sypro Ruby-stained titin signals.\(^{9,10,15,16}\)

*Titin/Phosphotitin immunoblots*

1.8% SDS-PAGE and Western blot were performed to measure site-specific phosphorylation and expression of titin using custom-made, affinity-purified, phosphosite-specific antibodies against phospho-S3991 (N2Bus), phospho-S12742 (PEVK) and phospho-S12884 (PEVK) (positions in mouse (Mus musculus) titin according to UniProtKB identifier A2ASS6), and antibodies recognizing the corresponding nonphosphorylated sequence around these sites.\(^{16}\)

**Statistical analysis**

Groups were compared by two-way repeated measures ANOVA whenever serial acquisitions were obtained for the same animal, and by one-way ANOVA for single acquisitions. Pressure-volume loop analysis was analyzed using LabChart 7 Pro v7.3.1. Values are given as mean ± SEM. A 2-tailed test with a probability of 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 repeated measure ANOVA. Statistical analysis was performed with SPSS (Version 15.0; SPSS Inc, Chicago, Ill).

RESULTS

Cardiometabolic risk in obese ZSF1 rats
ZSF1-Obese and ZSF1-Obese+HFD rats had higher weight gain at 20 weeks of age (Figure 1A). Energy intake was initially also higher in these animals, but leveled off at 20 weeks of age (Figure 1A). In both obese groups, glycemia levels, glucose tolerance and insulin resistance were higher (Figure 1B, Table 1). Hyperglycemia caused glycosuria, increased urine output and compensatory water intake. Proteinuria suggested presence of diabetic nephropathy despite preserved creatinine clearance and plasma protein levels (Table 1).

![Figure 1](image)

**Figure 1.** Body weight, energy intake and metabolism. **A.** Body weight and energy intake in all groups. **B.** Glycemia, oral glucose tolerance (OGT) and insulin resistance (IR) in all groups at 10, 14 and 18 weeks of age. *P<0.05 vs. WKY, †P<0.05 vs. ZSF1-Lean, ‡P<0.05 vs ZSF1-Obese.

Echocardiography, hemodynamics and morphometrics
Serial echocardiographic studies at 10, 14 and 18 weeks of age demonstrated normal systolic function in all groups (Figure 2A, 2B and Table 2). Concentric LV
remodeling was present throughout the entire study in both obese groups (Figure 2A, 2B and Table 2), which progressively developed diastolic LV dysfunction, evident from a restrictive LV-inflow signal, higher E/E’ and increased left atrial area (LAA) at 14 and 18 weeks (Figure 2B and Table 2).

At 20 weeks of age, hemodynamic evaluation confirmed normal LV systolic performance, evident from LVEF, LVdP/dtmax and ESPVR EESI (Figure 2C and Table 3). Diastolic LV dysfunction was again evident from a prolonged, elevated LVEDP, an upward shift of the LV diastolic pressure-volume relationship and a higher LV diastolic chamber stiffness constant (β) (Figure 2C and Table 3). At sacrifice, lung and heart weights were increased in both obese groups (Figure 3A and Table 4). There was evidence of visceral adiposity with more perirenal and perigonadal fat (Table 4).

**Histology**

Cardiomyocyte hypertrophy was confirmed histologically in both ZSF1-Obese groups (Figure 3B). Collagen volume fraction, collagen cross-linking, procollagen carboxyl-terminal proteinase type I (PCP) and PCP enhancer (PCPE) were similar in all groups (Figure 4). In line with these findings, the relative mRNA expression of collagen 1A1 and collagen 3A3 were also similar among all groups (Figure 4). No significant differences of Lysyl oxidase (LOX) expression were observed between all groups (WKY (1.75±0.44), ZSF1-Lean (1.76±0.19), ZSF1-Obese (1.50±0.35) and ZSF1-Obese+HFD (1.70±0.12)).

**F_{passive} in small muscle strips and cardiomyocytes**

The relative contributions of collagen and titin were determined in small muscle strips (Figure 5). F_{passive}-SL relations were constructed for SL ranging from 1.9 to 2.3μm. F_{passive} was higher in both obese groups from a SL of 2.075μm onwards (Figure 5A). To discern the contribution of extracellular matrix (E-matrix), F_{passive}-SL relations were also constructed following extraction with KCl/KI (Figure 5B). The contribution of titin was calculated by subtracting at each SL E-matrix based F_{passive} from total F_{passive} (Figure 5C). F_{passive} attributable to E-matrix and titin were higher in both obese groups respectively from a SL of 2.175 and 2.025μm onwards. At the upper limit of the physiological SL-range (2.2μm) titin accounted for 82 and 78% of F_{passive} in respectively ZSF1-Obese and ZSF1-Obese+HFD groups.

F_{passive}-SL relations of isolated skinned cardiomyocytes were steeper in ZSF1-Obese and ZSF1-Obese+HFD (Figure 5D). Incubation with PKG returned the


F_{passive-SL} relations to control levels (Figure 5D). No significant differences of active tension were observed between groups in single skinned small strips as well as skinned cardiomyocytes.

**Titin hypophosphorylation**

N2B titin isoform expression relative to WKY was similar in all groups (WKY 100±9.4%; ZSF1-Lean 100.5±9.1%; ZSF1-Obese 95.03±9.6%; ZSF1-Obese+HFD 71.58±11.4%), but titin phosphorylation decreased by 67 and 82% in ZSF1-Obese and ZSF1-Obese+HFD rats respectively (Figure 6A). Ex vivo phosphorylation by PKG significantly increased all-titin phosphorylation in ZSF1-Obese and ZSF1-Obese+HFD, up to the level measured in WKY and ZSF1-Lean (Figure 6B). Using affinity-purified phosphospecific antibodies phosphorylation status was assessed by Western blot at a conserved serine within the N2B segment (S3991 of full-length mouse titin) and at two conserved serines within the PEVK segment (S12742 and S12884) (Figure 6C, D and E). In the obese groups, significant hypophosphorylation was observed at the phospho-N2B S3991 site and at the phospho-PEVK S12884 site but comparable phosphorylation at the phospho-PEVK S12742 site. Protein loading was checked by a sequence-specific antibody that corresponded with the phosphospecific antibody.

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Figure 4. Collagen-volume fraction and cross-linking. A. Representative images of myocardial fibrosis (Picrosirius red; 200x magnification) in all groups. B-G. Collagen volume fraction, collagen cross-linking, procollagen carboxyl-terminal proteinase type I (PCP), PCP enhancer (PCPE), relative mRNA expressions of Collagen1A1 and Collagen3A1 in all groups.
Table 3. Hemodynamics

<table>
<thead>
<tr>
<th></th>
<th>WKY</th>
<th>ZSF1-Lean</th>
<th>ZSF1-Obese</th>
<th>ZSF1-Obese+HFD</th>
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<tr>
<td>BSA (cm²)</td>
<td>468 ± 5</td>
<td>508 ± 7*</td>
<td>651 ± 5†</td>
<td>616 ± 4‡</td>
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<td>SAP (mmHg)</td>
<td>117 ± 4</td>
<td>146 ±6*</td>
<td>181 ± 6†</td>
<td>170 ± 6†</td>
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<td>MAP (mmHg)</td>
<td>94 ± 6</td>
<td>127 ± 6*</td>
<td>149 ± 5†</td>
<td>140 ± 5*</td>
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<td>DAP (mmHg)</td>
<td>75 ± 7</td>
<td>106 ± 7*</td>
<td>125 ± 6*</td>
<td>113 ± 5†</td>
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<td>HR (bpm)</td>
<td>346 ± 10</td>
<td>391 ± 9*</td>
<td>354 ± 12†</td>
<td>336 ± 8†</td>
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<td>EDP (mmHg)</td>
<td>5 ± 0</td>
<td>4 ± 1</td>
<td>9 ± 1‡</td>
<td>7 ± 1†</td>
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<td>dP/dt max (mmHg.s⁻¹)</td>
<td>9430 ± 770</td>
<td>11700 ± 964</td>
<td>13000 ± 680</td>
<td>11000 ± 497</td>
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<tr>
<td>dP/dt min (mmHg.s⁻¹)</td>
<td>-7880 ± 838</td>
<td>-12600 ± 686</td>
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<td>τ (ms)</td>
<td>8.3 ± 0.3</td>
<td>7.6 ± 0.4</td>
<td>10.5 ± 0.6†</td>
<td>9.5 ± 0.4†</td>
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<tr>
<td>EF (%)</td>
<td>60 ± 4</td>
<td>55 ± 3</td>
<td>59 ± 4</td>
<td>63 ± 3</td>
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<td>ESPVR E₀,I (mmHg. µL⁻¹.cm⁻²)</td>
<td>0.64 ± 0.15</td>
<td>2.49 ± 0.71*</td>
<td>2.00 ± 0.39*</td>
<td>2.04 ± 0.39³</td>
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<tr>
<td>EDPVR β I (µL⁻¹.cm⁻²)</td>
<td>0.016 ± 0.002</td>
<td>0.023 ± 0.002</td>
<td>0.028 ± 0.002*</td>
<td>0.029 ± 0.004*</td>
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BSA, body surface area; SAP, systolic arterial pressure; MAP, mean blood pressure; DAP, diastolic arterial pressure; HR, heart rate; EDP, end-diastolic pressure; dP/dt max, maximum rate of pressure rise; dP/dt min, maximum rate of pressure fall; τ, time constant of isovolumetric relaxation; EF, ejection fraction; E₀,I, slope of linear ESPVR for indexed volumes; EDPVR, end-diastolic pressure-volume relationship; β I, chamber stiffness constant for indexed volumes, derived from exponential EDPVR. For the purpose of volume indexation, BSA was estimated as 9.1*(body weight in g)²/³. Values are mean ± SEM, n=11, each group. *P<0.05 vs WKY; †P<0.05 vs ZSF1-Lean; ‡P<0.05 vs ZSF1-Obese.

Table 4. Morphometrics

<table>
<thead>
<tr>
<th></th>
<th>WKY</th>
<th>ZSF1-Lean</th>
<th>ZSF1-Obese</th>
<th>ZSF1-Obese+HFD</th>
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<tr>
<td>TL (mm)</td>
<td>41.5 ± 0.1</td>
<td>42.1 ± 0.4</td>
<td>40.9 ± 0.4†</td>
<td>39.6 ± 0.3‡</td>
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<tr>
<td>LV+IVS weight/TL (mg.mm⁻³)</td>
<td>15.4 ± 0.6</td>
<td>16.6 ± 0.8</td>
<td>19.7 ± 1.1†</td>
<td>20.2 ± 1.3³</td>
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<td>RV weight/TL (mg.mm⁻³)</td>
<td>4.8 ± 0.4</td>
<td>4.5 ± 0.3</td>
<td>5.5 ± 0.3</td>
<td>5.6 ± 0.2†</td>
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<td>Lung weight/TL (mg.mm⁻³)</td>
<td>51 ± 2</td>
<td>53 ± 2</td>
<td>75 ± 3†</td>
<td>75 ± 2†</td>
</tr>
<tr>
<td>Liver weight/TL (mg.mm⁻³)</td>
<td>252 ± 9</td>
<td>314 ± 12</td>
<td>906 ± 55†</td>
<td>769 ± 29†</td>
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<tr>
<td>Kidney weight/TL (mg.mm⁻³)</td>
<td>23.3 ± 0.3</td>
<td>26.0 ± 0.8</td>
<td>37.1 ± 0.9*</td>
<td>33.9 ± 1.5†</td>
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<tr>
<td>Perirenal fat weight/TL (mg.mm⁻³)</td>
<td>64 ± 4</td>
<td>58 ± 6</td>
<td>362 ± 12†</td>
<td>415 ± 14†</td>
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<tr>
<td>Perigonadal fat weight/TL (mg.mm⁻³)</td>
<td>62 ± 3</td>
<td>56 ± 5</td>
<td>161 ± 6†</td>
<td>145 ± 5‡</td>
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<tr>
<td>Gastrocnemius weight/TL (mg.mm⁻³)</td>
<td>56 ± 1</td>
<td>63 ± 2</td>
<td>53 ± 1†</td>
<td>48 ± 1†</td>
</tr>
</tbody>
</table>

TL, tibial length; LV, left ventricle; IVS, interventricular septum; RV, right ventricle. Values are mean ± SEM, n=11, each group. *P<0.05 vs WKY; †P<0.05 vs ZSF1-Lean; ‡P<0.05 vs ZSF1-Obese.
DISCUSSION

The present study identified cardiac titin hypophosphorylation to be associated with high myocardial stiffness and HFPEF in an obese ZSF1 rat model with high metabolic risk.
Metabolic risk-related HFPEF model
At the time of sacrifice at 20 weeks of age, high metabolic risk was clearly evident in the obese ZSF1 rats fed either regular diet or high fat diet. Compared to lean ZSF1 rats or WKY rats, obese ZSF1 rats showed many features of high metabolic risk such as visceral obesity evident from elevated perirenal and perigonadal fat, insulin resistance, hyperglycemia and physical inactivity evident from striated muscle wasting. Arterial blood pressure was elevated in both obese and lean ZSF1 rats. At the time of sacrifice, HFPEF was however only present in the obese ZSF1
rats and high metabolic risk therefore seemed to be a prerequisite in this model for HFPEF development. As such, the current model differs from previous experimental HFPEF models, which largely disregarded metabolic risk as they were carried out in old, hypertensive dogs or in Dahl salt sensitive hypertensive rats. The current model however closely resembles clinical HFPEF where metabolic risk is highly prevalent as evident from numerous HFPEF registries or large outcome trials.

The HFPEF presentation observed in this metabolic risk model also shares characteristic features with clinical HFPEF presentation. After 18 weeks, during closed chest echocardiographic evaluation, the E/E’ ratio was diagnostic of diastolic LV dysfunction (ZSF1-Obese: 17.2±0.8; ZSF1-Obese+HFD: 15.8±1.1). At sacrifice, lung weight was 60% higher in obese animals. The latter probably resulted from episodes of pulmonary edema occurring during physical activity. A similar situation occurs in HFPEF patients who frequently have moderate abnormalities in diastolic LV function at rest but striking elevations of left ventricular filling pressure during exercise because of a steep diastolic LV pressure-volume relation. Steep diastolic LV pressure-volume and myocardial Fpassive-length relations were also present in the ZSF1 obese rats. The limited elevation of LVEDP in ZSF1 obese rats during open chest hemodynamic evaluation probably resulted from a reverse effect: thoracotomy and anaesthesia reduced venous return to the heart, which led to a prompt fall in LV filling pressures because of steep diastolic LV pressure-volume and myocardial Fpassive-length relations. Apart from elevated E/E’, high diastolic LV chamber stiffness and high myocardial stiffness, obese ZSF1 rats also had other evidence of diastolic LV dysfunction such as progressive LA enlargement and a significant increase in t. The latter could however also be partially accounted for by the higher SAP in ZSF1-obese rats.

Systolic LV function in the ZSF1 obese rats closely resembled systolic LV function of HFPEF patients as global indices of LV systolic performance (LV dP/dtmax, LVEF and Ees) were all preserved. In ZSF1 obese rats, Ees was even higher than in control WKY rats because of a steep end-systolic LV pressure-volume relation. The simultaneous presence of steep end-systolic and end-diastolic LV pressure-volume relations forces the left ventricle to function as a fixed stroke volume pump and explains the swings from pulmonary edema to low output frequently observed in HFPEF patients.
Titin versus extracellular-matrix

ZSF1-Obese rats had a steeper myocardial $F_{\text{passive}}$-sarcomere length relation (Figure 5A). After extraction of the cardiac muscle strips with KCl/KI, which depolymerised thick and thin filaments thereby leaving titin unanchored, the contribution of the E-matrix to myocardial $F_{\text{passive}}$ became evident (Figure 5B). Subsequently, the contribution of titin could be calculated by subtracting at each SL the contribution of the E-matrix from the measured $F_{\text{passive}}$ (Figure 5C). For SLs ranging from 2.0 to 2.2μm, the contribution of titin greatly exceeded the contribution of the E-matrix. At 2.0μm, the contribution of titin was 6.8 and 9.3 times larger than of E-matrix for ZSF1-obese and ZSF1-obese+HFD rats. At 2.2μm, the contribution of titin was still 4.6 and 3.6 times larger. SLs ranging from 2.0 to 2.2μm covered the physiological range of LV filling pressures (from 5 to 40 mmHg). Using a thick wall ellipsoidal model of the LV and the measured LVEDP of 5 mmHg (Table 3; WKY rats), the calculated LV end-diastolic wall stress ($\approx 1.25$ kN/m$^2$) corresponded with measured $F_{\text{passive}}$ (1.26 kN/m$^2$) at a 2.0μm SL. Similarly, after adjusting the values of LVEDVI and dLVPW-thickness for a 2.2μm SL and substituting LV end-diastolic wall stress by the measured $F_{\text{passive}}$ at 2.2μm SL (13.70 kN/m$^2$ in ZSF1-obese+HFD), the same thick wall ellipsoidal model yielded a LVEDP of 41 mmHg. Hence, up to filling pressures exceeding 40 mmHg, titin accounted for 82 and 78% of $F_{\text{passive}}$ in ZSF1-obese and ZSF1-obese+HFD rats respectively. High titin-based stiffness is therefore the main contributor to high myocardial stiffness and likely also to HFPEF development in this metabolic risk-related rat HFPEF model. The importance of intrinsic cardiomyocyte $F_{\text{passive}}$-SL relation of isolated skinned cardiomyocytes (Figure 5D), which was steeper and shifted upward in both ZSF1- obese and ZSF1-obese+HFD rats. Furthermore, in-vitro administration of PKG to the isolated cardiomyocytes corrected the $F_{\text{passive}}$-SL relations. This in-vitro reversibility suggests the high $F_{\text{passive}}$ of cardiomyocytes of obese ZSF1 rats to result more from altered phosphorylation status, than from structural changes of titin, such as isoform shifts or oxidative damage. In-vitro reversibility of high $F_{\text{passive}}$ was also observed in the ZSF1-obese+HFD rats, which were exposed to the highest systemic oxidative stress.

Limited involvement of the E-matrix in the high $F_{\text{passive}}$ of the obese ZSF1 rats was evident also from histological/biochemical analyses of myocardial tissue. Global myocardial collagen volume fraction, collagen cross linking, collagen 1A1 or collagen 3A3 gene expression and PCP or PCPE activity were unaltered in the obese ZSF1 rats (Figure 4). Despite these findings, there was a small increase in
myocardial $F_{\text{passive}}$ attributable to the E-matrix at SL $> 2.175 \mu m$ in ZSF1-obese and ZSF1-obese+HFD rats (Figure 5B). This increase could have resulted from subtle alterations in endomysial collagen which remained undetected by histological analysis or by components of the E-matrix other than collagen.

**Titin hypophosphorylation**

Titin stiffness can be modulated mainly through isoform shifts or alterations of the phosphorylation status. In patients presenting with eccentric LV remodeling after myocardial infarction or with dilated cardiomyopathy, a titin isoform shift from the stiff N2B to the compliant N2BA isoform has been reported. In patients with concentric LV remodeling related to HFPEF or AS, most studies failed to observe a major shift in titin isoform expression. Rat hearts predominantly express N2B titin isoform, the proportion of which remained unaffected in the present study by the concentric LV remodeling observed in lean and obese ZSF1 rats.

As previously observed in HFPEF patients, AS patients with type 2 diabetes and old hypertensive dogs with HFPEF, overall N2B titin isoform phosphorylation was greatly reduced in the obese ZSF1 rats, especially when exposed to HFD (Figure 6A). Using site-specific antibodies, the S3991 site and the S12884 of the N2Bus and PEVK segments of titin were identified as being hypophosphorylated (Figure 6C and E). The S3991 site can be phosphorylated by both PKA and ERK2 (extracellular regulated kinase 2) and was recently also shown to be hypophosphorylated in old hypertensive HFPEF dogs. Phosphorylation of the S12742 site of the PEVK segment was unaltered in contrast to old hypertensive HFPEF dogs where it was hyperphosphorylated. Increased phosphorylation of the N2Bus segment is reported after PKA or PKG administration and shown to lower $F_{\text{passive}}$ whereas increased phosphorylation of the S12742-PEVK occurs after PKC administration and raises $F_{\text{passive}}$. Both PKC and CaMKII can phosphorylate the S12884-PEVK site. Phosphorylation of the S12884-PEVK site by CaMKII leads to a reduction of $F_{\text{passive}}$. The elevated $F_{\text{passive}}$ observed in the present study in the obese ZSF1 rats is consistent with the observed hypophosphorylation of the S3991 site within the N2Bus segment and the observed hypophosphorylation of the S12884 site within the PEVK segment.
CONCLUSIONS

Obese ZSF1 rats with a high metabolic risk profile developed HFPEF at 20 weeks of age. The diagnosis of HFPEF was based on lung congestion, preserved global LV systolic function and diastolic LV dysfunction. The latter was evident from elevated E/E', LA enlargement, high LV diastolic chamber stiffness and high myocardial stiffness. High myocardial stiffness was largely (±80%) attributable to high cardiomyocyte stiffness, which resulted from hypophosphorylation of titin.

SOURCES OF FUNDING

Supported by a grant from the Dutch Heart Foundation (2006B035)(W.J.P.) and from the European Commission (FP7-Health-2010; MEDIA-261409)(W.J.P.)

DISCLOSURES

None.

CLINICAL PERSPECTIVE

Heart failure with preserved ejection fraction (HFPEF) accounts for >50% of all heart failure cases. Both arterial hypertension and metabolic comorbidities, such as overweight/obesity and type 2 diabetes mellitus, are prevalent in HFPEF. Hitherto, experimental studies mainly tried to reproduce HFPEF in arterial hypertension models such as old dogs with bilateral renal wrapping or Dahl salt-sensitive rats and largely overlooked the prominent involvement of metabolic comorbidities. The present experimental study, therefore, investigated ZSF1 rats that are first generation hybrids between the Zucker diabetic fatty and spontaneously hypertensive heart failure rats. Lean and obese ZSF1 rats are hypertensive as they inherited the hypertension gene from male spontaneously hypertensive heart failure rats. Obese ZSF1 rats also inherited 2 different leptin receptor mutations from female Zucker diabetic fatty and male spontaneously hypertensive heart failure rats. At 20 weeks, the obese, but not the lean ZSF1, rats
had developed HFPEF, which was evident from increased lung weight, preserved left ventricular ejection fraction, normal left ventricular end-diastolic volume index, elevated left ventricular filling pressures, left atrial enlargement, and a high diastolic left ventricular stiffness modulus. High myocardial stiffness was also obvious in isolated cardiac muscle strips and could be attributed after myofilamentary extraction to stiffer titin and not to collagen deposition. High titin stiffness resulted from hypophosphorylation of its elastic segments. Titin hypophosphorylation was, therefore, identified as the main contributor to HFPEF in an experimental animal model that comes close to the clinical HFPEF presentation as it combines metabolic comorbidities with arterial hypertension.

REFERENCES


ONLINE DATA SUPPLEMENT: METHODS AND MATERIALS

Animal model
Nine-week old male ZSF1 lean (ZSF1-Lean, n=11), ZSF1 obese (ZSF1-Obese, n=22) and Wistar-Kyoto rats (WKY, n=11) were obtained from Charles River (Barcelona, Spain) and fed with Purina Diet (#5008). After a 1 week laboratory adaptation period, animals underwent phenotypic evaluation consisting of metabolic cage studies, blood sample collection and echocardiographic evaluation. From this point onward, a subgroup of ZSF1 obese rats (ZSF1-Obese+HFD, n=11) was randomly allocated to receive HFD (Research Diet Inc. #D12468). Weight gain and energy intake were recorded every third day. Phenotypic evaluation was repeated at the 14th and 18th week of life. At 20 weeks of age, animals underwent hemodynamic evaluation under anaesthesia and were subsequently sacrificed with procurement of myocardial tissue samples for histological, biochemical and biomechanical studies. Animals were kept in individually ventilated chambers in a controlled environment with a 12-h-light/-dark cycle at 22°C room temperature and had unlimited access to food. Investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the NIH (NIH Publication no. 85–23, revised 1996) and was approved by the ethics committee of the Faculty of Medicine of Porto.

ZSF1 rats (Charles River, Barcelona, Spain) are first generation hybrids between the ZDF (Zucker diabetic fatty) and SHHF (spontaneously hypertensive heart failure) rats, which carry two different leptin receptor mutations (fa and fa<sup>cp</sup>). A female ZDF (+/fa) is crossbred with a male SHHF (+/fa<sup>cp</sup>) rat, leading to either lean ZSF1 or obese ZSF1 rats (fa/fa<sup>cp</sup>).<sup>1</sup> Both ZSF1 rats are hypertensive, since they inherit the hypertension gene from the SHHF rats but only obese ZSF1 rats develop diabetes and dyslipidemia.<sup>2</sup>

Metabolic studies and renal function
After a 24h acclimatization period, water and energy intake, weight gain and urine output were recorded, and a 24h urine sample was collected in metabolic cages (Techniplast, Buguggiate). After successive 24h rest intervals, all rats underwent oral glucose and insulin resistance testing, at the end of 12h feed-deprivation periods. Glycemia was recorded at baseline and 15, 30, 60, 90 and 120 min (Freestyle-Mini) after a 1 g.Kg<sup>−1</sup> glucose gavage or a 0.5 U.Kg<sup>−1</sup> intraperitoneal
insulin injection, respectively. Echocardiographic studies were conducted, after another 24h rest period, and a blood sample (1.5 mL) was collected from the subclavian vein under anaesthesia, at the end of the procedure.

**Echocardiography**
Rats (n=11 per group) were anaesthetized by inhalation of 8% sevoflurane in vented containers, orotracheally intubated and mechanically ventilated. Anaesthesia was maintained with sevoflurane (1- 2.5%) titrated to avoid the toe pinch reflex. Rats were placed in left-lateral decubitus on a heating pad, the ECG was monitored (lead II) and their temperature was kept at 38°C. The skin was shaved and depilated. After applying prewarmed echocardiography gel a linear 15MHz probe (Sequoia 15L8W) was gently positioned on the thorax. Systolic and diastolic wall thickness and cavity dimensions were recorded, in M-mode and 2D echocardiography, at the level just above the papillary muscles in the parasternal short axis view. The long axis diastolic dimensions of the left ventricle and transverse aortic root diameter were recorded by 2D and M-mode echocardiography, respectively, in the parasternal long axis view. Aortic flow velocity was recorded by pulsed-wave Doppler just above the aortic valve. Mitral flow velocity tracings were obtained with pulsed-wave Doppler just above the mitral leaflets, peak systolic tissue velocity and E’ were measured with tissue Doppler at the medial mitral annulus and lateral mitral annulus, respectively, and left atrial dimensions were measured, at their maximum, by 2D echocardiography in the four chamber view. Acquisitions were done while transiently suspending mechanical ventilation and recordings were averaged from three consecutive heartbeats (Siemens Acuson Sequoia CS12). Left ventricular (LV) mass and volumes were calculated by the 2D area-length method. Myocardial performance index was retrieved from the mitral flow pattern.

**Haemodynamic evaluation**
After sedation (100 μg.kg⁻¹ and 5 mg.kg⁻¹ intraperitoneal fentanyl and midazolam, respectively), anaesthesia (8 and 2.5–3% sevoflurane for induction and maintenance, respectively; Penlon Sigma Delta), endotracheal intubation, mechanical ventilation (TOPO, Kent scientific), 8 mL.kg⁻¹.h⁻¹ intravenous warm Ringer’s solution infusion (NE-1000, New Era Pump Systems), temperature maintenance at 38°C on a heating pad, left thoracotomy, LV and right ventricular (RV) pressure-volume catheter insertion through the apex (SPR-838 and PVR-1045
Millar Instruments, respectively), and ascending aorta probe placement (Transonics) that allowed CO measurement (Active Redirection Transit Time Flowmeter, Triton Technology), signals were continuously acquired (MPVS 300, Millar Instruments), recorded at 1000 Hz (ML880 PowerLab 16/30, ADInstruments), and analyzed (PVAN 3.5, Millar Instruments). Recordings were obtained at suspended end-expiration. The LV catheter was advanced to record systemic arterial pressure. Parallel conductance was assessed with hypertonic saline. After euthanasia (100 mg.kg\(^{-1}\) intravenous pentobarbital), blood (4mL) was collected for storage (-80°C) and for volume calibration (910–1048, Millar instruments). LV volumes were varied using transient inferior vena cava constrictions by adjusting a sling around the inferior vena cava. Organs were weighed, RV and LV + interventricular septum (IVS) were weighed after dissection, and tibia length (TL) was measured. Samples were either snap-frozen and stored at -80°C or processed for histology. Weights were normalized to TL due to the large body weight differences between groups.

Histomorphological analysis was performed on elastica-von-Giesson and hematoxylin-eosin stained, 4 μm thick, sections of tissue placed in 4% buffered formaldehyde solution. As previously validated, MyD was determined perpendicularly to the outer contour of the cell membrane at nucleus level.

**Collagen- volume fraction and cross-linking**

The collagen volume fraction (CVF) was determined by quantitative morphometry with an automated image analysis system in sections stained with collagen-specific picro-sirius red, as previously reported in ZSF1 lean (ZSF1-Lean, n=11), ZSF1 obese (ZSF1-Obese, n=22) and Wistar-Kyoto rats (WKY, n=11). All measurements were performed in duplicate by 2 independent observers. The inter- and intra-observer coefficients of variation were <4%. To distinguish between cross-linked (insoluble) and non-cross-linked (soluble) collagen a colorimetric procedure was employed. First, a fast green-sirius red assay was performed to identify and quantify total collagen. In a second step, a sircol-based assay was performed to obtain and quantify soluble collagen. The amount of insoluble collagen was calculated by subtracting the amount of soluble collagen from the amount of total collagen. The degree of cross-linking was calculated as the ratio between the insoluble and the soluble forms of collagen. All measurements were performed in duplicate. The inter- and intra-assay coefficients of variation were 5 and 3%, respectively.
**RNA isolation and gene expression analysis**

Frozen tissue sections ZSF1 lean (ZSF1-Lean, n=5), ZSF1 obese (ZSF1-Obese, n=11) and Wistar- Kyoto rats (WKY, n=5) were minced in Trizol and further disrupted during 10 minutes of vigorous shaking. To extract the RNA, chlorophorm was added, mixed, and centrifuged. The aqueous phase containing the RNA was collected in a separate tube, and isopropanol was added. For precipitation, the RNA solution was centrifuged 15 minutes at 4°C at high speed. The RNA pellet was then further purified using the RNeasy Mini Kit (Qiagen) according to manufacturer’s protocol. One μg of RNA was reverse transcribed into cDNA using the High Capacity Kit (Applied Biosystems) and then further diluted to a final concentration of 5 ng/μL cDNA.

The relative quantification of mRNA levels was carried out on a 7900 HT (Applied Biosystem). To assess the mRNA expression of the target genes, real-time PCR was performed using 5 μL of the gene expression master mix (Applied Biosystems) and 0.5 μL of the gene expression assay for Col1A1 (Rn01463848_m1) and Col3A1 (Rn01437683_m1) (each includes forward and reverse primers as well the fluorescently FAM-labelled probe) from Applied Biosystems, and 1 μL of cDNA in a final volume of 10 μL. Quantification of the house keeping gene GAPDH (Rn99999916_s1) as an internal control was performed for each sample. Data were normalized to 18S RNA level as an endogenous control and are expressed using the formula \(2^{-\Delta\Delta Ct}\) in comparison to the corresponding untreated controls.

**Force measurements on skinned cardiomyocytes and strips**

*Single skinned cardiomyocytes*

Force measurements were performed on single skinned cardiomyocytes as described.\(^3\) Cardiomyocytes were isolated from WKY, ZSF1-Lean, ZSF1-Obese and ZSF1-Obese+HFD rat hearts (n=15/4 cardiomyocytes/group). Briefly, samples were defrozen in relaxing solution (in mmol/L: free Mg, 1; KCl, 100; EGTA, 2; Mg-ATP, 4; imidazole, 10; pH 7.0), mechanically disrupted and incubated for 5 min in relaxing solution supplemented with 0.5% Triton X-100. The cell suspension was washed 5 times in relaxing solution. Single cardiomyocytes were selected under an inverted microscope (Zeiss Axiovert 135, 40x objective) and attached with silicone adhesive between a force transducer and a piezoelectric motor as part of a "Permeabilized Myocyte Test System" (1600A; with force transducer 403A; Aurora Scientific, Aurora, Ontario, Canada).
Cardiomyocyte $F_{\text{passive}}$ was measured in relaxing buffer at room temperature within a sarcomere-length range between 1.9 and 2.3 μm. Force values were normalized to myocyte cross-sectional area calculated from the diameter of the cells, assuming a circular shape. As a test of cell viability, each cardiomyocyte was also transferred from relaxing to maximally activating solution (pCa4.5), at which isometric force developed. Once a steady state force was reached, the cell was shortened within 1 ms to 80% of its original length to determine baseline force. Only cells developing active forces >20 kN/m² were included in the analysis. The passive tension was measured under steady state shear (viscous and elastic properties). Subsequently cardiomyocytes were incubated in relaxing solution supplemented with the PKG1α (0.1 U/mL; Sigma, batch 034K1336), guanosine cGMP (10 μmol/L, Sigma) and dithiothreitol (6 mmol/L; Sigma). After 40-min-long incubation with PKG1α, $F_{\text{passive}}$ measurements were again performed in relaxing solution at SL 1.9-2.3 μm.

**Skinned muscle strips**

Left papillary muscles were dissected after sacrificing the animals (n=16/4 muscle strips/group). Small muscle strips were created and chemically permeabilized in a 1% Triton X-100 solution for 30 minutes. After clipping both ends, the strips were mounted between a length motor and a force transducer on top of an inverted microscope. Strips were activated at 20°C with solutions containing a saturating Ca²⁺ concentration to determine maximal active tension (force/cross-sectional area) at a sarcomere length (SL) of 2.0 μm. Subsequently, strips were stretched from SL 1.9 μm to 2.3 μm, with a velocity of 10% muscle length per second and while in a solution with low Ca²⁺ (pCa9), to determine passive tension generation. 7 Afterwards, the strips underwent an extraction protocol of 45 minutes in 0.6M KCl followed by 45 minutes in 1.0M KI to depolymerize thick and thin filaments respectively, leaving titin unanchored, and the strips were again passively stretched as described above. The remaining tension after this procedure is extraction-insensitive and caused by extracellular matrix, i.e. collagen. The passive tension was measured under steady state shear (viscous and elastic properties).

**Titin isoform separation and titin phosphorylation by Pro-Q Diamond/Sypro Ruby**

Titin isoforms were separated as described. Briefly, tissue samples from ZSF1 lean (ZSF1-Lean, n=5), ZSF1 obese (ZSF1-Obese, n=10) and Wistar-Kyoto rats (WKY,
n=5) were solubilized in 50 mM Tris-sodium dodecyl sulfate (SDS) buffer (pH 6.8) containing 8 μg/mL leupeptin (Peptin Institute, Japan) and phosphatase inhibitor cocktail (PIC [P2880], 10 μL/mL; Sigma). Samples were heated for 3 minutes at 96°C and centrifuged. Then, samples (20 μg; equal concentration checked by spectroscopic methods) were separated by agarose-strengthened 1.8% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Gels were run at 5 mA constant current for 16 hours. The phosphorylation state of cardiac titin was determined using Pro-Q Diamond phosphoprotein. Titin gels were stained for one hour with Pro-Q Diamond, and then overnight with Sypro Ruby (Molecular Probes). Staining was visualized using the LAS-4000 Image Reader (Fuji Science Imaging Systems) and signals were analyzed using Multi Gauge V3.2 or AIDA software. Finally signals obtained from Pro-Q Diamond staining were normalized to signals obtained from Sypro Ruby staining.

**Titin and phospho-titin analysis by Western blot**

1.8% SDS-PAGE followed by Western blot was performed to measure expression and site-specific phosphorylation of titin. Samples from ZSF1 lean (ZSF1-Lean, n=5), ZSF1 obese (ZSF1-Obese, n=10) and Wistar-Kyoto rats (WKY, n=5) were applied at a concentration that was within the linear range of the detection system (30 μg dry weight; checked by spectroscopic methods). Sequence-specific or phosphosite-specific anti-titin antibodies were custom-made by Eurogentec, Belgium (positions in mouse (*Mus musculus*) titin according to UniProtKB identifier A2ASS6)). The following affinity-purified antibodies were used:

- Anti-titin- mouse N2Bus against EEGKSLSFPLA (rabbit polyclonal; 1:1000).
- Anti-phospho-N2Bus (S3991 in mouse titin and S4010 in human titin) against EEGKS(PO3H2)LSFPLA (rabbit polyclonal; 1:500).
- Anti-PEVK-domain against (cross-species conserved) sequence EVVLKSVLRK (1:1000) - Anti-phospho-PEVK-domain (S12742 in mouse titin and S11878 in human titin) against EVVLKS(PO3H2)VLRK (1:500)
- Anti-PEVK-domain against (cross-species conserved) sequence KLRPGSGGEKPP (1:100) - Anti-phospho-PEVK-domain (S12884 in mouse titin and S12022 in human titin) against KLRPGS(PO3H2)GGEKPP (1:500)

The amino acid sequences of rat are identical to the amino acid sequences of mouse. Titin antibodies gave specific signals on Western blots with cardiac...
tissue. Following SDS-PAGE, proteins were transferred to Hybond ECL nitrocellulose membranes. Blots were pre-incubated with 3% bovine serum albumin in Tween Tris-buffered saline (TTBS); 10 mmol/L Tris-HCl; pH 7.6; 75 mmol/L NaCl; 0.1% Tween) for 1 hour at room temperature. Then, blots were incubated overnight at 4°C with the primary antibodies against the respective (phospho) protein. After washing with TTBS, primary antibody binding was visualized using secondary horseradish peroxidase-labeled, goat-anti- rabbit/mouse antibodies (dilution 1:1000; DakoCytomation) and enhanced chemiluminescence (ECL Western blotting detection; Amersham Biosciences). Staining was visualized using the LAS-4000 Image Reader and analyzed with Multi Gauge V3.2 or AIDA software. PVDF stains were saved for comparison of protein load. Loading was also controlled by comparing signals of the phospho-specific antibodies with those of the respective sequence-specific antibodies. Finally signals obtained from phospho-specific antibodies were normalized to signals obtained from sequence-specific antibodies.

**Data analysis**

Circumferential LV end-diastolic wall stress (σ) was computed using a thick wall ellipsoid model of the LV:

\[
\sigma = \frac{PD}{2h} \times [1-(h/D)-(D^2/L^2)]
\]

where P is LV end-diastolic pressure, h is LV wall thickness, and D and L are LV short axis diameter and long axis length at the midwall. Groups were compared by two-way repeated measures ANOVA whenever serial acquisitions were obtained for the same animal, and by one-way ANOVA for single acquisitions. Pressure-volume loop analysis was analyzed using LabChart 7 Pro v7.3.1. Values are given as mean±SEM. A 2-tailed test with a probability of 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 repeated measure ANOVA. Statistical analysis was performed with SPSS (Version 15.0; SPSS Inc,Chicago,III).
Supplemental References


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_Circ Heart Fail._ 2013;6:1112-1115
doi: 10.1161/CIRCHEARTFAILURE.113.000825
_Circulation: Heart Failure_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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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/6/6/1112

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Mechanisms of Diastolic Dysfunction in Heart Failure With a Preserved Ejection Fraction

If It’s Not One Thing It’s Another

Martin M. LeWinter, MD; Markus Meyer, MD

It has been nearly 30 years since the first series of patients with the syndrome of heart failure with a preserved ejection fraction (HFpEF) was reported. It has proven to be a controversial topic. Because left ventricular (LV) EF is preserved, it was assumed that HFpEF results from altered diastolic properties. However, some argued that these patients did not truly have HF or had subtle forms of dilated HF. Symptomatic of this debate is reluctance to use the term diastolic HF (we prefer HFpEF because diastolic dysfunction is also present in HF with a reduced EF) as well as disagreement over the exact EF cutoff, that is, should a perfectly normal EF be required to diagnose HFpEF or does a modest reduction qualify?

HFP EF Substrates

Although a small number of patients have HFpEF in association with specific cardiac diagnoses, for example, hypertrophic and infiltrative cardiomyopathy, constrictive pericarditis, all of which have profound effects on diastolic compliance, the vast majority have a history of HTN. In many patients, especially elderly women, HTN is exclusively systolic, resulting from reduced arterial compliance rather than changes in resistance vessels. Moreover, although there is considerable variation between patient cohorts, the great majority of patients with HTN-associated HFpEF have concentric LV remodeling, defined as either concentric hypertrophy (increased LV mass with normal or reduced chamber volume) or, in the absence of increased mass, increased mass:volume ratio or relative wall thickness. In population studies, the progression from HTN to HFpEF is paralleled by declines in diastolic function. These observations strongly support the concept that diastolic dysfunction is in fact a major underlying mechanism of this progression, resulting in the hemodynamic hallmark of HF, a depressed Frank–Starling relation.

HTN is not the only substrate in many if not most patients with HFpEF. Approximately one third have type 2 diabetes mellitus (DM2). It is likely that a substantial additional number have insulin resistance in the absence of overt DM2. Insulin resistance/DM2 and associated hyperinsulinemia have pleiotropic effects on the myocardium, including stimulation of hypertrophy, increased oxidative stress, and a proinflammatory/proliferative state, which can modify cardiomyocyte function in multiple ways as well as extracellular matrix collagen, all of which can affect diastolic function. Obstructive sleep apnea and obesity are common in HFpEF and also associated with a proinflammatory state and cardiac hypertrophy. HTN, DM2/insulin resistance, and obesity are components of the metabolic syndrome (MS). Recognition of the association between MS and HFpEF has led to the concept that in many patients HFpEF can be considered metabolic heart disease, although the detailed mechanisms whereby metabolic derangements and associated oxidative stress and proinflammatory/proliferative states cause diastolic dysfunction remain to be elucidated. Abnormal myocardial triglyceride accumulation associated with echocardiographic evidence of diastolic dysfunction in patient with elements of the MS provides direct evidence of this link.

Mechanisms of Diastolic Dysfunction in HFpEF

The exact mechanisms leading to diastolic dysfunction in concentric remodeling and HFpEF have begun to be elucidated during the past 5 to 10 years. In discussing the article by Hambani et al in this issue of Circulation: Heart Failure, we will focus on its relationship to what is known about these mechanisms from studies on myocardial tissue from patients.

One well-documented mechanism studied in biopsy tissue from patients with HFpEF is hypophosphorylation of protein kinase (PK) A and PKG sites on cardiac titin, the giant myofilament protein responsible for cardiomyocyte passive tension. Titin’s N terminus is anchored in the z-disc of the sarcomere, and its C terminus is anchored in the M-band. When the cardiomyocyte is stretched, titin lengths and...
functions as a complex molecular spring, developing passive tension with a curvilinear length-tension relationship. Chemical disruption of titin’s anchors in the M-band eliminates virtually all cardiomyocyte passive stiffness over the physiological sarcomere length range.\textsuperscript{20} Using these chemical methods, the proportion of myocardial passive tension ascribable to titin versus extracellular matrix collagen has been dissected.\textsuperscript{20} Although there are differences in absolute levels of passive tension, in all species studied, including humans, titin accounts for the majority of myocardial passive tension at short sarcomere lengths. With further lengthening, the relative contribution of collagen increases such that it accounts for \( \approx 50\% \) or more of passive tension at sarcomere lengths at the upper end of the physiological range.\textsuperscript{20,21} Titin is also a key biomechanical sensing and signaling molecule and the most commonly mutated gene in human dilated cardiomyopathy. These and other functional and disease-specific aspects of titin have been discussed in recent reviews.\textsuperscript{20,21}

Titin stiffness is modulated by isoform variation accomplished by alternative splicing and changes in phosphorylation state.\textsuperscript{20,21} Two isoforms (N2B and N2BA) are present in the postnatal heart; N2B is smaller and markedly stiffer than N2BA. The N2BA:N2B ratio is \( \approx 40:60 \) in normal adult human LV myocardium. In both ischemic and nonischemic dilated cardiomyopathy as well as HFpEF, a shift toward the more compliant N2B isoform occurs, which reduces cardiomyocyte resting tension. Changes in phosphorylation can rapidly alter myocardial passive stiffness, for example, during exercise. In HFpEF, the net effect of increased N2BA titin and hypophosphorylation of PKA/PKG sites is increased cardiomyocyte resting tension.\textsuperscript{18} In addition to PKA/PKG, PKC-\( \alpha \) has been shown to phosphorylate other titin sites.\textsuperscript{7,9,12} CalM kinase targets these same sites. In contrast to PKA/PKG sites, PKC-\( \alpha \) phosphorylation increases resting tension.

In their elegant study, Hamdani et al\textsuperscript{17} used Zucker rats to demonstrate that the combination of obesity, DM, and HTN (with or without a high-fat diet) leads to HFpEF in association with increased passive myocardial stiffness and markedly reduced phosphorylation of titin’s PKA/PKG sites compared with controls. There were no changes in isoforms or phosphorylation of one of the PKC-\( \alpha \) sites. Importantly, phosphorylation of PKA/PKG sites was unchanged in lean, nondiabetic, but hypertensive Zucker rats. Thus, components of the MS besides HTN seem sufficient to cause changes in passive stiffness attributable to reduced titin phosphorylation in this experimental model. Although the underlying mechanism(s) of reduced phosphorylation was not elucidated, this article provides important insights into the pathophysiology of HFpEF that could play a role in patients.

Hamdani et al\textsuperscript{17} have not shown that reduced titin phosphorylation is sufficient in and of itself to cause HFpEF in obesity–diabetic–hypertensive rats. Lean hypertensive rats developed significant but modest increases in LV mass at the last, 18th week, measuring point, which were not associated with changes in diastolic function indexes. In contrast, increases in mass were much larger and occurred much earlier in obese–diabetic–hypertensive rats and were associated with abnormal diastolic function. Thus, it is important to consider determinants of diastolic function other than titin that could contribute to the development of diastolic dysfunction and HFpEF.

A modest amount of such information obtained in human tissue is now available, although it has not been specifically focused on metabolic heart disease. One determinant is changes in extracellular matrix collagen, but Hamdani et al\textsuperscript{17} report that collagen volume fraction and cross-linking were unchanged. However, this differs from HFpEF in patients,\textsuperscript{13} in whom collagen volume fraction and cross-linking are increased and underscores the potential for animal models to provide information that does not apply to patients. Using the chemical methods noted above, Hamdani et al\textsuperscript{17} also report that collagen-dependent passive tension was unchanged. However, an unexplained finding is that collagen-dependent tension accounted for only \( \approx 10\% \) to \( 20\% \) of total passive tension in all groups. As noted above, this is much smaller than what has been reported previously in several species.\textsuperscript{25}

In addition to passive diastolic properties, LV relaxation is abnormal in patients with LV hypertrophy and HFpEF \textsuperscript{7,9,13} but the mechanisms have received less attention. At the level of the LV, increased arterial load, when present, slows relaxation rate. At the myocardial level, the speed and completeness of relaxation are dependent on deactivation of cross-bridges formed during contraction, which in turn depends on both the mechanisms that restore systolic [\( \text{Ca}^{2+} \)]\textsubscript{i} to diastolic levels and the kinetics of cross-bridge dissociation.

We recently reported the first evidence of abnormal calcium handling in patients with pressure overload–induced concentric remodeling.\textsuperscript{26} In excitable tissue from LV epicardial biopsies obtained from patients with normal EF undergoing coronary bypass grafting, we found that isometrically contracting strips from patients with concentric remodeling (some of whom had HFpEF) displayed a progressive increase in diastolic tension beginning at stimulation frequencies in the 100 to 110 per minute range, that is, incomplete relaxation occurred at rates present during low-level physical activity. Additional experiments revealed a defect in sarcolemmal calcium extrusion. In more recent, unpublished work, we found that cytoplasmic [\( \text{Ca}^{2+} \)]\textsubscript{i} is indeed increased at these same rates. These results may provide a mechanism whereby patients with HFpEF increase filling pressures and become dyspneic with physical activity.\textsuperscript{27} Correspondingly, patients with HFpEF display a reduced ability to maintain end-diastolic volume and cardiac output during increases in heart rate, which could reflect the same mechanism.\textsuperscript{26}

In another recent report using demembranated (skinned) myocardial strips,\textsuperscript{28,29} we showed that the kinetics of cross-bridge dissociation are slowed in patients with concentric remodeling compared with controls. Using the method of sinusoidal length perturbation, the apparent rate constant of cross-bridge dissociation was reduced at submaximal [\( \text{Ca}^{2+} \)]\textsubscript{i} and its mathematical inverse, cross-bridge on-time (the time the cross-bridge is attached and generating force) was prolonged. These changes in dissociation kinetics serve to slow relaxation. We also found that total phosphorylation of both cardiac troponin I and myosin binding protein C is reduced in concentrically remodeled LV myocardium. Recent, unpublished studies using site-specific phosphoantibodies reveal
that PKA/PKG sites on both proteins are hypophosphorylated. Because phosphorylation of these sites speeds actomyosin kinetics, hypophosphorylation may contribute to slowed relaxation.

In summary, although studies are limited, in patients with HFP EF or pressure overload–induced concentric remodeling abnormalities of every component of LV diastolic function, arterial load, mass:volume ratio, passive stiffness (titin and collagen), and cross-bridge deactivation (calcium handling and actomyosin kinetics) have been demonstrated or implicated. In future, it will be important to understand the relative importance and time course of these abnormalities in relation to the progression to HFP EF as well as the influence of substrates other than HTN.

Therapeutic Considerations

There are currently no therapies for HFP EF that have been shown to improve long-term outcomes. Perhaps the diverse abnormalities of diastolic function identified, which could have great interpatient variability, make it difficult for treatments to yield significant effects in clinical trials. Guidelines for treatment are, therefore, largely empirical, emphasizing Na restriction, diuretics as needed, and blood pressure control. In patients with HFP EF with MS, common sense suggests that weight loss and perhaps exercise should be therapeutic goals. Small trials show that weight loss can improve diastolic function, but the effects of exercise have been variable.

The study by Hamdani et al links a specific component of diastolic dysfunction in HFP EF, that is, titin hypophosphorylation, to DM2 and obesity and suggests a potential mechanism whereby lifestyle changes can improve diastolic function. Our knowledge of the mechanisms of diastolic dysfunction in concentric remodeling and HFP EF, although admittedly rudimentary, has other therapeutic implications. It is intriguing that a specific alteration at the level of the myofilaments, hypophosphorylation of PKA/PKG sites may contribute to increased passive stiffness (titin) and slowed relaxation (cardiac troponin I/myosin binding protein C). Accordingly, pharmacological approaches that target this molecular abnormality offer promise. Unfortunately, the RELAX (Phosphodiesterase-5 Inhibition to Improve Clinical Status and Exercise Capacity in Heart Failure with Preserved Ejection Fraction) trial of sildenafil in HFP EF did not demonstrate efficacy despite the fact that phosphodiesterase-5 inhibition has several effects that, in addition to potential normalization of titin and cardiac tropion I/myosin binding protein C phosphorylation, should be beneficial. In the RELAX trial, sildenafil did not significantly increase plasma cGMP activity, suggesting that PKG activity may not have been effectively augmented. This in turn suggests that other approaches to increasing NO availability and PKG activity should be considered. Nitrates are an obvious choice. Other considerations arise concerning exercise and the common use of β-blockers in HFP EF. Hypophosphorylation of PKA/PKG sites should be ameliorated during exercise in conjunction with increased adrenergic stimulation, that is, their importance may decrease with physical activity. β-Blockers could potentiate these same abnormalities at rest and during exercise. In contrast, rate-dependent incomplete relaxation and inadequate maintenance of end-diastolic volume are obviously more pronounced during exercise and could therefore be more important as a mechanism of exercise limitation. In that case, β-blockers may help by blunting increases in heart rate during exercise. These divergent heart rate effects might make it difficult to detect beneficial effects of β-blockers.

Targeting the extracellular matrix is obviously also a promising therapeutic approach. Aldosterone inhibition is potently antifibrotic and has other potentially beneficial effects. The recent Aldo-DHF (Aldosterone Receptor Blockade in Diastolic Heart Failure) phase 2 trial of spironolactone revealed improvements in resting diastolic function in HFP EF. The results of the larger, ongoing TOPCAT (Treatment of Preserved Cardiac Function with an Aldosterone Antagonist) trial of spironolactone in HFP EF are therefore eagerly awaited.

Summary

We are beginning to gain a better understanding of the mechanisms of diastolic dysfunction in HFP EF. By demonstrating reduced titin phosphorylation in obese–diabetic–hypertensive rats, Hamdani et al have provided important insights into the substantial number of patients with HFP EF with components of the MS, that is, metabolic heart disease. As progress is made in other mechanistic aspects of HFP EF, we will hopefully gain a more integrated understanding and a more rational basis for developing new treatments. In view of the multiple abnormalities of diastolic function identified, it may be particularly important to individualize and target treatment.

Sources of Funding

Dr LeWinter received National Institutes of Health (NIH) grants U10HL110342 and RO1HL089944. Dr Meyer received AHA Scientist Development Grant 0730056 N and NIH R21HL94807.

Disclosures

None.

References


