Hypothyroidism leads to increased collagen-based stiffness and re-expression of large cardiac titin isoforms with high compliance

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Abstract

Because long-term hypothyroidism results in diastolic dysfunction, we investigated myocardial passive stiffness in hypothyroidism and focused on the possible role of titin, an important determinant of diastolic stiffness. A rat model of hypothyroidism was used, obtained by administering propylthiouracil (PTU) for times that varied from 1 month (short-term) to 4 months (long-term). Titin expression was determined by transcript analysis, gel electrophoresis and immunoelectron microscopy. Diastolic function was measured at the isolated heart, skinned muscle, and cardiac myocyte levels. We found that hypothyroidism resulted in expression of a large titin isoform, the abundance of which gradually increased with time to become the most dominant isoform in long-term hypothyroid rats. This isoform co-migrates on high-resolution gels with fetal cardiac titin. Transcript analysis on myocardium of long-term PTU rats, provided evidence for expression of additional PEVK and Ig domain exons, similar to what has been described in fetal myocardium. Consistent with the expression of a large titin isoform, titin-based restoring and passive forces were significantly reduced in single cardiac myocytes and muscle strips of long-term hypothyroid rats. Overall muscle stiffness and LV diastolic wall stiffness were increased, however, due to increased collagen-based stiffness. We conclude that long term hypothyroidism triggers expression of a large cardiac titin isoform and that the ensuing reduction in titin-based passive stiffness functions as a compensatory mechanism to reduce LV wall stiffness.

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1. Introduction

Thyroid hormones have significant effects on cardiac contractility with hyperthyroidism associated with a general hyperdynamic state and hypothyroidism leading to hypodynamic hearts. The molecular basis for changes in contractility and relaxation include effects of thyroid hormone on transcription of myosin, SERCA, and the SERCA inhibitor phospholamban (for reviews and original citations, see [1,2]). Furthermore, thyroid hormone is a negative regulator of expression of collagen [3–5], but the functional consequence of changes in collagen expression in hyper(hypo)thyroidism have not been investigated. Except for a study in the rat that revealed that manipulating thyroid hormone levels results in significant changes in the left ventricular end-diastolic stress-strain relation [6], little is know about effects of thyroid hormonal status on passive myocardial stiffness. Here we studied LV chamber and myocardial stiffness in rats with short- and long-term hypothyroidism, and focused on titin, a well-recognized major contributor to passive stiffness of the heart.

Titin is an endosarcomeric protein that spans from the Z-disk to the M-line of the sarcomere [7]. Passive stiffness of sarcomeres is largely due to the extensible I-band region of titin that functions as a molecular spring, developing passive force in sarcomeres stretched above the slack length (length at which passive force is zero) and restoring force when sarcomeres...
shorten to below this length [7]. The sequence composition of titin’s molecular spring segment varies greatly in different titin isoforms (due to differential splicing) and titin’s stiffness varies accordingly [8]. Stiffest is the adult N2B isoform, less stiff is the adult N2BA isoform, and most compliant is fetal cardiac titin (fetal cardiac titin belongs to the N2BA class of cardiac isoforms), which is expressed during fetal and neonatal development [8–10]. Sarcomeres of adult myocardium co-express the N2BA and N2B isoforms and their expression ratio determines the level of titin-based passive stiffness [11,12]. This expression ratio is not fixed but instead responds to altered cardiovascular hemodynamics. For example in the canine rapid pacing model, 4 weeks of pacing results in elevated N2B titin expression and down regulation of N2BA titin [13]. A similar change (i.e., increased expression of stiff N2B titin) has been reported in hypertensive rats [14]. Interestingly, opposite changes are present in patients with CAD or DCM: compliant N2BA isoforms (due to differential splicing) and titin’s stiffness varies accordingly [8]. The sequence composition of titin, and MHC (for details, see [9]). MHC isoform expression was determined by SDS-PAGE as explained in Warren et al. [19].

2.3. Transcript studies

The method that was followed has been described in detail in Lahmers et al. [9] Briefly, total RNA was isolated from left ventricular myocardium from control and PTU treated rats and was converted to biotinylated cDNA. A 50-mer oligonucleotide array containing 385 probes was used representing all of titin’s human gene exons, plus various controls. Biotinylated target was mixed, heat denatured and hybridized to the oligonucleotide array. Detection chemistries and signal amplification were achieved using the TSA™ biotin System (PerkinElmer, Boston, MA). A minimum of three independent experiments from each group was conducted. Results from different groups were compared with a two-tailed t-test for samples of unequal variance and \( P<0.05 \) was used as criterion for statistical significance. (For additional details, see [9]).

2.4. Immunoelectron microscopy (IEM)

Myocardial samples from control and PTU treated rats were skinned, fixed in 3% para-formaldehyde/PBS solution for 20 min, then washed and blocked in 1% BSA/PBS solution for 1 h. The samples were labeled with anti-N2B-Uc antibody [20] and then secondary antibodies for 48 h each. The samples were fixed in 3% glutaraldehyde/tannic acid and osmium tetroxide solutions, and embedded in araldite. Sections were cut using a Leica microtome, and stained with 2% potassium permanganate and lead citrate. For additional details, see [20].

2.5. Collagen

For collagen analysis, the left ventricle was quick frozen in liquid nitrogen. Sections of 12 \( \mu \)m were cut that were fixed with 100% acetone at 4 °C. Sections were double labeled using primary antibodies against collagen type I (goat polyclonal antibody raised against collagen type I of mouse, Cat. Sc-8788, Santa Cruz Biotechnology) and collagen type III (rabbit polyclonal antibody raised against C-terminus of collagen Type III of human, Cat. Sc-28888, Santa Cruz Biotechnology). Secondary antibodies were goat anti-rabbit Alexafluor 594 conjugated IgG and chicken anti-goat Alexafluor 488-conjugated IgG (Molecular Probes). Sections were analyzed on a Bio-Rad MRC 1024 Confocal Laser Scanning Imaging System.

2.6. Measurement of passive force in skinned myocardium

The methods that were used have been published [12]. Briefly, small wall muscle strips (diameter \( \sim 0.2 \) mm) were carefully dissected from control and 4 month PTU treated rat LV and skinned overnight at 4 °C in relaxing solution [12] containing 1% w/v Triton X-100. The strips were then washed thoroughly with relaxing solution. To prevent degradation, all
solutions contained protease inhibitors [12]. Experiments were performed at room temperature (20–22 °C). SL was measured with laser-diffraction. Passive force was divided by the muscle’s cross-sectional area, to obtain passive tension. The passive tension–SL relationship was measured in relaxing solution. The perfusate was then changed to relaxing solution containing 0.6 M KCl (for 45 min) followed by relaxing solution containing 1.0 M KI (for 45 min). The passive tension–SL relationship was then measured again in relaxing solution. The passive tension decrease that results from perfusion with relaxing solution containing KCl and KI is titin based, whereas the residual tension is collagen based [12].

2.7. Measurement of restoring force

Left ventricular cardiac myocytes were isolated from control and 4 months PTU treated rats [21]. Cells were skinned for 6 min in relaxing solution with 0.3% Triton, followed by washing with relaxing solution. To prevent titin degradation, all solutions contained protease inhibitors [21]. The restoring force was measured by using the method described in Helmes et al. [22]. Briefly, relaxed cell segments which had been glued to a force transducer and motor were first buckled, and the chamber was then perfused with rigor solution (relaxing solution without ATP and CPK and with additional K-propionate to adjust ionic strength to 180 mM). After a delay, sarcomeres shortened until the buckle disappeared to reach a length of ∼1.7 μm. The perfusion buffer was then switched from rigor to relaxing solution, resulting in a relaxed cell with a SL of ∼1.7 μm. We subsequently stretched the relaxed cell to a sarcomere length of ∼2.05 μm while measuring force. The force measured below the slack sarcomere length (∼1.90 μm) is defined as restoring force.

2.8. Isolated heart experiments

Hearts were isolated from PTU treated and age-matched control rats. Upon excision of the heart, the aorta was quickly cannulated and the heart was perfused with oxygenated Krebs–Henseleit solution (Ca2+ concentration, 1.25 mM). Hearts were mounted onto an experimental system that consisted of a constant-pressure perfusion subsystem, an environmental control subsystem, a pacing-control subsystem, and a volume servo subsystem. A latex balloon was inserted into the LV chamber through the mitral orifice. The balloon was inflated to a reference volume \( V_{\text{ref}} \), which was defined as the volume necessary to achieve an end-diastolic pressure of 5 mm Hg. LV pressure was measured with a 3-Fr catheter-tip Millar pressure transducer that was passed through a port in the volume servo system and positioned in the balloon within the LV chamber. Experiments were conducted at 37 °C. The pace period was set at 400 ms. Once stable, isovolumic beating was achieved at \( V_{\text{Bl}} \), a single-beat Frank–Starling protocol was administered to evaluate both systolic and diastolic LV functions: Volume was commanded to be 1 of 8 volumes (diastolic pressure from ∼10 mm Hg to ∼20 mm Hg), and records were collected for the full set of commanded volumes. Pressures (\( P \)) were measured from the first beat after each commanded volume. Passive \( P \) was measured as the lowest LV pressure measured during diastole after the first beat. Peak systolic \( P \) was measured from the test beat, and developed \( P \) was calculated as peak systolic pressure minus passive \( P \). LV equilibrium volume (volume where diastolic pressure is zero), was calculated by fitting the diastolic pressure–volume relationship with a polynomial curve (\( R^2 \geq 0.95 \)). LV developed pressure (LVDP) at 1.0 \( V_{\text{eqw}} \), 1.1 \( V_{\text{eqw}} \) and 1.2 \( V_{\text{eqw}} \) were determined from the LVDP–volume relationship. The diastolic LV pressure was converted into diastolic LV wall stress (\( \sigma \)) using a thick-walled spherical model: \( \sigma = P / \left( [LV_w / 1.05(V+1)]^{2/3} - 1 \right) \), where \( LV_w \) is the weight of the LV wall (for details, see [23]). The mid-wall radius (\( R \)) was used to compute midwall strain (\( (R - R_{\text{eqw}})/R_{\text{eqw}} \)), where \( R_{\text{eqw}} \) is the mid-wall radius at zero diastolic pressure (\( V_{\text{eqw}} \)), and we determined \( \sigma \)-strain relationships. The obtained results were fit by multiple linear regression analysis.

2.9. Statistics

Results are reported as mean±SEM unless indicated otherwise. Differences between mean values were evaluated using Student’s \( t \)-tests or one-way ANOVA, with \( P < 0.05 \) indicating a significant difference. Multiple linear regression analysis was used to analyze and compare the \( \sigma \)-strain relationships. Data were fit with the following regression model: \( Y = b_0 + b_1 X + b_2 X^2 + b_3 D + b_4 D*X + b_5 D*X^2 \), where \( Y \) is the response variable (\( \sigma \)), \( X \) is the predictor variable (\( R \) strain), \( D \) is a dummy variable to encode for control (\( D = 0 \)) or PTU treatment (\( D = 1 \)), \( b_0 \) is the intercept, and \( b_i \) are regression coefficients.

3. Results

3.1. Titin expression

PTU treatment resulted in a reduction of body weight, that was large during the first month of treatment and subsequently the reduction was more gradual (Table 1). Long-term PTU treatment did not affect the ventricular weight/body weight ratio

<table>
<thead>
<tr>
<th>Duration of PTU</th>
<th>Body weight (g)</th>
<th>Ventricle/body weight (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 m</td>
<td>2 m</td>
</tr>
<tr>
<td>Control</td>
<td>329.6±10.5</td>
<td>370.5±9.0</td>
</tr>
<tr>
<td>PTU</td>
<td>244.2±12.5</td>
<td>221.8±7.4</td>
</tr>
<tr>
<td>( P ) value</td>
<td>0.002</td>
<td>&lt; 0.001</td>
</tr>
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</table>
The first 2 months of treatment resulted in a slight reduction of the ratio, but this subsequently normalized (Table 1). High resolution agarose gel electrophoresis of control LV myocardium revealed N2B titin as the major titin isoform and two N2BA titins as minor isoforms (Fig. 1A left, lane C and Fig. 1B, inset). In PTU treated rats we found a large titin isoform that gradually became more prominent with PTU treatment duration (Fig. 1A, left). The mobility of this large titin isoform appeared to be similar to one of the fetal cardiac titin isoforms expressed in fetal myocardium (Fig. 1A left panel, right lane). By mixing PTU myocardium and fetal myocardium we found that there is no discernable mobility difference between the large isoform expressed in PTU samples and the fast mobility fetal cardiac titin isoform (Fig. 1A, right panel), although, due to the limited resolution of the gel system in the 3–4 MDa range, this does not firmly establish that this upregulated isoform in PTU myocardium is indeed a fetal cardiac titin isoform. In conclusion, long-term PTU treatment results in high-level expression of a large cardiac titin isoform.

3.2. Analysis of cardiac titin expression in PTU rats

The ratio of N2BA titins to N2B titin increased with the duration of PTU treatment and reached ~0.8 after 4 month of PTU treatment (Fig. 1B left). These changes take place without impacting the total titin/MHC ratio (Fig. 1B right). We conclude that during PTU treatment, stiff N2B titin is replaced by more...
compliant N2BA titin, with the largest observed change requiring
between 3 and 4 months of PTU treatment. (We do not exclude
that even larger changes occur for more extended treatment
durations.) Interestingly, the titin isoform switch occurs much
slower than the MHC isoform switch which is complete within
the first month of PTU treatment (Fig. 1C).

3.3. Microarray analysis

We used a titin microarray to obtain insights in the exon
composition of the large cardiac isoform expressed in PTU treated
myocardium. RNA from 4 month PTU treated and age-matched
control LV myocardium was isolated, reverse transcribed,
biotinylated, and hybridized to the exon microarray (see Materials
and methods). Clear differences existed when comparing PTU
myocardium with control myocardium (Fig. 2A). Statistical
analysis revealed that 16 titin exons were significantly up-
regulated in PTU treated rat myocardium (Fig. 2B). All up-
regulated titin exons are located either in the tandem Ig or PEVK
regions (Fig. 2B bottom panel), similar to what was found earlier
for fetal cardiac titin [9]. These results support the notion that the
large titin isoform in PTU treated rat myocardium is a fetal titin
isoform.

3.4. Immunoelectron microscopy (IEM)

IEM was used to examine titin expression at the level of
the sarcomere. Myocardium was labeled with N2B-Uc, an

![Image]

Fig. 2. Analysis of RLV myocardium transcripts by an oligonucleotide array representing all exons of the titin gene. RNA was isolated, reverse transcribed,
biotinylated, and hybridized to the exon microarray. (A) Examples of results of 4 month PTU RLV (top), and age-matched control RLV (bottom). (B) Bar graphs with
mean and SEM values of differentially expressed exons in PTU and control RLV. A total of 16 exons were significantly ($P < 0.01$) up-regulated in PTU RLV by at least
3 fold (exon numbers in red). The up-regulated exons are all Ig and PEVK exons. None of the other exons were significantly (up)down-regulated. Examples of
constitutively expressed exons are also shown (exon number in black).
antibody that recognizes an epitope C-terminal of the N2B unique sequence [20]. Importantly this epitope is in compliant titin isoforms (such as fetal cardiac titin) localized closer to the Z-disk than in the stiff N2B titin (this differential localization is due to the extra spring elements of compliant isoforms: see [20] for details). In control myocardium a major epitope was found far away from the Z-disc (Fig. 3A, top); in PTU treated myocardium (Fig. 3A, bottom) this epitope was also found (albeit weaker than in control myocardium). We determined the location of this near A-band epitope (epitope 2) in PTU samples as a function of sarcomere length and show the results in Fig. 3B, closed symbols. The broken line is the fit to the data and the solid line in Fig. 3B represents the fit to the result of a previous study on N2B titin expressing rat cardiac myocytes [11]. The data obtained in the present study on PTU myocardium overlap with this previous result indicating that the near A-band epitope in PTU samples is derived from N2B titin. In addition, a second epitope was seen in PTU myocardium that was present closer to the Z-disk (Fig. 3A, bottom, epitope 1 and 3B, open symbols). Within the sarcomere length range that was studied, the distance of this epitope to the Z-disk varied modestly with sarcomere length. Although the N2B-Uc behavior has not been studied in N2BA and fetal cardiac titin of the rat, the epitope behavior shown in Fig. 3B (open symbols) is consistent with being derived from a compliant titin isoform. (The long extensible region of compliant titin isoforms results in a low fractional extension and a short Z-disk to N2B-Uc distance, relative to that of stiffer titins; for details, see also [11]). Overall, the immuno EM study supports the view that PTU rats co-express at the level of the half-sarcomere a large titin isoform together with N2B titin.

3.5. Functional studies: muscle and myocyte

To gain insights in the functional significance of re-expressing fetal cardiac titin we determined the contribution of both titin and collagen to passive tension of muscle strips dissected from LV of 4 months PTU treated rats and age-matched controls (for details, see Materials and methods). Titin-based passive tensions were significantly lower in PTU myocardium (Fig. 4A). In contrast, the collagen-based passive tensions were higher in myocardium of PTU rats (Fig. 4B). Staining of collagen types I and III showed that both collagen types were upregulated in PTU rats (inset of Fig. 4B), providing a possible explanations for the increased collagen-based passive tension.

Because titin is not only responsible for generating passive tension during sarcomere stretch beyond the slack length, but also for restoring tension during sarcomere shortening to below slack [22], we determined the effect of fetal cardiac titin re-expression on restoring tension (see Materials and methods). Examples of results are shown in Fig. 4C. We determined the ‘restoring stiffness’ using the slope of the linear range of the restoring tension-SL relation (see Fig. 4C). Results show that restoring stiffness is significantly lower in PTU rats (inset of Fig. 4C).

3.6. Functional studies: isolated heart

To understand the possible significance of fetal cardiac titin for systolic and diastolic heart function we studied short- (1 month) and long-term (4 months) hypothyroid rats using an isolated heart preparation. A single beat Frank–Starling protocol was used (examples are shown in Fig. 5A) to determine the diastolic and developed pressures (defined in Fig. 5A). From the developed pressure–volume relationships, we determined the equilibrium volume, $V_{\text{eq}}$ (volume at which diastolic pressure is zero) and developed pressure at 1.0, 1.1, and 1.2 $V_{\text{eq}}$ (schematically shown in Fig. 5B, left). PTU treatment had no effect on $V_{\text{eq}}$ (Fig. 5B, middle). Developed pressure was significantly reduced both after 1 and 4 months of PTU treatment, and to a similar extend at all volumes tested (Fig. 5B, right).

We focused on diastolic function, and converted diastolic pressure–volume relationships into diastolic wall stress ($\sigma$)–strain relations (see Materials and methods for details). After 1 month PTU treatment, diastolic wall stress was indistinguishable from that of control rats, but after 4 months PTU treatment,
diastolic wall stress was significantly higher (Fig. 5C). The conversion from pressure to wall stress normalizes for possible size differences of hearts (LV volume, wall thickness) and, thus, it is unlikely that the increased diastolic wall stress after 4 months of PTU treatment is due to geometric factors. The possible mechanisms underlying these findings will be discussed below.

4. Discussion

4.1. Expression of a large cardiac titin isoform in hypothyroid adult rats

High resolution agarose gels revealed that hypothyroid rats express a large titin isoform, the abundance of which gradually increases with PTU treatment duration to become a prominent isoform after 4 months of treatment. Titin isoforms are the result of differential splicing of a single titin gene [24]. The classical cellular thyroid hormone effects are based on regulating cell specific gene transcription [1,2], and whether differential splicing of titin is regulated by thyroid hormone remains to be established. Alternatively, the changes in splicing that underlie the expression of the large titin isoform might be secondary effects that result from changes in contractility. Consistent with this notion is our finding that the re-expression of fetal cardiac titin is relatively slow and does not reach a steady state after 4 months of treatment (for comparison, the MHC switch is completed after ~1 month). Results also indicate that PTU treatment results in a large reduction in weight and although it is possible that factors involved in this weight reduction play also a role in the titin isoform switch, the fact that the weight reduction occurs much faster than the titin isoform switch argues against their involvement. Further work is required to establish the mechanism(s) underlying the isoform switch.

To gain insights in the sequence of this large titin isoform expressed in long-term PTU rats, antibodies specific for fetal cardiac titin.

4.2. Passive mechanics of cardiac myocytes

Important findings of this work are that passive and resorting forces of cardiac myocytes are significantly reduced in long-term PTU treated rats (Figs. 4A and C). These results can be understood as follows. The constant total titin/MHC ratio in myocardium of PTU treated rats (Fig. 1B, right) indicates that the large cardiac titin isoform is expressed at the expense of N2B titin. Immunoelectron microscopy shows that in hypothyroid animals the large cardiac titin isoform is co-expressed with N2B titin at the level of the half sarcomere (Fig. 3). Thus it is possible that the large titin isoform that is expressed in long-term hypothyroid rats represents fetal cardiac titin. Conclusive results require development of antibodies specific for fetal cardiac titin.

Fig. 4. Skinned cardiac muscle and skinned myocyte mechanics of PTU and control rat LV. (A) Titin-based passive tension–SL relationships. PTU treated rat LV myocardium has significantly lower titin-based passive tension. (B) Collagen-based passive tension-sarcomere length (SL) relationship. PTU treated rat LV myocardium has significantly higher collagen-based passive tension. Results from 6 control and 6 PTU treated (4 months) RLV muscle strips. Inset shows frozen sections labeled with anti-collagen type I and type III antibodies. Labeling is more intense in PTU myocardium (calibration bar is 25 μm). (C) Examples of restoring force measurements on myocytes. Data at SLs from 1.75 to 1.95 μm were fit with a linear equation (solid lines) and the slope of the fit was determined. Inset shows the summary of the obtained slopes in 10 control and 14 PTU treated myocytes. The slope (mN/(mm² μm/sarcomere) is significantly lower in myocytes from PTU rats (P=0.02).
large isoform and, as a result, passive force will be lower [11]. Considering that titin functions as a bi-directional spring [25] that develops restoring force when shortened to below the slack length (in addition to passive force when stretched) the longer spring element of the large isoform is also expected to lower restoring force in PTU animals. Thus, the finding that both passive forces and restoring forces are much lower in PTU rats (Figs. 4A and C) is consistent with the upregulation of a large N2BA titin isoform.

It has been proposed that upregulation of N2BA titin, which has been reported in ischemic human heart disease [17] and dilated cardiomyopathy patients [15,16], compensates for increased collagen-based stiffness. Thus, this compensation based on reduced titin-based passive stiffness may also occur in myocardium of hypothyroid animals (see also below).

4.3. Passive stiffness of myocytes vs. diastolic stiffness of the LV

The lower titin-based passive stiffness contrasts with the increased diastolic stiffness of the LV chamber in long-term PTU
treated animals (Fig. 5C). The elevated diastolic stiffness can be explained by the increased collagen-based stiffness that was revealed by our mechanical experiments on muscle strips isolated from long-term PTU treated rats and increased collagen type I and III levels (inset of Fig. 4B) (Fig. 4B). These findings are consistent with the fact that thyroid hormone is a negative regulator of expression of collagen [3–5]). The elevated diastolic LV stiffness in PTU animals might also in part be due to dysfunction of the calcium handling system. In normal adult rat hearts, reducing cytosolic calcium following contraction relies largely on the sarcoplasmic reticulum (SR) with SERCA transporting calcium into the lumen of the SR, resulting in relaxation. Hypothyroid rats express significantly lower levels of SERCA and, in addition, upregulate the SERCA inhibitor phospholamban [26,27]. Both changes are expected to slow calcium reuptake, and this might contribute to increased diastolic stiffness of PTU hearts. However, it is unlikely that this is a major factor in our isolated heart studies, because the hearts were paced at a relatively low frequency (atrioventricular node ablation made this possible) and pressure had decayed to a steady-state value when diastolic pressure was measured. Furthermore, the twitch relaxation parameters (such as time for pressure to reduce from 75% to 25% of peak pressure) were not different between the short-term and long-term hypothyroid rats, with normal and elevated diastolic wall stress, respectively (results not shown). Thus, dysfunction of the calcium handling system may only make a minor contribution to the high diastolic wall stress in the long-term hypothyroid hearts. The main reason for the high wall stress is likely to be an increase in collagen-based stiffness, explaining why diastolic LV stiffness is higher despite that the titin-based passive stiffness of the skinned myocardium is reduced.

4.4. Conclusion

Our study demonstrates that during long-term hypothyroidism collagen-based myocardial stiffness is increased whereas titin-based passive stiffness is reduced. The reduction in titin-based stiffness can be explained by the expression of a large cardiac titin isoform, which might represent fetal cardiac titin. The change in titin splicing that occurs during long-term hypothyroidism may be viewed as a beneficial adaptation to reduce diastolic stiffness and improve diastolic filling.

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