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

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


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-μm 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±0.4 kN/m^2) than in CON (2.3±0.3 kN/m^2; P<0.01) or in AS (2.2±0.2 kN/m^2; P<0.001). Titin isoform expression differed between HF (N2BA/N2B=0.73±0.06) and CON (N2BA/N2B=0.39±0.05; P<0.001) and was comparable in HF and AS (N2BA/N2B=0.59±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±0.05) than in AS (P-N2BA/P-N2B=0.54±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). In HFREF, diastolic LV dysfunction correlates with exercise intolerance; in HFNEF, diastolic LV dysfunction is an essential diagnostic feature. Diastolic LV passive have been attributed to the giant cytoskeletal protein titin, which can modulate alterations in cardiomyocyte $F_{\text{passive}}$ have been attributed to the giant cytoskeletal protein titin, which can modulate alterations in cardiomyocyte $F_{\text{passive}}$ have been attributed to the giant cytoskeletal protein titin, which can modulate alterations in cardiomyocyte $F_{\text{passive}}$ have been attributed to the giant cytoskeletal protein titin, which can modulate alterations in cardiomyocyte $F_{\text{passive}}$ have been attributed to the giant cytoskeletal protein titin, which can modulate alterations in cardiomyocyte $F_{\text{passive}}$ have been attributed to the giant cytoskeletal protein titin, which can modulate alterations in cardiomyocyte $F_{\text{passive}}$ have been attributed to the giant cytoskeletal protein titin, which can modulate alterations in cardiomyocyte $F_{\text{passive}}$ have been attributed to the giant cytoskeletal protein titin, which can modulate alterations in cardiomyocyte $F_{\text{passive}}$ have been attributed to the giant cytoskeletal protein titin, which can modulate.
Phosphorylation of the cardiac-specific N2B spring element of titin by PKA reduces $F_{\text{passive}}$ of rat cardiomyocytes and of rat cardiac myofibrils. 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 $\approx0.1$, and the smallest effect in bovine atrial myocardium, which has an N2BA/N2B ratio of $\approx0.16$. Apart from phosphorylation by PKA, the cardiac-specific N2B spring element of titin can also be phosphorylated by PKG, with a similar reduction of myofibrillar stiffness. 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. 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 significant 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.

The HF 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: $0.5\pm0.3$ cm$^2$). Patients had syncope, angina, and/or dyspnea ($\approx$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 peroperatively 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.

**Table. Clinical and Hemodynamic Patient Data**

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<th>AS (n=25)</th>
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<td>24±1†</td>
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<tr>
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<td>132±6*</td>
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<tr>
<td>LV Stiff Mod, kN/m²</td>
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<td>LVEDWS, kN/m²</td>
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<td>4.0±0.3‡</td>
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</tr>
</tbody>
</table>

*P<0.05 vs CON, †P<0.01 vs CON, ‡P<0.001 vs CON, §P<0.001 vs AS, ¶P<0.001 vs HFREF.

### 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 derived from 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
to 200 μL Tris–sodium dodecyl sulfate buffer (pH 6.8) containing 8 μg/mL leupeptin (Peptin Institute, Japan) and phosphatase inhibitor cocktails (PIC I [P2850] and PIC II [P5726], 10 μL/mL from each; Sigma). Samples were heated (3 minutes at 99°C) and centrifuged (10 minutes, 13,000g at 0°C). Each sample (~40 μg dry weight) was applied on agarose-strengthened 2% sodium dodecyl sulfate–polyacrylamide gels. The gel was run at 4 mA constant current for ~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).

Myofilamentary Protein and Titin Isoform Phosphorylation

Myofilamentary 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 μL/mL, P 8340, Sigma), heated (3 minutes at 99°C) and centrifuged (10 minutes, 13,000g at 0°C), and a sample (~35 μg dry weight in 25 μL) 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). 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±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

Fpassive 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 μm before measuring Fpassive (Figure 1a). Fpassive in HF cardiomyocytes (6.1±0.4 kN/m²) was higher than in CON cardiomyocytes (2.3±0.3 kN/m²; P<0.01) or in AS cardiomyocytes (2.2±0.2 kN/m²; P<0.001) (Figure 1b). Following administration of PKA, the high Fpassive of HF cardiomyocytes fell (Figure 1c). The high Fpassive of HF cardiomyocytes also fell on administration of PKG and remained unaltered on subsequent treatment with PKA (Figure 1d). The high Fpassive of HF cardiomyocytes was unaltered after 40 minutes of exposure to gelsolin despite maximal Ca2⁺ activated force falling below 10% of the baseline value. Subsequent administration of PKA continued to induce a significant fall in Fpassive (P<0.05; n=4). Gelsolin exposure confirms involvement of titin in the high Fpassive because it removes the thin filament from the cardiomyocytes. A contribution of crossbridge cycling to Fpassive was ruled out by experiments exposing HF cardiomyocytes to BDM. In these experiments BDM failed to alter cardiomyocyte Fpassive (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±0.06; P<0.001) and AS patients (0.59±0.06; P<0.05) had an N2BA/N2B ratio higher than the CON group (0.39±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 Fpassive between HF and CON.
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±0.02 versus 0.30±0.02; $P=0.84$). Similar ratios of phosphorylated to total protein were also observed for myosin binding protein-C (0.21±0.02 versus 0.26±0.03; $P=0.23$), desmin (0.35±0.05 versus 0.34±0.05; $P=0.93$), troponin T (0.48±0.04 versus 0.55±0.05; $P=0.32$), and myosin light chain-2 (0.27±0.02 versus 0.31±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±0.01 versus 0.16±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/N2B ratio was higher in HF (0.77±0.05) than in AS (0.54±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. 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.

**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. The present study observed a phosphorylation 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 of N2B titin isoform phosphorylation explains both their higher $F_{\text{passive}}$ and their larger PKA-induced fall of $F_{\text{passive}}$.  

**HFNEF Versus HFREF**

In previous studies, $F_{\text{passive}}$ was especially elevated in cardiomyocytes isolated from LV myocardium of HFNEF patients. $F_{\text{passive}}$, 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±0.07) (Figure 4b). LVEDWS was also highest in HFREF (8.7±0.7 kN/m²) (Figure 4c). $F_{\text{passive}}$ (7.4±0.7 kN/m²) and the PKA-induced decrease in $F_{\text{passive}}$ (−3.2±0.6 kN/m²) were largest in HFNEF (Figure 4d). 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 $F_{\text{passive}}$.  

$F_{\text{passive}}$ 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. 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. Such an effect of weakly bound crossbridges on $F_{\text{passive}}$ was however ruled out by the unchanged $F_{\text{passive}}$ following exposure to BDM, which inhibits crossbridge 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. Effective thin filament removal was evident from the fall in maximal Ca²⁺-activated force to less than 10% of its baseline value. The ability of PKA to lower $F_{\text{passive}}$ after thin filament removal
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 titin isoform phosphorylated in HF than in AS myocardium. Less N2B titin isoform phosphorylation was previously shown to raise $F_{\text{passive}}$, 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. 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. 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 and in the titin/Tcap/muscle LIM protein complex at the Z-disc.

**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. 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...
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 $F_{\text{passive}}$ and the larger response of $F_{\text{passive}}$ 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.30,31 The N2B unique sequence contains the phosphorylation site, which mediates the fall in $F_{\text{passive}}$.13,15,32 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.26 PKA is targeted by A-kinase anchoring proteins (AKAP) to transverse tubules,30 which are also adjacent to the Z disc. In HF, this targeting of PKA by AKAP is disrupted,33 and this leads to a fall in phosphorylation of surrounding myofilamentary proteins.30 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.34

Conclusions
Because of comparable expression of titin isoforms and similar overall phosphorylation of titin, the 2-fold rise in $F_{\text{passive}}$ of HF cardiomyocytes compared to AS cardiomyocytes 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 $F_{\text{passive}}$ in human HF cardiomyocytes. High cardiomyocyte $F_{\text{passive}}$ 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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