Titin Isoform Switch in Ischemic Human Heart Disease

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Background—Ischemia-induced cardiomyopathy usually is accompanied by elevated left ventricular end-diastolic pressure, which follows from increased myocardial stiffness resulting from upregulated collagen expression. In addition to collagen, a main determinant of stiffness is titin, whose role in ischemia-induced left ventricular stiffening was studied here. Human heart sarcomeres coexpress 2 principal titin isoforms, a more compliant N2BA isoform and a stiffer N2B isoform. In comparison, normal rat hearts express almost no N2BA titin.

Methods and Results—Gel electrophoresis and immunoblotting were used to determine the N2BA-to-N2B titin isoform ratio in nonischemic human hearts and nonnecrotic left ventricle of coronary artery disease (CAD) patients. The average N2BA-to-N2B ratio was 47:53 in severely diseased CAD transplanted hearts and 32:68 in nonischemic transplants. In normal donor hearts and donor hearts with CAD background, relative N2BA titin content was ∼30%. The titin isoform shift in CAD transplant hearts coincided with a high degree of modifications of cardiac troponin I, probably indicating increased preload. Immunofluorescence microscopy on CAD transplant specimens showed a regular cross-striated arrangement of titin and increased expression of collagen and desmin. Force measurements on isolated myofibrils revealed reduced passive-tension levels in sarcomeres of CAD hearts with high left ventricular end-diastolic pressure compared with sarcomeres of normal hearts. In a rat model of ischemia-induced myocardial infarction (left anterior descending coronary artery ligation), 43% of animals, but only 14% of sham-operated animals, showed a distinct N2BA titin band on gels.

Conclusions—A titin isoform switch was observed in chronically ischemic human hearts showing extensive remodeling, which necessitated cardiac transplantation. The shift, also confirmed in rat hearts, caused reduced titin-derived myofibrillar stiffness. Titin modifications in long-term ischemic myocardium could impair the ability of the heart to use the Frank-Starling mechanism. (Circulation. 2002;106:1333-1341.)

Key Words: preload ■ connectin ■ ischemia ■ diastole ■ coronary artery disease

Heart failure is associated with depressed myocardial contractile function1 manifested by, among other things, reduced cross‐bridge cycling rate or altered calcium homeostasis.2,3 These effects result from changes of many proteins directly involved in activation and contraction of cardiac muscle. Conversely, cardiomyopathic and failing hearts also show alterations of various cytoskeletal proteins,4 including tubulin, desmin, vinculin, and collagen. Degeneration of cytoskeletal structures leading to cellular atrophy and interstitial fibrosis may contribute importantly to myocardial dysfunction. However, before the onset of strong degeneration processes in heart failure, expression of cytoskeletal proteins can be upregulated or protein isoform expression can be altered. Elevated collagen and desmin expression is frequently observed in diseased myocardium, eg, in ischemic heart, and is thought to increase left ventricular (LV) wall stiffness.4,5 In addition to collagen and (to a lesser degree) desmin, a principal contributor to LV wall stiffness is titin.6,7 Some studies reported changes of titin expression in failing human myocardium, detected by immunohistochemistry8 or low-resolution gel electrophoresis,9 but these scattered hints did not allow firm conclusions on whether titin contributes to stiffness changes in diseased heart.

See p 1302

Titin, the largest protein known to date (3000 to 3700 kDa), bridges half-sarcomeres from the Z disk to the M line. It is essential for myofibrillar assembly and determines the elasticity of myofibrils.10,11 The elastic region of titin is located in the I band of the sarcomere. In human myocardium, different-length isoforms of I-band titin (Figure 1A) are generated by differential splicing.10,12 Two principal isoforms have been described, a shorter (stiffer) N2B isoform and a longer (more compliant) N2BA isoform.12

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1333
Figure 1. Titin detection by low-percentage SDS-PAGE. A, Domain structure of elastic I-band titin in HH coexpressing 2 principal isoforms, N2B and N2BA. Positions of titin antibodies used in this study (boxed) are indicated by arrowheads. HH titin isoforms were usually separated on 1.8% (B), 2.8% (C), or 2.0% SDS-polyacrylamide gels (E). Rabbit soleus and rat heart served as molecular-weight standards. D, Calibration gels to determine linear loading range of proteins. N2BA-to-N2B titin isoform ratio measured on 2.8% gels changed little with protein load (top) and was similar to that measured on 2% to 10% polyacrylamide gradient gels. Titin-to-MHC ratio on 2.8% gels was constant at loads $\leq 55 \mu g$ (bottom). E, Normal HH25 sample was allowed to degrade at room temperature in absence of leupeptin (symbols on graph are mean of 6 data points). T2 indicates titin degradation band.
Here, we studied whether changes in titin protein expression, analyzed by gel electrophoresis and immunohistochemistry, could contribute to changes of stiffness in hearts of patients with coronary artery disease (CAD). We find that nonnecrotic LVs of heart transplantation patients with extensive CAD exhibit a shift in titin isoform expression, which is confirmed in a rat heart model. The shift leads to reduced titin-derived stiffness measured by single-myofibril mechanics, although whole CAD hearts have highly elevated LV end-diastolic pressure (LVEDP). Interestingly, the titin isoform shift in CAD transplant hearts coincided with modifications of cardiac troponin I (cTnI), a protein reported to be altered during myocardial stunning and/or in response to increased (long-term) preload. Thus, in long-term ischemic hearts entering severe congestive heart failure, cytoskeletal remodeling includes adjustments in coexpression of titin isoforms, resulting in more compliant myofibrils.

Methods

Human Heart Tissue
LV samples from 25 human hearts (HHs) were analyzed and classified into 4 groups: (1) transplant hearts from patients diagnosed with extensive CAD (multiple infarcts), showing clear evidence of LV dysfunction and severe congestive heart failure (CAD transplants); (2) donor hearts showing significant CAD but little myocardial infarction or LV failure (CAD donors); (3) hearts transplanted for reasons other than CAD (nonischemic transplants); and (4) nonfailing HH samples obtained from brain-dead human donors, for which normal LV function had been confirmed by echocardiographic evidence (normal donors). Generally, we studied nonnecrotic regions of the heart, as detected by gross examination of the ventricles cut in transverse fashion. Only the anterolateral midwall region was analyzed, because titin isoform expression varies with location in the heart. Transplant HHs were transported for ≈ 1 hour and donor HHs for 1 to 5 hours in cardioplegia (University of Wisconsin solution) before being stored at −80°C. The study was approved by the Subcommittee on Human Research at Massachusetts General Hospital.

Experimental Animals
Myocardial infarction was induced in 4-week-old female Sprague-Dawley rats by the supplier (Charles River Laboratories, Wilmington, Mass) by the procedure of left anterior descending coronary artery (LAD) ligation. Sham-operated controls were treated like- wise, except that the suture around the coronary artery was not closed. Animals were euthanized 12 and 24 weeks after ligation. The Experimental Animals section starts here.

Antibodies

Gel Electrophoresis and Immunoblotting

Acrylamide gradient gels. Titin was visualized by 1.8%, 2%, or 2.8%, sometimes 2% to 10%, SDS-PAGE. Lanes were loaded with equal amounts of protein (70 to 80 µg) determined spectrophotometrically. Gels stained with Coomassie brilliant blue R or silver were digitized, and the optical volume of protein bands (integrated optical density) was determined with TotalLab software (Phoretix). Immunoblotting was done with a chemiluminescent reaction kit (ECL system, Amersham Pharmacia) according to standard protocols.

Immunofluorescence Microscopy

Paraformaldehyde-fixed LV HH sections (20 µm thick) were cut at −20°C in parallel with or perpendicular to the fiber axis and were processed for IF according to standard methods (eg, see Elsasser et al12). IF on isolated myofibrils was done as described in Reference 18. Images were recorded with a color CCD camera (Sony).

Myofibril Mechanics

Cardiac myofibrils were prepared as described from frozen HH tissue. Under a Zeiss Axiovert-135 microscope, myofibrils were attached to a micromotor (Physik Instrumente) and a home-built force transducer (sensitivity = 5 nN). LabView software was used for motor control and data acquisition and analysis. Force measurements were carried out at 25°C in relaxing buffer supplemented with 2,3-butanedione monoxime. A typical experimental protocol consisted of stretching a relaxed myofibril stepwise from slack sarcomere length (SL) to a maximum SL of 2.4 µm. Steps were completed within 1 second; hold periods lasted 19 seconds. The force-sampling rate was 1 kHz. Measured force values were related to myofibrillar cross-sectional area.

Statistical Analysis

If not indicated otherwise, data are expressed as mean±SEM. Statistical significance was evaluated by Student’s t test.

Results

Human Cardiac Titin Expression

Clear separation of bands corresponding to the 2 principal HH titin isoforms (Figure 1A) was apparent on 1.8% SDS-polyacrylamide gels (Figure 1B). The lower N2B titin band was sharp (≈3000 kDa), whereas the upper N2BA titin band was fuzzy, indicating the presence of multiple isoforms in the range of 3300 to 3500 kDa. Titin and MHC together were usually detected by 2.8% SDS-PAGE, which still separated the HH titin isoforms sufficiently well (Figure 1C). On calibration gels (example in Figure 1D), the optical volume ratios for N2BA-to-N2B titin and titin-to-MHC were determined; all ratios were load-independent for loads greater than ≈55 µg. The apparent titin-to-MHC ratio measured by 2.8% SDS-PAGE was generally lower (≈0.1; Figure 1D) than that measured, for comparison (Figure 1D), on 2% to 10% polyacrylamide gradient gels. The differences must arise from the use of different gel types, because samples were solubilized the same way. The titin (N2B+N2BA)-to-MHC ratio on 2.8% gels was indistinguishable in the 4 HH groups analyzed (CAD transplants, n=5; nonischemic transplants, n=4; CAD donors, n=4; normal donors, n=12), ≈0.10±0.01. To test whether the N2BA-to-N2B titin ratio can be affected by protein degradation, samples were left standing at 25°C in the absence of leupeptin (Figure 1E). Already after a few minutes, a strong titin-degradation band (T2) appeared, but the N2BA-to-N2B ratio changed little. After 30 to 60 minutes, the ratio was clearly decreased. We conclude that under normal study conditions (ie, in the presence of leupeptin, which largely prevents the...
appearance of T2), the measured N2BA-to-N2B ratio is independent of possible minor protein degradation processes.

N2BA-to-N2B Titin Isoform Ratio Is Increased in CAD Transplant Hearts

Titin isoform expression in normal and diseased, nonnecrotic, LV samples was quantified on 2% SDS-polyacrylamide gels (Figure 2A) and confirmed by Western bloting using isoform-specific antibodies (Figure 2B). N2BA titin frequently was stained more strongly in CAD transplant hearts than in normal HH by antibodies to both N2BA titin and all titin isoforms (examples in Figure 2B). The Table (right column) lists the average N2BA titin content (relative to N2B/N2BA) of each heart. The means calculated for each HH group (Figure 2C) indicate a relative N2BA titin content of 47.0±2.4% in CAD transplants, statistically significantly higher (P<0.002) than in nonischemic transplants (32.1±1.4%), CAD donors (29.5±2.6%), or normal donors (28.1±0.8%). Figure 2D groups the relative N2BA titin content of individual hearts in 5-percentage-point bins. Nonischemic transplants and normal donor hearts showed a narrow distribution around 30%. One of 4 CAD donor hearts had slightly increased N2BA content, whereas this content was elevated by various degrees in all CAD transplant hearts.

The total amount of titin (N2B+N2BA) per unit tissue did not change in a statistically significant manner (P>0.05): relative to normal donors (100.0±10.3%), it was 101.1±14.7% in CAD transplants, 96.2±7.1% in nonischemic transplants, and 95.4±15.9% in CAD donors.

Titin Isoform Shift in CAD Transplant Hearts Coincides With cTnI Modification

cTnI is a regulatory protein modified during ischemia/reperfusion injury and in long-term ischemic hearts with elevated preload. Because preload is thought to depend partly on titin function, we tested whether cTnI proteolysis may coincide with the observed titin isoform shift in CAD transplant HHs. cTnI modification was assayed by Western blotting using TnI 8I-7 antibody (Figure 3A), which detects the major proteolysis bands. The applicability of the method was confirmed by allowing a sample of normal HH to degrade at 25°C for 1 hour in the absence of protease inhibitor (Figure 3A, left 3 lanes). During forced degradation, several cTnI degradation products and cTnI-containing complexes appeared. Subsequent analyses of all (undegraded) HH samples (examples in Figure 3A) were done on supernatants, which tended to reveal sharper cTnI modification bands (as a result of lower background signals) than pellet fractions. Of
Results of Gel-Electrophoretic Analyses of Individual HHs

<table>
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<tr>
<th>Heart No.</th>
<th>Age, y/Sex</th>
<th>cTnI Degradation/cTnI Complexes</th>
<th>N2BA Titin (%), mean± SD (n)</th>
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<tr>
<td>CAD transplants</td>
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<td>HH27</td>
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<td>++/+</td>
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<td>??/F</td>
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<td>??/F</td>
<td>±/±</td>
<td>31.9±4.5 (11)</td>
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Modifications of cTnI were analyzed on Western blots (10% to 20% polyacrylamide gradient gels), with the use of TnI antibody 8I-7 (cf. Figure 3). Symbols indicate TnI band intensity: −, no; ±, faint; +, normal; and ++, strong band. Average relative N2BA titin content (N2BA / N2B + N2B = 100%) was measured as the optical volume of bands on Coomassie-stained 2% SDS gels (n indicates number of lanes analyzed).

Figure 3. Cardiac TnI analysis. A, Western blots (from 10% to 20% gradient gels) with anti-TnI B1-7 antibodies to study modifications of native cTnI in HH samples. d indicates cTnI degradation product; c1, c2, cTnI-containing complexes. Third lane, cTnI degradation in purposely degraded normal HH (1 hour, 25°C, no leupeptin). B, Summary for HH groups. Equation was used to estimate degree of cTnI modification on Western blots.

The 4 HH groups studied, CAD transplants exhibited by far the highest incidence and degree of cTnI modification (Figure 3B); differences from both nonischemic transplants and normal donor hearts were statistically significant (P<0.05). Higher-than-normal cTnI modification was also found in CAD donors. The Table compiles results for individual HH samples. It appeared that cTnI modification coincides with a high level of N2BA titin expression in CAD transplant hearts.

Titin and Collagen Expression in Myocardial Tissue

Expression of titin and collagen was visualized by IF on tissue sections (examples in Figure 4) prepared from normal hearts with low N2BA titin content (HH9+5) and CAD transplants with high N2BA content (HH1+17). In both normal and diseased tissue, antibodies to the N2BA titin isoform (Figure 4A) and to all titin isoforms (Figure 4B) stained in a regular cross-striated pattern. The uniformity of staining across the tissue suggested that all sarcomeres express N2B+N2A titin. IF with antibodies to collagen type I (Figure 4C) and collagen type III (data not shown) revealed increased staining intensities in CAD transplant tissue. Upregulation of collagen leading to myocardial stiffening is well recognized in chronically diseased myocardium.

Titin and Desmin in Isolated Human Cardiomyofibrils

Titin is considered the main contributor to myocardial stiffness at the sarcomere level, but some contribution may also come from desmin. Expression of these 2 proteins was studied by IF on isolated, mechanically stretched,18,20 myofibrils (Figure 5, A and B) obtained from normal HHs (HH5+9+10) and CAD transplant hearts (HH1+17). CAD heart myofibrils showed greater desmin fluorescence intensity at the Z disk than normal heart myofibrils (examples in Figure 5A). In myofibrils double-stained with MG1 antibody to N2BA titin and with N2B antibody (Figure 5B), sarcomeres were always labeled by the 2 antibody types, indicating coexpression of N2B titin and N2BA titin isoforms at the sarcomere level.22 Image overlays showed that the 2 antibody epitopes are located close to one another in the middle I-band region (Figure 5B, bottom), as expected for intact titin filaments.
Passive-Force Measurements Reveal That Stiff Hearts Have More Compliant Myofibrils

Force measurements were carried out on nonactivated HH myofibrils (Figure 5, C and D). Specimens were obtained from normal HH and CAD transplant HH hearts. The latter hearts had been diagnosed with abnormally high LVEDPs of 32 and 25 mm Hg, respectively, compared with a normal LVEDP of 7 to 12 mm Hg (Figure 5C, inset). All myofibrils included in the analysis presented a regular sarcomere pattern, and their slack SL in relaxing buffer was 1.85 to 1.90 μm. Force measurements on stepwise sarcomere stretch (Figure 5C) showed a statistically significant depression of quasi–steady-state passive tension in both types of CAD transplant myofibrils, compared with normal HH myofibrils, in the SL range of 2.1 to 2.4 μm (Figure 5D). Hence, increased N2BA-to-N2B titin isoform ratios in myofibrils from CAD transplant hearts translate into lowered myofibrillar passive tension, although whole CAD hearts are stiffer than normal.

Failing Hearts of LAD Rat Models Express Significant Amounts of N2BA Titin

Myocardial infarction was induced in a rat model of heart disease, the LAD ligature, and cardiac titin expression was studied in chronically ischemic tissue. Nontumorotic RV tissue was examined in rats 12 and 24 weeks after surgery17 and in sham-operated rats. The severity of the chronic disease induced was indicated, eg, by increased LV diameters and RV weight-to–body weight ratios (Figure 6, top right). Rat heart is useful to assay possible changes of titin isoform composition, because only N2B titin is usually detectable in this species12; if a titin isoform shift took place, N2BA titin should appear as a newly expressed isoform in infarcted hearts.

On Coomassie-stained 2.8% SDS-polyacrylamide gels, a distinct N2BA titin band was indeed frequently observable in LAD hearts but almost never in sham-operated hearts (Figure 6A). Despite upregulation of N2BA titin, the titin (N2B+N2BA)-to-MHC optical volume ratio was unaltered in LAD versus sham-operated animals (Figure 6B). Titin tissue content, assayed by 2% SDS-PAGE, did not differ in a statistically significant manner between LAD and sham but was generally lower in older than in younger rats (Figure 6C). Because the newly appearing N2BA titin band was usually faint on Coomassie-stained gels, silver staining was used to increase the sensitivity of detection (Figure 6D). On digitized gels, expression of N2BA titin was considered positive if a clear minor peak was detected next to the major N2B titin peak (graphs, Figure 6D). The identity of titin bands was confirmed by immunoblot analysis with N2BA-specific antibody and antibody to all titin isoforms (Figure 6E). Altogether, 6 of 14 LAD hearts (7 each for 12-week and 24-week LAD), but only 2 of 14 sham-operated hearts, expressed significant amounts of N2BA titin (Figure 6F). No obvious differences in N2BA titin expression could be detected between early (12-week) and late (24-week) LAD rat hearts. In conclusion, the rat model confirmed a titin isoform shift in chronic ischemia–induced cardiac disease, but the change was independent of the advance of congestive heart failure.

Discussion

In ischemia-induced heart disease and failure, complex adaptive changes are launched that affect dozens of proteins. Morphological changes accompanying impaired heart function may also involve titin, the giant sarcomere protein.4 A few studies reported degeneration of titin in failing8,9 and hibernating21 human myocardium. The importance of titin for the elasticity, passive-tension development, and assembly of cardiac myofibrils10 implies that loss of titin and/or alterations of titin expression should have profound effects on mechanical heart function.
A characteristic of HH tissue is the coexpression of 2 principal titin isoforms, N2B and N2BA. A shift toward higher N2BA titin levels lowers cellular passive stiffness, as shown in dog hearts exposed to pacing tachycardia. In the present work, we investigated whether the titin isoform expression pattern can be altered in diseased HHs. Hearts from CAD patients were selected for the analysis because they frequently exhibit increased LV wall stiffness. A well-established scenario is that elevated wall stiffness results from increased expression of collagen and desmin. Confirmatory evidence for collagen and desmin upregulation in CAD transplant hearts was provided here (Figures 4C and 5A). Thus, increased fibrosis leading to high diastolic stiffness is a likely cause for the much-elevated LVEDP in the CAD transplants for which clinical parameters were available. (We caution that, without additional data, the correlation between elevated myocardial stiffness and high LVEDP is only presumed.) Perhaps surprisingly, myofibrils from CAD transplant hearts with greatly increased LVEDP were less stiff than normal HH myofibrils (Figure 5, C and D). The decreased myofibrillar stiffness in CAD transplants correlated with a shift in titin isoform pattern toward the more compliant N2BA isoform (Figure 2, A through D), whereas total titin content and titin-to-MHC ratio remained unaltered. Thus, the observed changes in titin isoform expression lead to reduced titin-derived stiffness; titin does not take part in the stiffening of long-term ischemic HH.

Because relevant clinical parameters, such as LVEDP, were available for only some HHs, we looked for an indirect method to obtain information about the status of the hearts. A recent report suggested that increased preload in long-term ischemic heart causes modification of cTnI, a myofibrillar protein also found to be degraded during myocardial stunning. The excellent correlation between increased preload and cTnI degradation demonstrated in that report led us to argue that cTnI modification may be a good marker to detect elevated diastolic stiffness in long-term ischemic CAD HHs. Indeed, increased cTnI modification was seen particularly in CAD transplant HHs, but not in nonischemic transplant or donor hearts (Figure 3). Thus, we tentatively propose that increased preload could be a decisive factor for the titin isoform shift observed in CAD transplant hearts.

We also found that most donor hearts with a CAD background did not have elevated N2BA titin levels. This less severely diseased HH group showed only slightly increased cTnI modification. It is not unreasonable to assume that long-term alterations in LVEDP characteristic of advanced congestive heart failure may be necessary to produce the titin isoform switch. Of interest is that chronically ischemic failing rat hearts (LAD model), which confirmed the titin isoform shift (Figure 6), showed no difference in the incidence of N2BA titin expression 12 weeks and 24 weeks after induction of the disease. We suggest that the shift in titin isoform pattern may take place over the course of several days to...
weeks. However, the exact time course and the signaling pathways involved remain to be established.

Finally, titin was proposed to be partly responsible for the Frank-Starling mechanism of the heart (see Sutko et al. and references therein). Titin could be the structural element that senses changes in SL, which cause altered myofilament Ca^{2+} sensitivity. If so, changes in myofibrillar passive tension caused by titin isoform shift (as in CAD transplant HHs) are likely to affect the molecular basis underlying the Frank-Starling law. Although speculative, this may contribute to depressed contractile function in failing hearts, a possibility that warrants follow-up. Our results are consistent with the idea that titin is a key element in the sarcomere responsible for passive, as well as active, mechanical properties of normal and diseased myocardium.

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**Figure 6.** Expression of a distinct N2BA titin isoform in RV samples of rats with myocardial infarction (model of LAD ligature). Some parameters indicating severity of disease are indicated (top right). A, Typical Coomassie-stained 2.8% SDS-polyacrylamide gel showing large protein bands of LAD rat RV and sham-operated sample. Detail: enlarged titin band region. B, A 2.8% SDS-PAGE revealed similar titin-to-MHC ratios in LAD and sham-operated specimens (n=8 per column). C, On silver-stained 2% SDS gels, total cardiac titin content was decreased with age in a statistically significant manner (P<0.05) but was similar in LAD and sham-operated animals both 12 and 24 weeks after surgery (n=8 per column). D, LAD rat hearts frequently showed a distinct N2BA titin band appearing as a minor peak on lane intensity profiles (arrow, right panel). E, Western blot confirming N2BA titin presence in LAD rat hearts. F, Relative frequency of appearance of N2BA titin band in LAD and control hearts.
References
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