Changes in Titin Isoform Expression in Pacing-Induced Cardiac Failure Give Rise to Increased Passive Muscle Stiffness

Yiming Wu, MD, PhD; Stephen P. Bell, BA; Karoly Trombitas, PhD; Christian C. Witt, PhD; Siegfried Labeit, MD; Martin M. LeWinter, MD; Henk Granzier, PhD

Background—Titin contains a molecular spring segment that underlies passive myocardial stiffness. Myocardium coexpresses titin isoforms with molecular spring length variants and, consequently, distinct stiffness characteristics: the stiff N2B isoform (short spring) and more compliant N2BA isoform (long spring). We tested whether changes in titin isoform expression occur in the diastolic dysfunction that accompanies heart failure.

Methods and Results—We used the tachycardia-induced dilated cardiomyopathy canine model (4-week pacing) and found that control myocardium coexpresses the N2B and N2BA isoforms at similar levels, whereas in dilated cardiomyopathy the expression ratio had shifted, without affecting the amount of total titin, toward more prominent N2B expression. This shift was accompanied by elevated titin-based passive muscle stiffness. Pacing also resulted in significant upregulation of obscurin, an 800-kDa elastic protein with several signaling domains.

Conclusions—Coexpression of titin isoforms with distinct mechanical properties allows modulation of passive stiffness via adjustment of the isoform expression ratio. The canine pacing-induced heart failure model uses this mechanism to increase myocardial stiffness. Thus, changes in titin isoform expression may play a role in diastolic dysfunction in heart failure. (Circulation. 2002;106:1384-1389.)

Key Words: diastole ■ elasticity ■ cardiomyopathy ■ heart failure ■ myocardium

Although diastolic dysfunction with reduced passive ventricular compliance has been recognized in a variety of patients with heart failure, its molecular basis is not fully understood. Diastolic pressure results from the tension that develops when passive myocardium is stretched beyond or shortened below its slack length. This passive tension is derived largely from the extracellular matrix protein collagen and the intrasarcomeric protein titin. Collagen normally dominates at the upper limit of the physiological sarcomere length (SL) range, whereas titin dominates at short to intermediate lengths.2,3

Titin spans the half-sarcomeric distance from the Z line to the M line, with an extensible I-band segment that functions as a molecular spring that develops passive tension (for a recent review, see Granzier and Labeit5). Titin is encoded by a single gene containing 363 exons that are differentially spliced, creating isoforms with different elasticity, with myocardium expressing the N2B and N2BA isoforms.5–7 The N2B isoform contains within its extensible I-band region the cardiac-specific N2B exon and short tandem Ig and PEVK segments. The larger N2BA isoform includes the N2B exon, N2A exons (hence the name N2BA), and longer tandem Ig and PEVK segments.5,7 Because the fractional extension (end-to-end length divided by maximal length) of the molecular spring determines passive tension levels,4 the shorter tandem Ig and PEVK segments of the N2B isoform result in passive stiffness (slope of the passive tension–SL relation) that is substantially higher than for N2BA titin. Cardiac muscle can express these splice variants alone, or they can be coexpressed.6

Several earlier studies reported that the amount of intact titin is reduced in patients with heart failure, suggesting that titin is involved in altered ventricular compliance of the cardiomyopathic heart. Furthermore, recent linkage and sequencing studies have shown that titin is responsible for familial forms of dilated cardiomyopathy (DCM).12–14

To better understand the role of titin in diastolic dysfunction, we studied the canine tachycardia-induced model of DCM. This model was selected because of its many similarities to human DCM, its temporal consistency, and the fact that a previous study with short-term (2-week) pacing suggested changes in titin expression as a possible explanation...
for reduced diastolic suction.\textsuperscript{15} In the present study, we investigated animals paced for 4 weeks, with additional progression of myocardial failure, and showed that relative to controls, the total amount of titin was unchanged but the ratio of N2BA to N2B isoforms was reduced. Mechanical studies on muscle revealed that titin-based passive tension is significantly elevated in DCM. We conclude that in addition to stressing and possibly losing animals at the end of the pacing period. Control animals underwent a thoracotomy without pacemaker implantation. After 4 weeks of pacing, the pacemaker was disabled, and paced and sham dogs were anesthetized, a fifth interspace thoracotomy was performed, and a full-thickness specimen of the LV anterior wall at the level of the minor equator was removed. To avoid the possibility that transmural gradients in isoform expression\textsuperscript{16,15} give rise to variable results, the studies described below were restricted to tissue obtained from the middle of the wall.

### Methods

#### Tachycardia-Induced Cardiac Failure

The model has been described.\textsuperscript{15} Briefly, a limited right thoracotomy was performed on purpose-bred mongrel dogs (\textasciitilde 25 kg), and a pacing electrode was screwed into the right ventricular free wall. On the day after surgery, the pacing rate was set at 235 bpm and maintained at that level for 4 weeks. Numerous studies have documented that 4 weeks of pacing is sufficient to consistently cause myocardial failure with chamber dilatation, reduced ejection fraction, reduced ventricular chamber compliance, and increased muscle stiffness (see, for example, Solomon et al\textsuperscript{16} and Neumann et al\textsuperscript{17} and references therein). Accordingly, we did not measure hemodynamics or left ventricular (LV) function at the end of this study to avoid stressing and possibly losing animals at the end of the pacing period.

#### Passive Tension and SL Measurement

The setup and protocols for force and SL measurement have been described.\textsuperscript{2,3,6,19} Maximum active force (at a pCa of 4.5) was measured at an SL of \textasciitilde 2.2 m for paced animals and \textasciitilde 2.35 m for controls, the total amount of titin was unchanged but the ratio of N2BA to N2B isoforms was reduced. Mechanical studies on muscle revealed that titin-based passive tension is significantly elevated in DCM. We conclude that in addition to stressing and possibly losing animals at the end of the pacing period. Control animals underwent a thoracotomy without pacemaker implantation. After 4 weeks of pacing, the pacemaker was disabled, and paced and sham dogs were anesthetized, a fifth interspace thoracotomy was performed, and a full-thickness specimen of the LV anterior wall at the level of the minor equator was removed. To avoid the possibility that transmural gradients in isoform expression\textsuperscript{16,15} give rise to variable results, the studies described below were restricted to tissue obtained from the middle of the wall.

#### Gel Electrophoresis and Western Blotting

Sample preparation and gel electrophoresis were performed as described earlier.\textsuperscript{2,6} Wet gels were scanned and analyzed with 1D scan software (Scanalytics Inc). The integrated optical density (OD) of N2BA titin, N2B titin, total titin (N2BA+N2B+T2), and myosin heavy chain (MHC) were determined at each loading. Using regression analysis, we calculated the slope of the linear range of the relation between integrated OD and loading for each protein. The N2BA/N2B ratio was calculated as the slope of N2BA titin divided by the slope of N2B titin. The slope ratio of total titin and MHC was also calculated (Table). To estimate the molecular weights of unknown proteins (Figure 1A), we coelectrophoresed skeletal muscle (soleus) samples and made a calibration curve of mobility versus molecular weight using as standards nebulin (800 kDa), titin (3700 kDa), and MHC (205 kDa).\textsuperscript{18} Western blotting was performed with the antibodies Z1/Z2 (which labels the N-terminus of titin) and F6/I7 and I48-F50 (which label the N- and C-termini of obscurin, respectively). For details see References 5 through 7.

#### Muscle

Muscle strips (length \textasciitilde 2.5 mm, diameter \textasciitilde 0.3 mm) were dissected from the LV midwall specimens, skinned overnight at 4°C in relaxing solution containing 1% vol/vol Triton X-100, and then washed thoroughly with relaxing solution. All solutions contained protease inhibitors (for details, see Reference 3).
controls. (Damage to collagen at long SLs explains the limited reversible SL range.2,3) Passive force was also measured after muscle was extracted in relaxing solution containing first 0.6 mol/L KCl and then 1 mol/L KI.3 The KCl/KI-sensitive tensions may be assumed to be titin-based and KCl/KI-insensitive tension to be collagen-based. (For details see Reference 3.) The cross-sectional area of muscle was measured2 and used to convert forces into tensions. Experiments were done at 20°C to 22°C.

Immunoelectron Microscopy
Muscles were stretched, fixed, immunolabeled with N2Bc antibody,20 embedded, and processed for immunoelectron microscopy (IEM) as described.20 Negatives were scanned and the integrated ODs of epitopes (see Results) determined with 1D scan software (Scanalytics Inc).

Statistics
Results are reported as mean±SEM. Differences were assessed with Student’s t test, with a value of P<0.05 indicating a significant difference. The results shown in Figures 3 and 4 were obtained from 5 control and 6 paced animals. For each animal, 2 to 5 muscle strips were studied, the results of which were averaged. The mean values of all animals were then pooled, to calculate the final mean and SEM.

Results
Protein Expression
Figure 1 shows a typical gel-electrophoresis result. Consistent with earlier studies,6 the control myocardium coexpresses N2B and N2BA titin isoforms with expression levels that are approximately equal. In paced animals, in contrast, the expression level of N2BA titin was reduced and N2B expression was more prominent (Figure 1, A through C), indicating that changes in titin expression had taken place. Densitometric analysis of gels revealed that the total titin-to-MHC ratio was similar in paced and control animals (Table). This is consistent with our earlier study of myocardium from species with isoform expression ratios that vary greatly, in which we found a constant titin thick-filament stoichiometry.6 The ratio of N2BA to N2B titins was reduced significantly, from 1.01 in controls to 0.80 in the paced group (Table). Thus, pacing does not influence the total amount of titin, but the expression level of the stiffer N2B isoform increases at the expense of the compliant N2BA isoform.

Minor proteins with estimated molecular weights ranging from ~250 to ~800 kDa were also visible on gels (Figure 1A). Of these proteins, the ~800-kDa protein was significantly upregulated by pacing (Table), whereas the other proteins were not significantly affected (results not shown). Western blotting revealed that the ~800-kDa protein is obscurin (Figure 1D), a large modular protein with various domains involved in signaling processes.7,21 Earlier, we suggested that obscurin is involved in the sarcomeric restructuring and adaptations that occur during

Figure 2. A, Sequence of I-band region of N2B and N2BA cardiac titins (based on Reference 5). N2BA has a longer PEVK segment (yellow) and additional immunoglobulin domains (red). B, Schematic of stretched sarcomere that coexpresses N2B and N2BA titins, explaining how IEM can be used to study coexpression. N2Bc antibody labels C-terminal of N2B unique sequence and gives rise to an epitope that is closer to Z line in N2BA titin than in N2B titin. (For details, see Reference 20.) C, Immunoelectron micrographs of sarcomeres of control and paced myocardium, labeled with N2Bc. Both tissues reveal 2 epitopes: 1 diffuse epitope near Z line (A) and another, sharper epitope toward A band (B). B epitope (derived from N2B titin) is more pronounced in paced animals, indicating that expression of N2B titin is upregulated.
muscle disease, and the present findings are consistent with this proposal.

Immunoelectron Microscopy

Coexpression of titin isoforms was also studied with the N2Bc antibody. This antibody labels an epitope found in both N2B and N2A isoforms. Because of the extra compliance provided by the additional PEVK residues and Ig domains of N2BA titin, in stretched sarcomeres the epitope is closer to the Z line in N2BA than N2B titan (Figure 2C, epitopes A and B). Thus, sarcomeres that coexpress isoforms will show two epitopes. Their relative staining intensities reflect the relative abundance of the isoform types. Both paced and control myocardium revealed two epitopes in the I band (Figure 2C), consistent with coexpression of isoforms at the level of the half-sarcomere. The near-Z-line epitope was relatively diffuse (epitope A), which may be a result of coexpression of N2BA subisoforms that contain variable numbers of Ig domains interspersed between the N2B and N2A exons. In sarcomeres of controls, the intensity of the 2 N2Bc epitopes is approximately equal. In paced animals, the epitope derived from the N2BA isoform appears weaker than the N2B isoform (Figure 2C, epitopes A and B), consistent with increased N2B expression at the expense of N2BA titin. The integrated OD of the N2BA and N2B epitopes was determined by use of quantitative densitometry of IEM negatives. This revealed that the ratio of N2BA to N2B epitopes was 0.87 ± 0.03 in paced animals (n = 51) (P = 0.03). Thus, both SDS-PAGE and IEM of the LV midwall region reveal equal levels of the isoforms in control specimens, whereas in paced animals, the N2BA/N2B expression ratio is reduced.

Passive Tension Measurements

To explore the functional significance of the reduced N2BA/N2B expression ratio, we performed mechanical experiments on skinned muscle strips, dissected from the LV midwall. No significant differences were found in the maximal active tension of the control and paced groups (Table). However, passive tension (Figure 3, top) is significantly elevated in paced animals. To determine the elements responsible for this increase, muscles were treated with relaxing solution containing high concentrations of KCl and KI (see Methods) to extract thick and thin filaments. This abolishes the sarcomeric anchors of titin and consequently its ability to develop passive tension. In earlier work, we showed that KCl/KI treatment does not affect collagen and that KCI/KI-insensitive tension may be assumed to be collagen-based and KCI/KI-sensitive tension to be titin-based. The present work reveals that elevated total passive tension in paced animals (Figure 3, top) is due to significant increases in both collagen-based (Figure 3, middle) and titin-based tension (Figure 3, bottom).

Discussion

To better understand the molecular alterations underlying diastolic dysfunction, we studied titin expression and passive
stiffness in the pacing-induced DCM model. Results indicate that the expression levels of the compliant N2BA and stiff N2B titins are approximately equal in the LV of control animals, whereas in DCM, N2B expression is upregulated at the expense of N2BA titin (Figures 1 and 2; Table). These findings suggest that the differential splice pathways of titin respond to pacing. We speculate that pacing-induced changes in strain amplitudes and/or strain rates of titin influence splice pathways, possibly via effects on the binding affinity of titin for ligands. Elucidation of the molecular mechanisms underlying shifts in differential splicing of titin is an important area for future research.

Considering that the isoforms are coexpressed at the level of the half-sarcomere (Figure 2), in control animals each half-sarcomere consists of an approximately equal number of stiff (N2B) and compliant (N2BA) titin molecules, organized in parallel. The titin-based stiffness will therefore be intermediate between that of sarcomeres that express solely N2B or solely N2BA titin. This intermediate stiffness allows for maximal adaptability. Thus, sarcomeres can either greatly increase compliance by increasing the N2BA/N2B expression ratio or greatly increase stiffness by reducing this ratio. Our previous study of 2-week paced animals showed that titin expression in the LV (midwall region) was not yet significantly different from that in the controls. This indicates that adaptation of titin expression requires many weeks, a conclusion consistent with the long half-life of titin in vitro. It is also worth noting that N2BA and N2B titins are the same in the isoform expression ratio is altered, the functions of these regions (for example, construction and maintenance of Z lines and M lines, thick-filament length control) will not be altered as long as the total amount of titin is constant (as was found here). Varying the coexpression ratio of titin isoforms is an effective means for modulating the passive properties of the cardiac sarcomere. The reduction in the N2BA/N2B expression ratio seen in DCM is expected to increase titin-based stiffness, consistent with our findings (Figure 3C). This increased stiffness may initially benefit the heart in that it reduces dilatation (together with the increase in the collagen-based stiffness; see below) but may ultimately be deleterious because it contributes to elevated diastolic pressures.

Comparison With Other Studies
Our results are different from those of earlier studies that reported that the amount of intact titin is reduced in myocardium of patients with end-stage DCM. As discussed above, decreasing the total amount of titin is a less than ideal mechanism for altering passive stiffness because it also affects the many functions of the nonextensible regions of titin. We speculate that the reduction of the total amount of titin in earlier studies may be a manifestation of end-stage heart failure that is preceded by an adaptive response with altered expression ratios of titin isoforms. Considering the high susceptibility of titin to proteolysis and the possibility that elevated intracellular Ca levels are present in the failing heart, which may activate proteases, titin degradation in end-stage heart failure would not be surprising. Elevated titin expression has been reported in guinea pigs with decompensated LV hypertrophy. Because elevated expression was detected with antibodies to a constitutively expressed region of titin, these results suggest that the total level of titin expression is enhanced in guinea pigs with heart failure, which is different from the canine pacing model, in which the total expression level is unchanged (Table). The myocardium of small rodents expresses almost exclusively N2B titin, with no or very low levels of N2BA titin, and passive stiffness therefore cannot be increased by switching expression from N2BA to N2B. Thus, the enhanced expression seen in the guinea pig model may be an attempt to increase stiffness by increasing total titin. Whether the extra titin molecules are correctly incorporated into the sarcomere and thus give rise to extra resting stiffness is an issue that remains to be addressed. Finally, an increase in the mean passive tension of cardiac myocytes isolated from paced canine ventricle has been reported by Wolf et al, although the increase did not reach statistical significance. Whether this is caused by titin degradation resulting from the absence of protease inhibitors in this study or other reasons remains to be established. Our experiments on muscle indicate that after pacing, titin-based passive tension is significantly increased.

Collagen
A remarkable result is the large increase in collagen-based stiffness in paced animals (Figure 3B). The elevated collagen-based muscle stiffness is consistent with the reported >5-fold increase in the steepness of the LV end-diastolic pressure-strain relation in canine pacing-induced heart failure, although possible differences in the diastolic SL range and changes in the geometry of the chamber during heart failure preclude a direct comparison between muscle stiffness and chamber stiffness. It seems unlikely that the large increase in collagen-based stiffness that we found results solely from increased collagen content, because this is only 50% increased after 5 to 6 weeks of pacing and is virtually unchanged after 3 weeks of rapid pacing. Thus, other changes in collagen that increase stiffness may take place during pacing, such as increased cross-linking and changes in the type. The extracellular matrix contains both type I and III collagens. Type I forms large, well-structured fibers that resist extension, whereas type III forms a more compliant fine reticular network. Thus, the type I–to–type III ratio is an important determinant of collagen-based stiffness. In human DCM, the collagen type I–to–type III ratio is increased dramatically, and such a change in type is a good candidate for explaining the elevated collagen-based stiffness in canine DCM.

The relative contributions of collagen and titin to total passive tension are graphically depicted in Figure 4. In control myocardium, titin is the main contributor to passive tension. Collagen contributes 20% at SLs up to 2.1, gradually increasing to 40% at an SL of 2.35 μm. The several-fold increase in passive tension in pacing DCM at SLs less than 2.1 μm is largely the result of increased titin-based tension, whereas at longer SLs, increased collagen-based tension is dominant. Thus, the increased total passive tension in pacing DCM is the result of a coordinated change in both collagen and titin, with titin dominating at
short to intermediate SLs and collagen at long SLs. By studying healthy myocardium from species that express different ratios of titin isoforms, we previously showed that in myocardium in which titin-based stiffness is high (low N2BA/N2B expression ratio), collagen-based stiffness is high as well. The present work shows that this positive correlation between titin- and collagen-based stiffness extends to diseased myocardium.

Summary

Coexpression of titin isoforms at equal levels in normal myocardium allows adjustments in isoform expression that can increase or lower passive stiffness. In the canine DCM model, elevating N2B expression at the expense of N2BA titin enhances passive stiffness. It is possible that the reverse adaptation (elevated N2BA expression at expense of N2B titin) decreases passive stiffness in other pathological situations. The increase in titin-based stiffness in the canine DCM model may be a response that, together with the increase in collagen-based stiffness, counteracts the increased myocardial strain during ventricular dilatation. Finally, it is also worth considering other cellular processes in which titin may play a role. For example, recent studies (reviewed in Reference 4) have shown that the passive force of titin enhances the calcium sensitivity of active force and that ligands link titin to the stretch-regulated Ks potassium channel. Thus, variable titin isoform expression allows for passive stiffness modulation and may also influence calcium sensitivity and channel activity.

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