

# Hypophosphorylation of the Stiff N2B Titin Isoform Raises Cardiomyocyte Resting Tension in Failing Human Myocardium

Attila Borbély,\* Ines Falcao-Pires,\* Loek van Heerebeek, Nazha Hamdani, István Édes, Cristina Gavina, Adelino F. Leite-Moreira, Jean G.F. Bronzwaer, Zoltán Papp, Jolanda van der Velden, Ger J.M. Stienen, Walter J. Paulus

**Abstract**—High diastolic stiffness of failing myocardium results from interstitial fibrosis and elevated resting tension ( $F_{\text{passive}}$ ) of cardiomyocytes. A shift in titin isoform expression from N2BA to N2B isoform, lower overall phosphorylation of titin, and a shift in titin phosphorylation from N2B to N2BA isoform can raise  $F_{\text{passive}}$  of cardiomyocytes. In left ventricular biopsies of heart failure (HF) patients, aortic stenosis (AS) patients, and controls (CON), we therefore related  $F_{\text{passive}}$  of isolated cardiomyocytes to expression of titin isoforms and to phosphorylation of titin and titin isoforms. Biopsies were procured by transvascular technique (44 HF, 3 CON), perioperatively (25 AS, 4 CON), or from explanted hearts (4 HF, 8 CON). None had coronary artery disease. Isolated, permeabilized cardiomyocytes were stretched to 2.2- $\mu\text{m}$  sarcomere length to measure  $F_{\text{passive}}$ . Expression and phosphorylation of titin isoforms were analyzed using gel electrophoresis with ProQ Diamond and SYPRO Ruby stains and reported as ratio of titin (N2BA/N2B) or of phosphorylated titin (P-N2BA/P-N2B) isoforms.  $F_{\text{passive}}$  was higher in HF ( $6.1 \pm 0.4 \text{ kN/m}^2$ ) than in CON ( $2.3 \pm 0.3 \text{ kN/m}^2$ ;  $P < 0.01$ ) or in AS ( $2.2 \pm 0.2 \text{ kN/m}^2$ ;  $P < 0.001$ ). Titin isoform expression differed between HF (N2BA/N2B =  $0.73 \pm 0.06$ ) and CON (N2BA/N2B =  $0.39 \pm 0.05$ ;  $P < 0.001$ ) and was comparable in HF and AS (N2BA/N2B =  $0.59 \pm 0.06$ ). Overall titin phosphorylation was also comparable in HF and AS, but relative phosphorylation of the stiff N2B titin isoform was significantly lower in HF (P-N2BA/P-N2B =  $0.77 \pm 0.05$ ) than in AS (P-N2BA/P-N2B =  $0.54 \pm 0.05$ ;  $P < 0.01$ ). Relative hypophosphorylation of the stiff N2B titin isoform is a novel mechanism responsible for raised  $F_{\text{passive}}$  of human HF cardiomyocytes. (*Circ Res.* 2009;104:780-786.)

**Key Words:** myocardium ■ heart failure ■ diastole ■ titin

Diastolic left ventricular (LV) dysfunction importantly contributes to heart failure (HF) with either reduced LV ejection fraction (EF) (HFREF) or with normal LV ejection fraction (HFNEF).<sup>1</sup> In HFREF, diastolic LV dysfunction correlates with exercise intolerance<sup>2</sup>; in HFNEF, diastolic LV dysfunction is an essential diagnostic feature.<sup>3,4</sup> Diastolic LV dysfunction has usually been attributed to interstitial fibrosis because of an imbalance between matrix metalloproteinases and their tissue inhibitors.<sup>5,6</sup> Recently high resting tension ( $F_{\text{passive}}$ ) of cardiomyocytes has also been implicated in diastolic LV dysfunction.<sup>7-9</sup> Cardiomyocytes from patients with HFNEF have higher  $F_{\text{passive}}$  than cardiomyocytes from patients with HFREF and cardiomyocytes from both HF groups have higher  $F_{\text{passive}}$  than cardiomyocytes from controls (CON).<sup>7-9</sup> In vitro administration of protein kinase (PK)A to HF cardiomyocytes lowers their elevated  $F_{\text{passive}}$  to the level

observed in CON cardiomyocytes,<sup>7,8</sup> whereas  $F_{\text{passive}}$  of CON cardiomyocytes does not respond to PKA.<sup>7</sup>

Alterations in cardiomyocyte  $F_{\text{passive}}$  have been attributed to the giant cytoskeletal protein titin,<sup>8</sup> which can modulate  $F_{\text{passive}}$  through isoforms shifts<sup>10-12</sup> and phosphorylation status.<sup>13-17</sup> In explanted hearts of HFREF patients with ischemic or nonischemic cardiomyopathy,<sup>10-12</sup> myocardial titin isoform expression shifts from the stiff N2B to the compliant N2BA isoform with a resultant rise of the N2BA/N2B ratio. In pooled endomyocardial biopsy samples of HFNEF patients, the N2BA/N2B ratio is lower than in HFREF patients,<sup>8</sup> and the corresponding higher expression of the stiff N2B isoform could explain the higher  $F_{\text{passive}}$  in HFNEF cardiomyocytes. Correction of the high  $F_{\text{passive}}$  of skinned HF cardiomyocytes by PKA suggests a phosphorylation deficit of titin or of other myofibrillar proteins to be also involved.

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From the Department of Physiology (A.B., I.F.P., L.v.H., N.H., J.v.d.V., G.J.M.S., W.J.P.) and Cardiology (J.G.F.B.), Institute for Cardiovascular Research, VU University Medical Center Amsterdam, The Netherlands; Institute of Cardiology (A.B., I.É., Z.P.), University of Debrecen Medical and Health Science Center, Debrecen, Hungary; and Department of Physiology (I.F.P., C.G., A.F.L.M.), Faculty of Medicine, University of Porto, Portugal.

\*Both authors contributed equally to this study.

Correspondence to Prof Dr Walter J. Paulus, MD, PhD, Laboratory of Physiology, VU University Medical Center Amsterdam, Van der Boechorststraat 7, 1081 BT Amsterdam, The Netherlands. E-mail [wj.paulus@vumc.nl](mailto:wj.paulus@vumc.nl)

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**Table. Clinical and Hemodynamic Patient Data**

	CON (n=7)	AS (n=25)	HFNEF (n=17)	HFREF (n=27)
Age, yr	61±5	67±3	66±3	56±3
LVEF, %	65±3	65±2	61±4	28±2‡§¶
LVEDVI, mL/m <sup>2</sup>	75±7	56±4	82±5	130±7‡§¶
LVEDP, mm Hg	11±1	21±1‡	24±1‡	22±2‡
LVMI, g/m <sup>2</sup>	98±4	132±6*	113±5	161±8‡¶
LV Stiff Mod, kN/m <sup>2</sup>	2.2±0.2	6.0±0.3‡	6.1±0.7‡	5.0±0.8
LVEDWS, kN/m <sup>2</sup>	2.9±0.2	4.0±0.3‡	5.5±0.4‡	8.7±0.7‡§¶

\* $P < 0.05$  vs CON, † $P < 0.01$  vs CON, ‡ $P < 0.001$  vs CON, § $P < 0.001$  vs AS, ¶ $P < 0.001$  vs HFNEF.

Phosphorylation of the cardiac-specific N2B spring element of titin by PKA reduces  $F_{\text{passive}}$  of rat cardiomyocytes<sup>13</sup> and of rat cardiac myofibrils.<sup>14</sup> This phosphorylation-induced reduction of  $F_{\text{passive}}$  is titin isoform-dependent, with the largest effect observed in rat ventricular myocardium, which has an N2BA/N2B ratio of  $\approx 0.1$ , and the smallest effect in bovine atrial myocardium, which has an N2BA/N2B ratio of  $\approx 9$ .<sup>16</sup> Apart from phosphorylation by PKA, the cardiac-specific N2B spring element of titin can also be phosphorylated by PKG, with a similar reduction of cardiomyofibrillar stiffness.<sup>15</sup> Lower cardiomyocyte  $F_{\text{passive}}$  after PKA or PKG can result not only from phosphorylation of titin but also from less diastolic actin-myosin interaction of weakly bound crossbridges. An effect of weakly bound crossbridges on cardiomyocyte  $F_{\text{passive}}$  is unlikely in normal myocardium but possible in failing myocardium because of its increased myofilamentary calcium sensitivity.<sup>18,19</sup>

The present study investigates the importance for the high  $F_{\text{passive}}$  in HF cardiomyocytes of: (1) titin isoform expression; (2) titin phosphorylation; (3) titin isoform phosphorylation; (4) phosphorylation of other myofilamentary proteins; and (5) formation of weakly bound crossbridges. All measurements were performed using human LV myocardial samples derived from transvascular biopsies, surgical biopsies, and explanted hearts. Transvascular procurement allowed HF patients with less advanced HF to be included in the study.

## Patients and Methods

### Patients

The HF group consisted of 48 patients and was composed of 2 subgroups: (1) 44 patients hospitalized for worsening HF (New York Heart Association [NYHA] class IV) and referred for cardiac catheterization and transvascular endomyocardial biopsy because of suspicion of infiltrative or inflammatory myocardial disease; and (2) 4 patients with end-stage dilated cardiomyopathy referred for cardiac transplantation. In all HF patients, coronary angiography revealed absence of significant ( $>50\%$  luminal diameter reduction) coronary artery stenoses and histological examination of the biopsies or of the explanted hearts ruled out active inflammatory or infiltrative myocardial disease. Treatment consisted of angiotensin converting enzyme inhibitors (48/48), diuretics (48/48) and  $\beta$ -blockers (25/48). The 44 HF patients referred for cardiac catheterization and endomyocardial biopsy consisted of 27 HFREF patients and of 17 HFNEF patients. Their hemodynamic data are listed in the Table. The 4 HF patients referred for cardiac transplantation all had HFREF. Their hemodynamic data were not included in the Table because of the long time interval between hemodynamic evaluation

and cardiac transplantation. All patients with HFREF had a LVEF  $< 45\%$ . All patients with HFNEF satisfied the criteria for the diagnosis of HFNEF as recently proposed in a consensus document of the Heart Failure and Echocardiography associations of the European Society of Cardiology.<sup>4</sup>

The CON group consisted of 15 patients and was composed of 3 subgroups: (1) 3 patients with normal LV function and major ventricular arrhythmias; (2) 4 patients with normal LV function and mitral stenosis; and (3) 8 explanted donor hearts. Transvascular LV biopsies were obtained in the first subgroup. In the second subgroup, perioperative transmural LV biopsies were obtained at the time of mitral valve replacement. The endomyocardial portion of the transmural biopsy was used to allow for comparison with the endomyocardial biopsies of the HF patients. No patient had significant ( $>50\%$  luminal diameter reduction) coronary artery disease. Donors did not undergo hemodynamic evaluation before explantation and their hemodynamic data were therefore not included in the Table.

The AS group consisted of 25 patients with significant and symptomatic aortic valve stenosis (mean aortic valve area:  $0.5 \pm 0.3$  cm<sup>2</sup>). Patients had syncope, angina, and/or dyspnea ( $\leq$ NYHA class II). Their hemodynamic data are also listed in the Table. No patient had significant ( $>50\%$  luminal diameter reduction) coronary artery disease. In the AS group, LV myocardial samples were procured perioperatively at the time of aortic valve replacement by transmural biopsy. The endomyocardial portion of the transmural biopsy was used to allow for comparison with the endomyocardial biopsies of the HF patients.

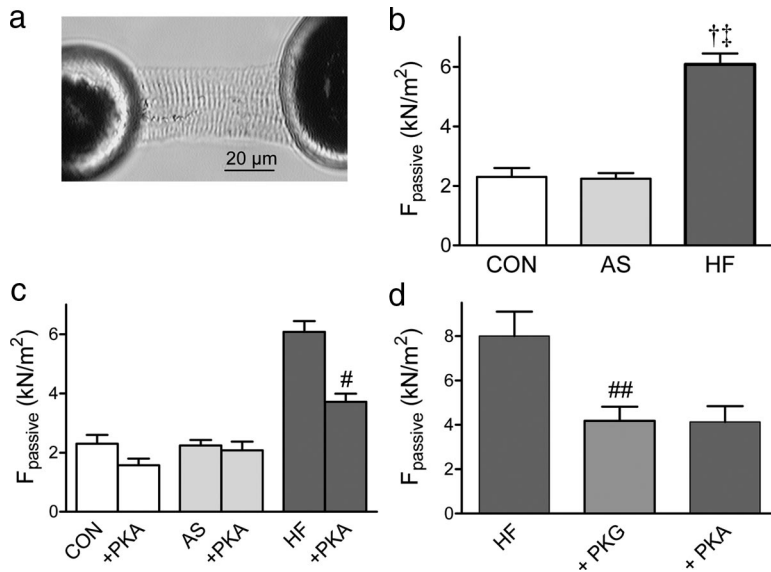
The local ethics committee approved the study protocol. Written informed consent was obtained, and there were no complications related to catheterization or biopsy procurement.

### $F_{\text{passive}}$ Measurements in Isolated Cardiomyocytes

Force measurements were performed in single, mechanically isolated cardiomyocytes as described previously.<sup>7-9</sup> Force measurements were obtained in cardiomyocytes isolated from transvascular biopsies (CON:  $n=3$ ; HF:  $n=25$ ) and perioperative biopsies (CON:  $n=4$ ; AS:  $n=18$ ). 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 subsequently attached with silicone adhesive between a force transducer and a piezoelectric motor ( $2.7 \pm 0.4$  cardiomyocytes per patient). Sarcomere length of isolated cardiomyocytes was adjusted to  $2.2 \mu\text{m}$ . To assess the effect of PKA on the  $F_{\text{passive}}$ , myocytes were incubated for 40 minutes in relaxing solution supplemented with the catalytic subunit of PKA (100 U/mL; Sigma, batch 12K7495) and dithiothreitol (6 mmol/L; Sigma). To study the effect of PKG,  $F_{\text{passive}}$  measurements were also performed after incubation of HF cardiomyocytes in relaxing solution containing PKG1 $\alpha$  (0.1 U/mL; Sigma, batch 034K1336), guanosine cGMP (10  $\mu\text{mol/L}$ , Sigma) and dithiothreitol (6 mmol/L; Sigma). After 40 minutes of incubation with PKG,  $F_{\text{passive}}$  measurements were repeated. Thereafter, cardiomyocytes were incubated with PKA, as described above, and their  $F_{\text{passive}}$  were reassessed. To study the contribution of the thin filament to  $F_{\text{passive}}$ , HF cardiomyocytes were incubated for 40 minutes with the actin-capping protein gelsolin (0.05 mg/mL; clone FX-45, kindly provided by Prof Dr Henk Granzier [University of Arizona, Tucson]<sup>20</sup>) and subsequently exposed to PKA. To investigate the involvement of weak crossbridge interaction,  $F_{\text{passive}}$  of HF cardiomyocytes was also measured in relaxing solution containing 2,3-butanedione monoxime (BDM) (40 mmol/L for 5 minutes; Sigma). Force values were normalized for myocyte cross-sectional area.

### Titin Isoform Separation

Titin isoform separation was performed in myocardial samples of 10 HF patients, 9 AS patients, and 8 CON subjects. For 6 HF patients, the myocardial sample was procured by transvascular biopsy and for 4 HF patients the myocardial sample was derived from an explanted heart. For all CON subjects, the myocardial sample was derived from an explanted donor heart. Tissue samples were homogenized in 100



**Figure 1.** Measurement of cardiomyocyte  $F_{\text{passive}}$ . a, Single cardiomyocyte attached to a force transducer and an adjustable lever used to stretch the cardiomyocyte to 2.2- $\mu\text{m}$  sarcomere length. b, Higher  $F_{\text{passive}}$  in cardiomyocytes of HF patients compared to CON group ( $\dagger P < 0.01$  vs CON) and AS patients ( $\dagger\dagger P < 0.001$  vs AS). c, After in vitro administration of PKA,  $F_{\text{passive}}$  fell in HF cardiomyocytes but not in CON and AS cardiomyocytes ( $\# P < 0.0001$  vs HF). d, PKG administration also significantly lowered  $F_{\text{passive}}$  of HF cardiomyocytes ( $\#\#\# P < 0.0001$  vs HF;  $n = 18$ ); however, no further decrease in  $F_{\text{passive}}$  was detected on subsequent PKA treatment.

to 200  $\mu\text{L}$  Tris-sodium dodecyl sulfate buffer (pH 6.8) containing 8  $\mu\text{g}/\text{mL}$  leupeptin (Peptin-Institute, Japan) and phosphatase inhibitor cocktails (PIC I [P2850] and PIC II [P5726], 10  $\mu\text{L}/\text{mL}$  from each; Sigma). Samples were heated (3 minutes at 99°C) and centrifuged (10 minutes, 13,000g at 0°C). Each sample ( $\approx 40 \mu\text{g}$  dry weight) was applied on agarose-strengthened 2% sodium dodecyl sulfate-polyacrylamide gels. The gel was run at 4-mA constant current for  $\approx 16$  hours. Gels were washed and stained with SYPRO Ruby (Molecular Probes, Eugene, Ore) according to the instructions of the manufacturer. Staining was analyzed with a LAS-3000 system (Fuji Science Imaging Systems) and AIDA Image analyzer software (Isotopenmeßgeräte GmbH, Staubenhardt, Germany).<sup>21</sup>

### Myofibrillary Protein and Titin Isoform Phosphorylation

Myofibrillary protein separation and phosphorylation were determined in myocardial samples procured by transvascular biopsy in 8 HF patients and by perioperative biopsy in 7 AS patients. Tissue was dissolved in 1D sample buffer containing 100 mmol/L dithiothreitol, PIC I, PIC II, and protease inhibitor cocktail (10  $\mu\text{L}/\text{mL}$ , P 8340, Sigma), heated (3 minutes at 99°C) and centrifuged (10 minutes, 13,000 g at 0°C), and a sample ( $\approx 35 \mu\text{g}$  dry weight in 25  $\mu\text{L}$ ) was applied on a 3% to 8% gradient gel (Criterion, Bio-Rad). The gel was run at 100 V for 30 minutes followed by 200 V for 50 minutes. The gels were stained for 90 minutes with ProQ Diamond (Molecular Probes). Thereafter, the gels were washed and subsequently stained with SYPRO Ruby (Molecular Probes).<sup>21</sup> The intensity of the myosin binding protein-C band was used to normalize for differences in protein loading.

Titin isoform phosphorylation was determined in perioperative biopsies of 9 AS patients and in transvascular biopsies of 6 HF patients. Tissue samples were handled as for titin isoform separation. The gels were subsequently stained with ProQ Diamond and with SYPRO Ruby and analyzed as described above.

### Statistical Analysis

Values are given as means  $\pm$  SEM. Statistical significance was set at  $P < 0.05$  and was obtained for multiple comparisons between groups by ANOVA followed by a Bonferroni test. Relationships between 2 continuous variables were assessed with linear regression analysis. Statistical analysis was performed with SPSS (version 9.0, SPSS Inc, Chicago, Ill).

## Results

### $F_{\text{passive}}$ of Isolated Cardiomyocytes

Cardiomyocytes were isolated from myocardial samples of the CON ( $n = 7$ ), AS ( $n = 18$ ), and HF ( $n = 25$ ) groups and

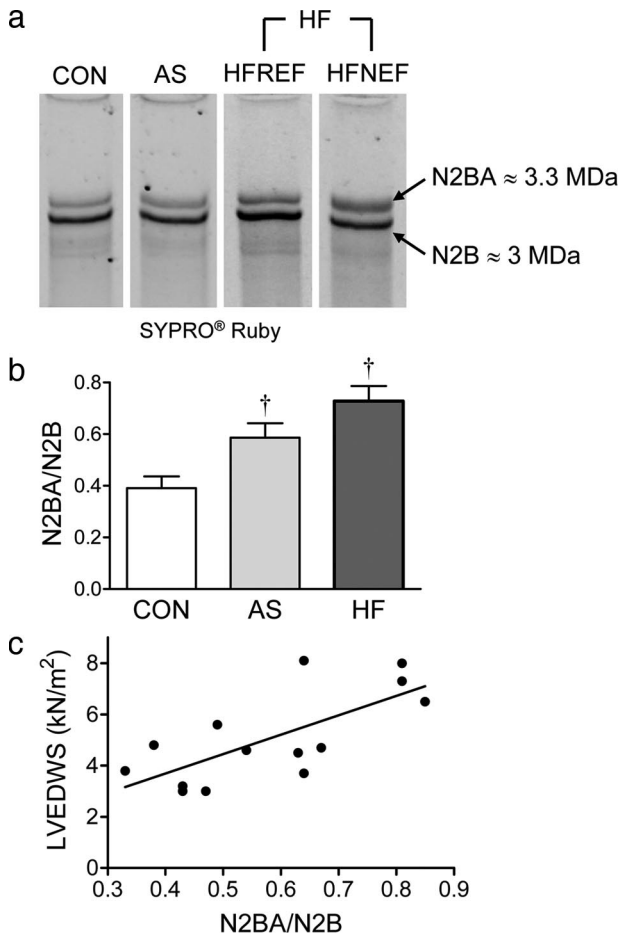
stretched to an identical sarcomere length of 2.2  $\mu\text{m}$  before measuring  $F_{\text{passive}}$  (Figure 1a).  $F_{\text{passive}}$  in HF cardiomyocytes ( $6.1 \pm 0.4 \text{ kN}/\text{m}^2$ ) was higher than in CON cardiomyocytes ( $2.3 \pm 0.3 \text{ kN}/\text{m}^2$ ;  $P < 0.01$ ) or in AS cardiomyocytes ( $2.2 \pm 0.2 \text{ kN}/\text{m}^2$ ;  $P < 0.001$ ) (Figure 1b). Following administration of PKA, the high  $F_{\text{passive}}$  of HF cardiomyocytes fell (Figure 1c). The high  $F_{\text{passive}}$  of HF cardiomyocytes also fell on administration of PKG and remained unaltered on subsequent treatment with PKA (Figure 1d). The high  $F_{\text{passive}}$  of HF cardiomyocytes was unaltered after 40 minutes of exposure to gelsolin despite maximal  $\text{Ca}^{2+}$  activated force falling below 10% of the baseline value. Subsequent administration of PKA continued to induce a significant fall in  $F_{\text{passive}}$  ( $P < 0.05$ ;  $n = 4$ ). Gelsolin exposure confirms involvement of titin in the high  $F_{\text{passive}}$  because it removes the thin filament from the cardiomyocytes.<sup>20</sup> A contribution of crossbridge cycling to  $F_{\text{passive}}$  was ruled out by experiments exposing HF cardiomyocytes to BDM. In these experiments BDM failed to alter cardiomyocyte  $F_{\text{passive}}$  ( $n = 4$ ).

### Titin Isoform Expression

Titin isoform expression was determined in myocardial samples of CON ( $n = 8$ ), AS ( $n = 9$ ) and HF patients ( $n = 10$ ) (Figure 2a). HF patients ( $0.73 \pm 0.06$ ;  $P < 0.001$ ) and AS patients ( $0.59 \pm 0.06$ ;  $P < 0.05$ ) had an N2BA/N2B ratio higher than the CON group ( $0.39 \pm 0.05$ ) (Figure 2b). For 6 HF and all AS patients, myocardial biopsy occurred, respectively, at the time of hemodynamic data acquisition or shortly thereafter. Correlations between N2BA/N2B ratio and hemodynamic variables were limited to this group. A significant correlation ( $r = 0.71$ ;  $P < 0.01$ ) was observed between N2BA/N2B ratio and LV end-diastolic wall stress (LVEDWS) (Figure 2c).

### Overall Titin Phosphorylation and Titin Isoform Phosphorylation

Because titin isoform expression differed between HF and CON, unequal titin isoform expression could have contributed to the difference in  $F_{\text{passive}}$  between HF and CON



**Figure 2.** Titin isoform expression. a, Representative examples of titin isoform separation. b, Higher N2BA/N2B ratio in HF and AS patients than in the CON group ( $\dagger P < 0.05$  vs CON). c, Correlation between N2BA/N2B ratio and LVEDWS ( $r = 0.71$ ;  $P < 0.01$ ).

cardiomyocytes. However, because titin isoform expression was comparable in HF and AS, mechanisms other than a titin isoform shift had to account for raising  $F_{\text{passive}}$  in HF cardiomyocytes compared to AS cardiomyocytes. Overall phosphorylation of titin was therefore compared between HF and AS patients (Figure 3a). The ratio of phosphorylated titin to total titin was similar in HF and AS samples ( $0.29 \pm 0.02$  versus  $0.30 \pm 0.02$ ;  $P = 0.84$ ). Similar ratios of phosphorylated to total protein were also observed for myosin binding protein-C ( $0.21 \pm 0.02$  versus  $0.26 \pm 0.03$ ;  $P = 0.23$ ), desmin ( $0.35 \pm 0.05$  versus  $0.34 \pm 0.05$ ;  $P = 0.93$ ), troponin T ( $0.48 \pm 0.04$  versus  $0.55 \pm 0.05$ ;  $P = 0.32$ ), and myosin light chain-2 ( $0.27 \pm 0.02$  versus  $0.31 \pm 0.01$ ;  $P = 0.09$ ) (values indicate HF versus AS, respectively) (Figure 3a). Only troponin I had a lower ratio of phosphorylated to total protein in HF compared to AS ( $0.12 \pm 0.01$  versus  $0.16 \pm 0.02$ ;  $P = 0.048$ ).

Because titin isoform expression and overall titin phosphorylation were comparable in HF and AS patients, the phosphorylation status of the respective N2BA and N2B titin isoforms was subsequently investigated in myocardial samples of HF and AS patients (Figure 3b). The P-N2BA/P-N2B ratio was higher in HF ( $0.77 \pm 0.05$ ) than in AS ( $0.54 \pm 0.05$ ;  $P < 0.01$ ) (Figure 3c), consistent with lower phosphorylation of the stiff N2B and higher phosphorylation of the compliant

N2BA titin isoform. Lower phosphorylation of N2B explains the elevated  $F_{\text{passive}}$  of HF cardiomyocytes because titin phosphorylation was previously shown to lower  $F_{\text{passive}}$  in an isoform-dependent manner, with a larger fall in  $F_{\text{passive}}$  when more of the stiff N2B isoform was phosphorylated.<sup>16</sup> The P-N2BA/P-N2B ratio correlated with the N2BA/N2B ratio in AS patients ( $r = 0.68$ ;  $P < 0.05$ ) (Figure 3d). This correlation was absent in HF patients.

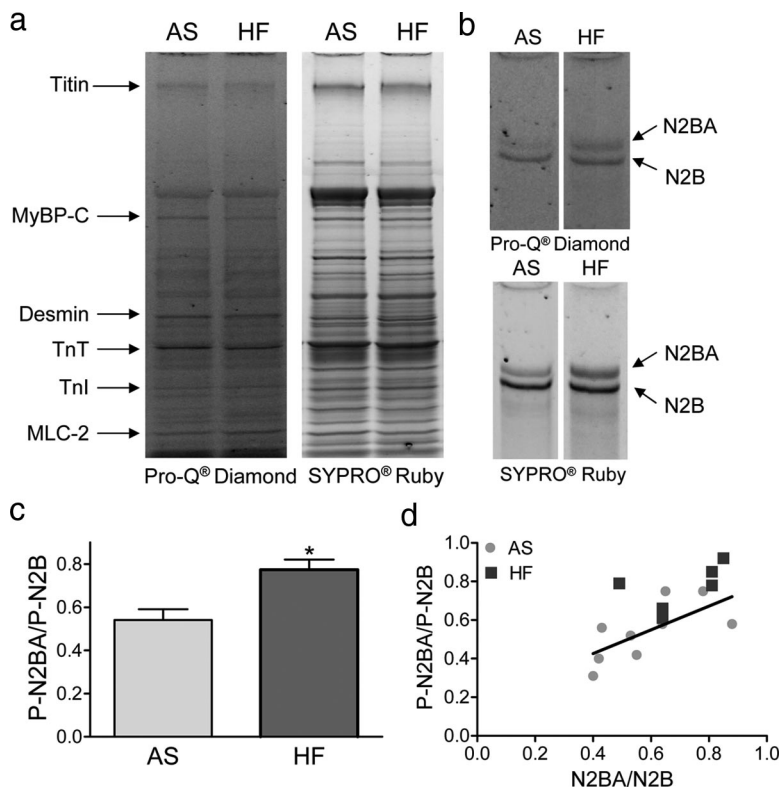
### HFNEF Versus HFREF

In previous studies,  $F_{\text{passive}}$  was especially elevated in cardiomyocytes isolated from LV myocardium of HFNEF patients.<sup>7-9</sup>  $F_{\text{passive}}$ , titin isoform expression and titin isoform phosphorylation were therefore compared in subgroups of HFNEF and HFREF patients (Figure 4a through 4e), whose hemodynamic characteristics are shown in the Table. HFREF patients had the highest N2BA/N2B ratio ( $0.77 \pm 0.07$ ) (Figure 4b). LVEDWS was also highest in HFREF ( $8.7 \pm 0.7$  kN/m<sup>2</sup>) (Figure 4c).  $F_{\text{passive}}$  ( $7.4 \pm 0.7$  kN/m<sup>2</sup>) and the PKA-induced decrease in  $F_{\text{passive}}$  ( $-3.2 \pm 0.6$  kN/m<sup>2</sup>) were largest in HFNEF (Figure 4d).  $F_{\text{passive}}$  and the PKA-induced absolute change in  $F_{\text{passive}}$  rose from AS to HFREF and to HFNEF (Figure 4e) ( $P < 0.05$  for trend). The deficit of N2B titin isoform phosphorylation followed a similar course also rising from AS to HFREF and to HFNEF (Figure 4e) ( $P < 0.05$  for trend). The deficit was expressed as the difference between observed P-N2BA/P-N2B ratio and predicted P-N2BA/P-N2B ratio. The predicted P-N2BA/P-N2B ratio was derived from the observed N2BA/N2B ratio and the equation describing the linear curve fit of Figure 3d. A larger N2B titin isoform phosphorylation deficit in HFNEF patients explains both their higher  $F_{\text{passive}}$  and their larger PKA-induced fall of  $F_{\text{passive}}$ .

## Discussion

### High $F_{\text{passive}}$ of HF Cardiomyocytes and Titin

In the present study,  $F_{\text{passive}}$  was twice as high in human HF cardiomyocytes than in human AS cardiomyocytes. The high  $F_{\text{passive}}$  of HF cardiomyocytes fell after in vitro administration of PKA or PKG but remained unchanged after in vitro administration of BDM or gelsolin. The PKA- and PKG-induced decrease in  $F_{\text{passive}}$  suggested a phosphorylation deficit of myofilamentary proteins, which are a target for both PKA and PKG. Only troponin I and titin have so far been identified as myofilamentary proteins targeted by both PKA and PKG.<sup>15,22</sup> The present study observed a phosphorylation deficit of troponin I in HF myocardium. The concomitant increase in myofilamentary calcium sensitivity could promote diastolic actin-myosin interaction and thereby raise  $F_{\text{passive}}$  in HF cardiomyocytes.<sup>19</sup> Such an effect of weakly bound cross-bridges on  $F_{\text{passive}}$  was however ruled out by the unchanged  $F_{\text{passive}}$  following exposure to BDM, which inhibits cross-bridge cycling. Involvement of the giant cytoskeletal protein titin in the high  $F_{\text{passive}}$  of HF cardiomyocytes was suggested by unaltered  $F_{\text{passive}}$  after gelsolin, which removes the thin filament from the cardiomyocytes.<sup>20,23,24</sup> Effective thin filament removal was evident from the fall in maximal  $\text{Ca}^{2+}$ -activated force to less than 10% of its baseline value. The ability of PKA to lower  $F_{\text{passive}}$  after thin filament removal



**Figure 3.** Overall titin and titin isoform phosphorylation. a, Comparison between a representative AS and HF patient of phosphorylation of myofibrillar proteins using sequential gel electrophoresis with ProQ Diamond phosphoprotein and SYPRO Ruby protein stain. b, Comparison between a representative AS and HF patient of phosphorylation of titin isoforms using sequential gel electrophoresis with SYPRO Ruby and ProQ Diamond phosphoprotein stain. c, Higher P-N2BA/P-N2B ratio in HF than in AS ( $*=P<0.01$ ). d, Correlation between P-N2BA/P-N2B ratio and N2BA/N2B ratio in AS patients ( $r=0.68$ ;  $P<0.05$ ). All HF patients fell above the regression line.

implied phosphorylation of titin to mediate the PKA-induced fall in  $F_{\text{passive}}$ .

The present study observed titin isoform expression to be comparable in HF and AS myocardium. Mechanisms other than a titin isoform shift therefore had to account for the difference in  $F_{\text{passive}}$  between HF and AS cardiomyocytes. Overall titin phosphorylation was also similar in HF and AS myocardium. However, relative titin isoform phosphorylation was different, with less N2B isoform phosphorylated in HF than in AS myocardium. Less N2B titin isoform phosphorylation was previously shown to raise  $F_{\text{passive}}$ ,<sup>16</sup> and the observed hypophosphorylation of the stiff N2B titin isoform relative to the compliant N2BA titin isoform could therefore explain the higher  $F_{\text{passive}}$  in HF cardiomyocytes.

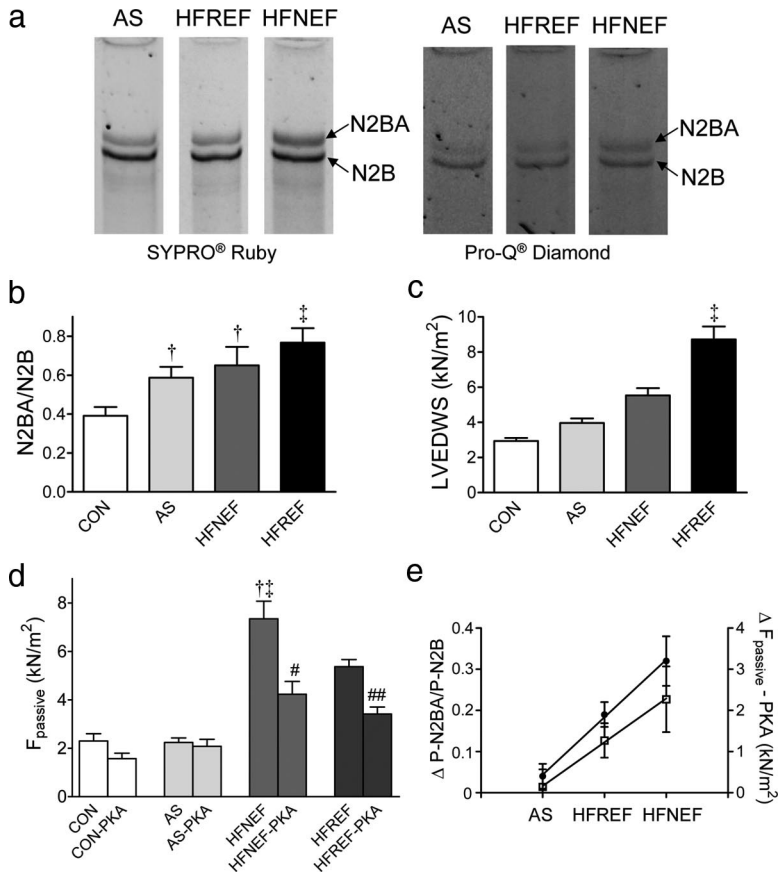
### Titin Isoform Expression

In a subgroup of HF and AS patients, hemodynamic data were obtained either at the time of transvascular biopsy or shortly before perioperative biopsy. In these patient groups, myocardial titin isoform expression could accurately be correlated with hemodynamic variables. The closest correlation observed was between N2BA/N2B ratio and LVEDWS (Figure 2c). The higher expression of the compliant N2BA titin isoform at higher LVEDWS implies titin isoform shifts to be a compensatory mechanism whereby higher expression of the compliant N2BA titin isoform compensates for an elevated LVEDWS. A compensatory role of titin isoform shifts has previously also been suggested in patients with dilated cardiomyopathy, in whom the N2BA/N2B ratio correlated with the LV end-diastolic volume/pressure ratio, a measure of end-diastolic LV distensibility, and with peak oxygen consumption during exercise.<sup>11</sup> The time span over

which LVEDWS rises is probably an important determinant for shifts in titin isoform expression because 2 canine dilated cardiomyopathy models with rapid elevation of LVEDWS induced by pacing tachycardia or by intracoronary microembolizations revealed an opposite shift with overexpression of the stiff N2B titin isoform.<sup>25–27</sup> Furthermore, the correlation observed in the present study between N2BA/N2B ratio and LVEDWS is also consistent with stress-sensing abilities of titin. Stress-sensing abilities of titin reside both in its kinase domain at the M-line<sup>28</sup> and in the titin/Tcap/muscle LIM protein complex at the Z-disc.<sup>29</sup>

### Titin Isoform Phosphorylation

Higher  $F_{\text{passive}}$  in HF than in AS cardiomyocytes was attributed to a shift in isoform phosphorylation with more of the N2BA and less of the N2B titin isoform phosphorylated in HF myocardium. Previous studies indeed demonstrated less reduction in  $F_{\text{passive}}$  when the compliant N2BA titin isoform is phosphorylated than when the stiff N2B titin isoform is phosphorylated.<sup>16</sup> The present study not only observed higher N2BA titin isoform phosphorylation in HF myocardium but also a loss in HF myocardium of the close relationship between titin isoform expression and titin isoform phosphorylation, which was present in AS myocardium (Figure 3d). The uniformly low  $F_{\text{passive}}$  observed in AS cardiomyocytes despite the N2BA/N2B titin isoform ratio ranging from 0.4 to 0.9 (Figure 3d), probably resulted from the concordant changes in titin isoform expression and phosphorylation in AS myocardium, whereby higher N2B titin isoform phosphorylation offsets a rise of  $F_{\text{passive}}$  induced by higher N2B titin isoform expression. In HF myocardium, this concordance between titin isoform expression and phosphorylation



**Figure 4.** Titin isoform expression, titin isoform phosphorylation, and cardiomyocyte  $F_{passive}$  in HFNEF and HFREF patients. a, Comparison between representative AS, HFREF, and HFNEF patients of phosphorylation of titin isoforms using sequential gel electrophoresis with SYPRO Ruby and ProQ Diamond phosphoprotein stain. b, Higher N2BA/N2B ratio in AS, HFNEF, and HFREF patients than in the CON group ( $\dagger P < 0.05$  vs CON,  $\ddagger P < 0.001$  vs CON). c, Higher LVEDWS in HFREF patients than in CON, AS, and HFNEF groups ( $\ddagger P < 0.001$  vs CON, AS, or HFNEF). d, Higher  $F_{passive}$  in HFNEF patients than in CON, AS, and HFREF patients ( $\ddagger P < 0.001$  vs CON or AS;  $\dagger P < 0.01$  vs HFREF). After in vitro administration of PKA,  $F_{passive}$  fell in HFNEF ( $\#\# P < 0.0001$  vs HFNEF) and HFREF cardiomyocytes ( $\#\#\# P < 0.0001$  vs HFREF). e, Parallel trends in AS, HFREF, and HFNEF patients of the N2B phosphorylation deficit ( $\Delta P-N2BA/P-N2B$ ) (open squares) and the PKA-induced fall in  $F_{passive}$  (filled circles).

was absent. This could be of special relevance to HFNEF myocardium, which had higher N2B titin isoform expression (Figure 4b) and a larger N2B titin isoform phosphorylation deficit (Figure 4e). The latter explained both the larger  $F_{passive}$  and the larger response of  $F_{passive}$  to PKA in HFNEF cardiomyocytes (Figure 4e).

A shift in titin isoform phosphorylation, as demonstrated in the present study in HF myocardium, can be explained by spatial separation of phosphorylation sites on the N2BA and N2B titin isoforms and by changes in compartmentalized activities of protein kinases and phosphodiesterases.<sup>30,31</sup> The N2B unique sequence contains the phosphorylation site, which mediates the fall in  $F_{passive}$ .<sup>13,15,32</sup> Immunoelectron micrographs with antibody labeling C-terminal N2B unique sequence revealed the site to be closer to the Z-disc in the N2BA isoform than in the N2B isoform.<sup>26</sup> PKA is targeted by A-kinase anchoring proteins (AKAP) to transverse tubules,<sup>30</sup> which are also adjacent to the Z disc. In HF, this targeting of PKA by AKAP is disrupted,<sup>33</sup> and this leads to a fall in phosphorylation of surrounding myofilamentary proteins.<sup>30</sup> The shift in titin isoform phosphorylation in HF myocardium could result from altered local availability of not only cAMP but also cGMP because in mouse, cardiac muscle phosphodiesterase 5 activity is also highly compartmentalized.<sup>34</sup>

**Conclusions**

Because of comparable expression of titin isoforms and similar overall phosphorylation of titin, the 2-fold rise in  $F_{passive}$  of HF cardiomyocytes compared to AS cardiomyo-

cytes was attributed to a shift in titin isoform phosphorylation with relative hypophosphorylation of the stiff N2B titin isoform. Relative hypophosphorylation of the stiff N2B titin isoform is a novel mechanism responsible for raising  $F_{passive}$  in human HF cardiomyocytes. High cardiomyocyte  $F_{passive}$  is an important contributor to the high diastolic LV stiffness of the failing heart, and the present findings could therefore have important therapeutic implications.

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**Disclosures**

None.

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