Thyroid Hormone Regulates Developmental Titin Isoform Transitions via the Phosphatidylinositol-3-Kinase/AKT Pathway

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Abstract—Titins, giant sarcomere proteins with major mechanical/signaling functions, are expressed in 2 main isoform classes in the mammalian heart: N2B (3000 kDa) and N2BA (>3200 kDa). A dramatic isoform switch occurs during cardiac development, from fetal N2BA titin (3700 kDa) expressed before birth to a mix of smaller N2BA/N2B isoforms found postnatally; adult rat hearts almost exclusively have N2B titin. The isoform switch, which can be reversed in chronic human heart failure, alters myocardial distensibility and mechanosignaling. Here we determined factors regulating this switch using, as a model system, primary cardiomyocyte cultures prepared from embryonic rats. In standard culture, the mean N2B percentage initially was 14% and increased by ≈60% within 1 week, resembling the in vivo switching. The titin isoform transition was independent of endothelin-1–induced myocyte hypertrophy and was not altered by pacing, contractile arrest, or cell stretch; however, it was modestly impaired by decreasing substrate rigidity and strongly dependent on serum components. Angiotensin II significantly promoted the transition. The mean N2B proportion in 1-week-old cultures dropped 20% to 25% in hormone-reduced medium, but addition of 3,5,3′-triiodo-L-thyronine (T3) nearly restored the proportion to that found in standard culture. This T3 effect was not prevented by bisphenol A, a specific inhibitor of the classic genomic pathway of T3 action. In contrast, the titin switch could be stalled by the phosphatidylinositol 3-kinase inhibitor LY294002, which decreased the proportion of N2B mRNA transcripts within hours and suppressed a rapid T3-induced increase in Akt phosphorylation. Also, angiotensin II, but not endothelin-1 or cell stretch, enhanced Akt phosphorylation. Thus, although matrix stiffness modulates developmental titin isoform transitions, these transitions are mainly regulated through phosphatidylinositol 3-kinase/Akt-dependent signaling triggered particularly by T3 via a rapid action pathway. (***Circ Res*****. 2008;102:439-447.)**

Key Words: cardiac development ■ growth factors ■ mechanosignaling ■ thyroid hormone

During perinatal heart development, various myocardial proteins show a switch in isoforms from the fetal to the adult type. This isoform switching is characteristic for contractile proteins such as α-actin and myosin heavy chain (MHC) and muscle regulatory proteins including tropomyosin, myosin light chain-1, and troponins.[1] Developmental isoform transitions also have been reported for the giant elastic protein titin.[2–5] Two main titin isoform classes are expressed in mammalian heart: N2B (3000 kDa) and N2BA (>3200 kDa); both are splice variants from a single titin gene.[6] The isoform diversity in titin results from alternative splicing in the elastic I band segment, which generates the relatively short and stiff N2B isoform and many long and more compliant N2BA isoforms. The size variability among the N2BA isoforms (3200 to 3700 kDa) arises from differential splicing of the mid-immunoglobulin domain region (consisting of serially linked immunoglobulin (Ig)-like domains and the PEVK domain). Because the N2BA and N2B isoforms are coexpressed in the cardiac half-sarcomere, the changes in titin isoform composition during heart development affect myofibrillar extensibility and passive force generation and alter the stiffness of the cardiac walls.[7]

The titin isoform transition during development is particularly dramatic in rat hearts. Fetal hearts at embryonic day (E)16 express exclusively a long and compliant N2BA titin isoform of ≈3700 kDa but no N2B titin.[3–5] Around birth, this fetal isoform becomes downregulated and shorter, less extensible N2BA isoforms appear together with N2B titin (Figure 1). After birth, the stiff N2B isoform is strongly upregulated and soon constitutes >90% of total titin, which is the N2B proportion found in adult rat hearts.[2–5] The perinatal isoform switch from 100% fetal N2BA to >90% N2B greatly increases titin-based passive stiffness in rat myocardium over the course of a few weeks. These modifications likely affect

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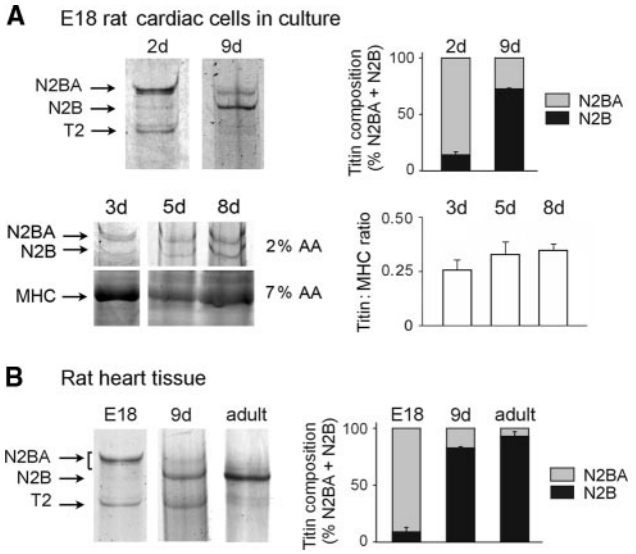

Figure 1. Titin isoform transition in cultured rat CMs and in rat heart tissue in vivo. A, Top, Examples of titin bands on 2% SDS-PAGE gels and titin isoform composition on culture days 2 (2d) and 9 (9d). Cells were isolated on E18 and grown under standard conditions (IMDM+20% FCS). Bottom, Total titin to MHC expression determined by 2-phase SDS-PAGE (2%/7% acrylamide [AA]). B, Titin switch in intact rat heart from fetal E18 to culture day 9 to adult stage. Data in graphs are means±SEM (n=3 to 7). N2BA and N2B indicate full-length titin isoforms; T2, proteolytic titin fragment.

other mechanical properties, such as the length-dependent Ca^{2+} sensitivity of active force development and mechanochemical signaling events in the myocyte.[7]

Fetal isoforms of myocardial proteins can be reexpressed in failing adult hearts.[8] With regard to titin, human hearts in end-stage systolic failure express increased proportions of N2BA isoforms compared with nonfailing control hearts, causing lower than normal myofibrillar passive stiffness.[9–11] In particular, the extremely large N2BA isoforms are upregulated in failing human myocardium,[11] suggesting there may be reexpression of fetal titin isoforms. Interestingly, experimentally induced hypothyroidism in adult rats caused reexpression of large cardiac N2BA titin isoforms, which was associated with a transition to cardiac failure.[12] Thus, the thyroid hormone metabolism may have a role in regulating titin isoform expression in heart disease; however, the mechanism of action is unknown. Many earlier studies on diseased or developing myocardium suggested a role for thyroid hormones in regulating the isoform expression of major cardiac proteins, including MHC and troponins.[13–16] Whether thyroid hormone affects the dramatic titin isoform switching during development had not been studied before.

In this work, we determined the role of growth-promoting hormones, particularly 3,5,3′-triiodo-L-thyronine (T3) but also endothelin (ET)-1 and angiotensin (Ang) II, contractile activity, and mechanical stress (stretch) for the developmental titin isoform transitions. We chose the model system of the primary cardiomyocyte (CM) cell culture, which allows for direct manipulation of hormonal components and mechanical parameters. CMs were prepared from late-embryonic (E18) rat hearts, a stage at which the rapid changes in titin isoform expression begin. To our knowledge, titin isoform composition had never been studied in a CM cell culture model before. Our results show that a developmental titin isoform switch from low to high proportions of N2B titin occurs in cultured cardiac cells and resembles that found in vivo. As a main determinant of the titin isoform transition, we identify the T3 hormone, with Ang II promoting the transition as well. The data suggest that T3 and Ang II act on titin switching via pathways critically involving phosphatidylinositol 3-kinase (PI3K)/Akt-dependent signaling.

Materials and Methods

Heart Tissue

Heart tissue was obtained from adult pregnant Sprague–Dawley rats and embryos after 18 days of gestation (E18). The animals, bred at the department animal house at the University of Muenster, Germany, were anesthetized with ether and killed by cervical dislocation. All procedures were conducted in accordance with the guidelines of the local Animal Care and Use Committee.

Primary Cultures of Embryonic Rat CMs

CMs were isolated from E18 hearts by enzymatic dissociation as described,[17] plated at a density of 8×10^5 cells/mm^2, and cultured at 37°C and 5% CO_2. To prevent overgrowth of CMs by fibroblasts, the cells were exposed once on culture day 3 to irradiation (15 Gy). In 1 set of experiments, CMs were cultured in Iscove's modified Dulbecco medium (IMDM) supplemented with 20% FCS for 2 days. From culture day 3 on, the cells were serum-starved (FCS-starved), and the medium was supplemented with 1 of the following (or a mix of 2 of these components): Ang II (1 μmol/L); ET-1 (10 nmol/L); KCl (50 mmol/L); T3 (75 nmol/L); bisphenol A (BPA) (100 μmol/L); and LY294002 (LY) (10 μmol/L). In a different set of experiments, CMs were cultured in hormone-reduced medium containing charcoal-filtered serum (IMDM+chf-FCS); the medium was supplemented with T3 (15 to 150 nmol/L) or T3 and either BPA (100 μmol/L) or LY (50 μmol/L).

Radial Stretching and Pacing of Cultured CMs

E18 CMs were plated onto 6-well Bioflex culture dishes and exercised by radial stretch–release cycles at a frequency of ≈0.02 or 1.0 Hz for up to 4 days using a cell stretcher apparatus. For pacing experiments, E18 CMs were plated onto 4-well plates and paced between culture days 4 and 8 (3-ms pulses, 27 V, 2 Hz).

Histomorphometry

Digital images of cultured cells were obtained with a CCD camera installed on an inverted light microscope using a ×40 objective in phase-contrast mode, and the area of the cells was calculated.

Preparation and Stretching of Engineered Heart Tissues

Circular engineered heart tissues (EHTs) were constructed from neonatal heart cells as described.[18] EHTs were kept under either phasic or static strain from day 7 to day 12 of culture and were then frozen in liquid N_2 and stored at −80°C.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis

Rat heart tissue, EHTs, and CM cultures were homogenized in modified Laemmli buffer.[19] Agarose-strengthened 2% SDS-PAGE to detect titin was performed as described.[3,9] Protein bands were visualized by Imperial protein stain, scanned, and analyzed densitometrically. Average titin isoform composition was calculated from a minimum of 3 per experimental condition. Titin:MHC ratio was determined on 2-phase (2%/7%) acrylamide gels.

Akt Phosphorylation Assay

Protein components were separated by 12.5% SDS-PAGE, transferred onto a poly(vinylidene difluoride) membrane, and probed by Western blot using monoclonal antibodies against Akt, phospho (p)-Akt$_{(Ser473)}$, and p-Akt$_{(Thr308)}$. Goat anti-rabbit IgG-conjugated with horseradish peroxidase served as secondary antibody. Protein amount was normalized to β-actin.

Real-Time RT-PCR

CMs were cultured in FCS-starved or hormone-reduced medium. Total RNA was isolated from cells harvested at various time points (between 2 and 72 hours) following addition of T3 or T3+LY. cDNA was synthesized using oligo(dT)$_{18}$ primers. Real-time RT-PCR was conducted in an Mx3000P thermocycler (Stratagene) using Brilliant SYBR Green QPCR Mastermix. For primer sequences, see the online data supplement, available at http://circres.ahajournals.org.

Statistics

To test for statistically significant differences, we used ANOVA and the unpaired Student's *t* test. Probability values of <0.05 were taken as indicating significant differences and are indicated by asterisks in the figures.

Results

Titin Isoform Switching in Primary CM Cultures Occurs Similarly as Observed In Vivo

The proportions of the 2 titin isoform classes, N2BA and N2B, were analyzed in rat CM samples prepared from embryonic E18 hearts and grown under standard culture conditions (IMDM+20% FCS) (Figure 1A). Results were compared with the titin isoform composition found in intact developing rat hearts at stages E18, 9 days, and adult (Figure 1B). On culture day 2, the mean percentage of N2B isoform was ≈14%, the remainder being 2 fetal N2BA isoforms of 3700 and ≈3600 kDa (Figure 1A, top). This composition is similar to that measured in intact E18 rat hearts (10% N2B, 90% N2BA). The identity of the titin isoforms was confirmed previously by us using Western blotting.[3] Until culture day 9, the mean N2B titin percentage increased to 73% (Figure 1A, top) and did not exceed this value in cells held in culture for up to 12 days (data not shown). In comparison, 9-day-old intact rat hearts had an average relative N2B content of 83% (Figure 1B). The titin isoform composition of adult rat hearts (≈93% N2B and low-abundant N2BA isoforms of ≈3400 and ≈3200 kDa) was never reached in primary CM cultures. Total titin relative to MHC expression remained unchanged during CM cultivation (Figure 1A, bottom).

Serum Components Influence Titin Isoform Transition in Culture

FCS contains a complex mixture of hormones and growth factors, which are likely to affect the titin isoform switching in CM culture. The presence of FCS in the IMDM was required for the first 2 culture days to ensure cell adhesion. Subsequently, cells could be grown in FCS-free medium (FCS-starved). The average proportion of N2B titin was 62% on culture day 9 under serum-free conditions, significantly lower (by ≈11%) than in CMs grown in IMDM+20% FCS (Figure 2A).

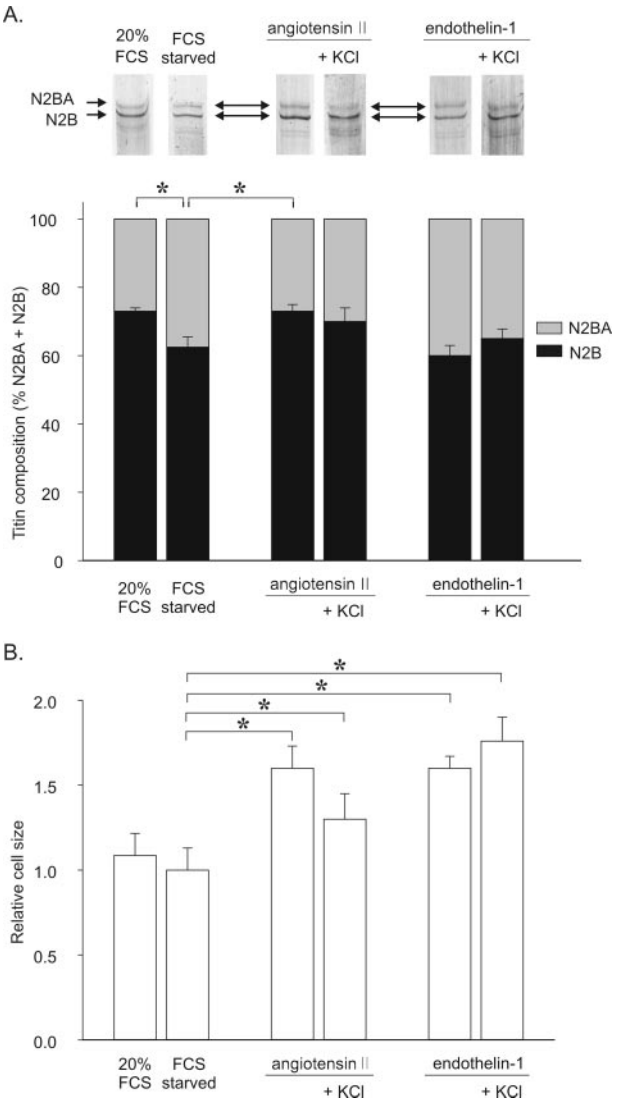

Figure 2. Influence of growth-promoting factors on titin isoform expression and CM hypertrophy. A, Titin isoform composition in CMs cultured for 9 days. One set of CMs was grown under standard conditions for the entire cultivation period (20% FCS); all other CMs were kept in medium depleted of FCS from culture day 3 on (FCS-starved). The effect of 1 μmol/L Ang II or 10 nmol/L ET-1 on titin expression was studied in absence or presence of 50 mmol/L KCl. Bars in the graph show means±SEM (n=4 to 7). B, Relative cell size in CM cultures measured on day 6. Bars show means±SEM (n=40 to 70 cells analyzed for circumferential size).

Cell Hypertrophy and Spontaneous Beating Are Not Essential for Titin Isoform Switching

Various serum components could potentially influence the titin isoform transition via inducing hypertrophy. We mimicked a hypertrophic environment by adding either Ang II or ET-1 to the culture medium on the first day of serum starvation (culture day 3). As expected, both hormones induced hypertrophic growth, detected as an increase in average cell size by a factor of ≈1.6, compared with FCS-starved CMs grown in the absence of Ang II and ET-1 (Figure 2B). The mean N2B titin percentage increased to 73% (culture day 9) in the presence of Ang II, up by ≈11% from

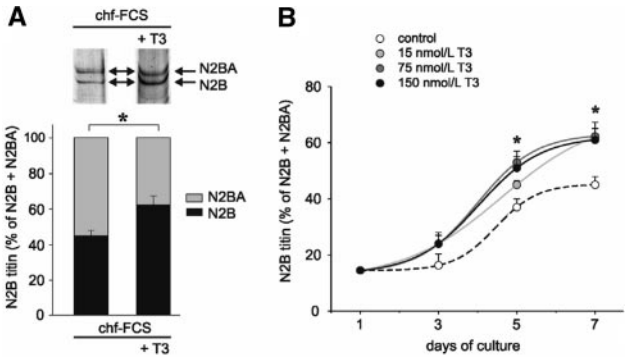

Figure 3. Effect of T3 on titin isoform composition in CMs cultured in hormone-reduced medium containing chf-FCS. A, Titin isoform expression on culture day 7 in the absence and presence of 75 nmol/L T3. B, Time course of changes in N2B titin percentage of CMs cultured in presence (filled circles) or absence (open circles) of T3. Bars/symbols show means±SEM (n=3 to 8). Curves are best sigmoidal fits to data.

the FCS-starved control (Figure 2A). In contrast, ET-1 had no effect on the titin isoform transition (Figure 2A).

Spontaneous CM contractions were permanently arrested by adding 50 mmol/L KCl (at culture day 3) to FCS-starved cultures grown either in the presence or absence of Ang II and ET-1. Contractile arrest reduced the average cell size in Ang II–treated CMs by 19% and increased the cell size in ET-1–treated cells by 10% (Figure 2B). However, contractile arrest did not significantly impact the titin isoform composition in Ang II and ET-1–treated cells (Figure 2A).

We also studied whether pacing of the CMs affects titin expression. CMs grown under standard conditions were subjected to electrical stimulation at 2 Hz (27 V) for 2 days (culture days 3 to 5). However, the mean N2B proportion was similar (67% to 73%) in paced and nonpaced cultures (data not shown). Taken together, these results suggest that Ang II significantly promotes the developmental titin isoform transition, although the transition essentially takes place independent of contractile activity or CM hypertrophy.

T3 Is a Key Factor in the Titin Isoform Transition

The serum concentrations of the thyroid hormone T3 greatly increase with the onset of thyroid function in the fetus. Whether T3 affects the titin isoform switching was tested by adding the hormone to CMs cultured in FCS-containing medium, in which hormone concentrations were selectively reduced by charcoal treatment (chf-FCS) (Figure 3). Hormone reduction lowered the mean N2B titin proportion substantially, eg, on culture day 7 from ≈70% in standard FCS to 45% in chf-FCS. In contrast, supplementing the chf-FCS medium with T3 (range of concentrations, 15 to 150 nmol/L) greatly enhanced the titin isoform switching, with a maximum effect already seen at 15 nmol/L T3 (Figure 3B). At culture day 7, at which the mean N2B percentage leveled out, T3-treated CMs (at all T3 concentrations) expressed on average ≈62% N2B titin, 17% more than controls (Figure 3A and 3B). The N2B titin percentage increased during CM culture even in control cells (Figure 3B, dashed curve), which could be related to the fact that T3 remained in the serum despite the charcoal filtering (our analysis showed a reduction in T3 content by ≈40% in chf-FCS, compared with standard FCS). In any case, we conclude that T3 is important for regulating the developmental titin isoform switching.

T3 Regulates Titin Isoform Expression via a Novel Pathway Involving PI3K/Akt Signaling

The classic genomic pathway of T3 action via thyroid hormone receptors is blocked by BPA (Figure 4A).[20,21] We added 100 μmol/L BPA to CMs cultured in the presence of T3 to test for possible effects on the titin isoform switching but found no effect (Figure 4B, left, and 4C). Thus, the T3 effect on titin is unlikely to be mediated via the classic genomic pathway.

Alternatively, T3 could affect titin isoform switching via a rapid novel pathway involving PI3K-dependent signaling (Figure 4A).[22] When activated by T3, PI3K induces phosphorylation of Akt/protein kinase B (PKB) at positions Ser473 and Thr308, thereby increasing Akt kinase activity. To test for the involvement of this pathway, T3-treated CMs were grown in FCS-free medium for an additional 6 days starting at culture day 3 (Figure 4B) or for the entire culture period in chf-FCS medium (Figure 4C) and were exposed to the PI3K inhibitor LY. Moreover, a possible effect of LY on titin isoform composition was tested in cells cultured in chf-FCS medium without added T3 (Figure 4C, left 2 columns).

In 5-day-old FCS-starved cultures treated with 75 nmol/L T3 for 3 days, the presence of 10 μmol/L LY reduced the mean N2B titin percentage from ≈54% to 41% (Figure 4B, left, asterisk). A similar, but much more dramatic, effect was seen in cells grown in chf-FCS medium supplemented with 50 μmol/L LY. Here, the mean N2B proportion (on culture day 5) was 38% in the absence of T3 and LY, ≈54% in the presence of 75 nmol/L T3, but 21% in T3+LY-treated cells and 16% in LY-only treated CMs (Figure 4C). The latter value is almost identical to the relative N2B level at the beginning of the culture period using E18 rat heart tissue. Thus, blocking the PI3K/Akt pathway by LY fully inhibits the titin isoform transition.

These effects again were tested for a possible causative relationship to cell hypertrophy or contractile activity. FCS-starved CMs treated with T3 showed a 1.6-fold increase in cell size compared with controls, which was somewhat reduced in the presence of 100 μmol/L BPA or 10 μmol/L LY and following contractile arrest by 50 mmol/L KCl during T3 treatment (Figure 4B, right). However, the titin isoform switching, being affected by LY, remained unaltered by KCl and BPA (Figure 4B, left). We conclude that the T3-induced effects on titin isoform expression are independent of cell hypertrophy or contractile status.

The T3 Effect on Titin via Changes in PI3K/Akt Signaling Occurs Rapidly

To test whether T3 and LY indeed affect the PI3K/Akt-signaling pathway, CMs were harvested 60 minutes and 24 hours following addition of 75 nmol/L T3 or T3+50 μmol/L LY to chf-FCS–containing medium and were analyzed for Akt and p-Akt expression by Western blot (Figure 5A). At 60 minutes, T3 significantly increased Akt phosphorylation at

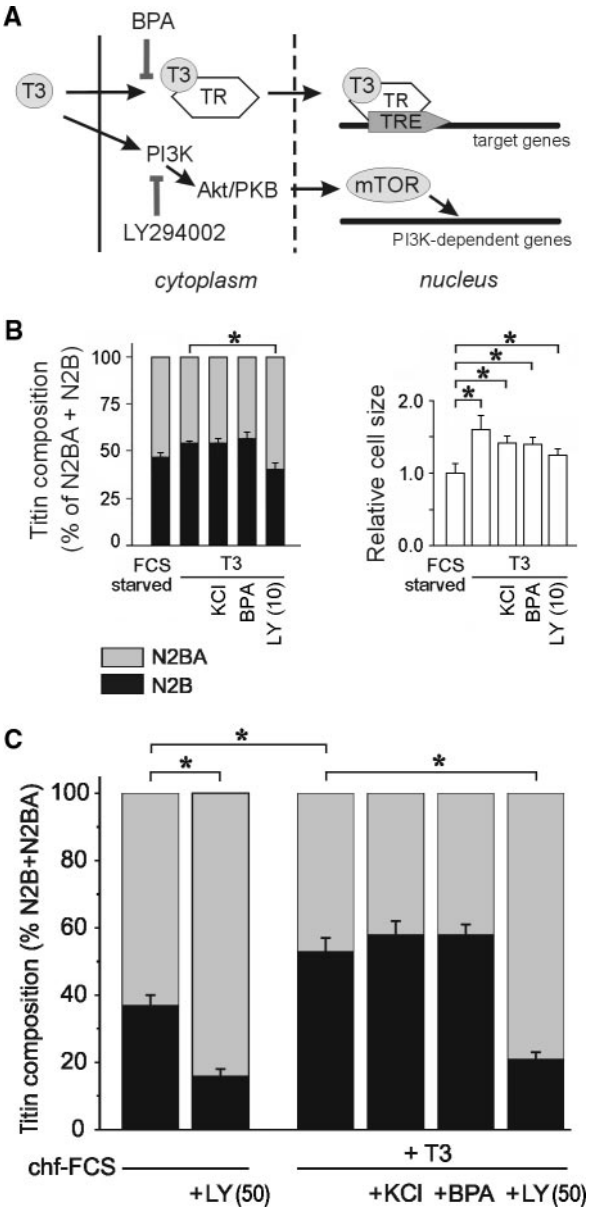

Figure 4. Probing the pathway of T3 action on titin isoform composition. A, Simplified scheme showing classic genomic pathway and novel rapid pathway of T3 action. TR indicates thyroid hormone receptors; TRE, thyroid hormone-responsive elements; Akt/PKB, Akt kinase/protein kinase B; mTOR, mammalian target of rapamycin. B, Titin isoform composition (culture day 5) (left) and relative cell size (culture day 8) (right) of CMs grown in FCS-starved medium. Some cultures were exposed, beginning at day 3 (time of serum removal), to T3 (75 nmol/L), T3+KCl (50 mmol/L), T3+BPA (100 μmol/L), or T3+LY (10 μmol/L). Bars in the right graph show means±SEM (n=40 to 70 cells). C, Titin isoform composition in CMs cultivated for 5 days in hormone-reduced medium supplemented with LY (50 μmol/L), T3 (75 nmol/L), T3+KCl (50 mmol/L), T3+BPA (100 μmol/L), or T3+LY. Bars in B (left) and C show means±SEM (n=3 to 8).

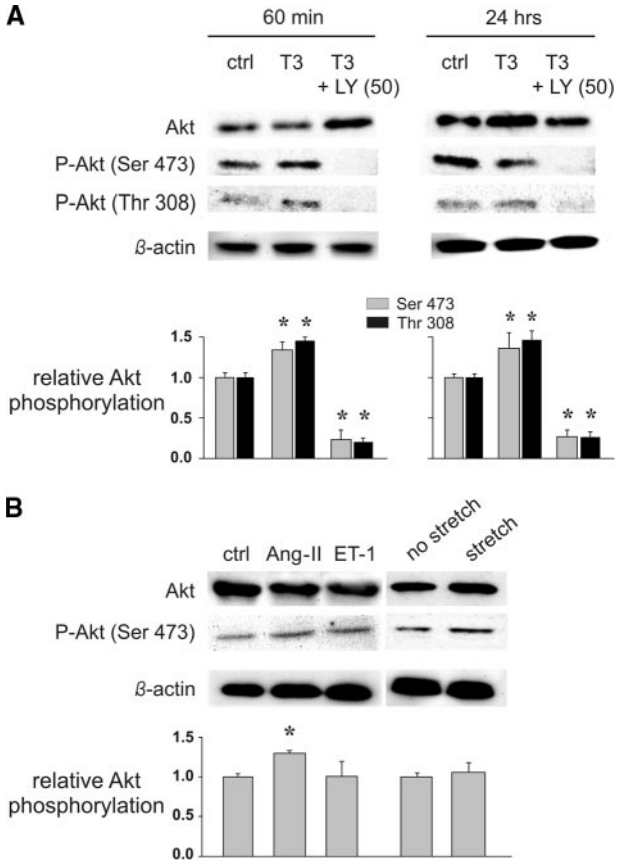

Figure 5. Akt phosphorylation tests. A, Changes in Akt phosphorylation in CMs treated with T3 (75 nmol/L) or T3+LY (50 μmol/L) determined by Western blot. Akt and p-Akt [P-Akt (Ser473), P-Akt (Thr308)] compared with β-actin levels were analyzed in CMs cultured for 2 days in hormone-reduced medium before T3 or T3+LY were added for 60 minutes or 24 hours. B, Influence of Ang II, ET-1, and stretch on Akt phosphorylation. CMs were cultured in standard culture medium for 2 days before radial stretch was applied or serum was removed and Ang II or ET-1 added to the culture medium for 24 hours. Bars show means±SEM (n=3).

Ser473 and Thr308 by 1.35- and 1.45-fold, respectively, compared with control CMs cultured without T3. This increase in Akt phosphorylation persisted for >24 hours. In contrast, Akt phosphorylation was effectively suppressed in cells treated with T3+LY for 60 minutes or 24 hours (Figure 5A), demonstrating inhibition of PI3K/Akt signaling and suggesting that T3 may induce some of its effects using the Akt pathway.

We also wanted to know whether Akt phosphorylation is altered by Ang II, ET-1, or radial cell stretch (Figure 5B). Whereas Akt phosphorylation remained unaffected in CMs exposed for 24 hours to ET-1 or repeated stretch–release cycles, it was increased 1.3-fold by Ang II (Figure 5B).

We reasoned that fast effects of T3 and LY on titin may first appear as rapid alterations at the mRNA level, rather than at the protein level. Therefore, real-time RT-PCR was used to quantify the levels of N2BA and N2B titin transcripts at various time points following addition of T3 or T3+LY using primer pairs each amplifying 1 of the 2 main titin splice pathways (Figure 6A). In FCS-starved CMs, the proportion of N2B transcripts significantly increased within 72 hours following treatment with 75 nmol/L T3, whereas both at 18 and 72 hours following exposure to T3+10 μmol/L LY, the N2B mRNA percentage decreased (Figure 6B, asterisks). Similarly, in cells grown in chf-FCS medium supplemented with 75 nmol/L T3 (Figure 6C), the N2B mRNA transcripts were

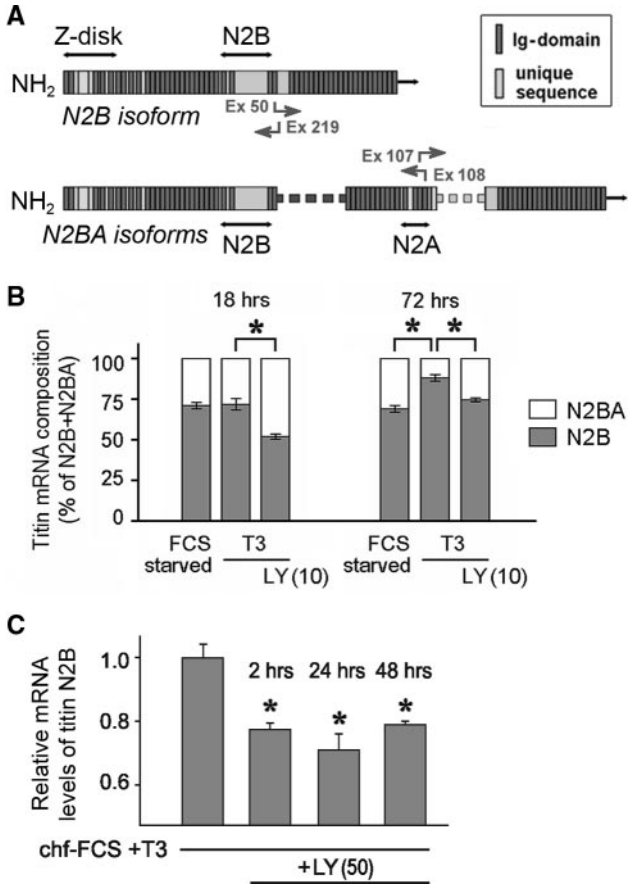

Figure 6. Rapid alterations in titin mRNA transcripts depending on PI3K/Akt signaling. A, Domain architecture of titin I band segments showing positions of primer pairs amplifying the N2B (exons [Ex] 50 to 219) or N2BA [exons 107 to 108) mRNA transcripts. B, Proportion of titin N2B and N2BA mRNA transcripts by real-time RT-PCR, measured in CMs grown in FCS-starved medium. Medium was supplemented with either T3 (75 nmol/L) or T3+LY (10 μmol/L) for a period of 18 or 72 hours. C, Proportion of titin N2B mRNA transcripts by real-time RT-PCR in CMs grown for 2 days in hormone-reduced medium (chf-FCS) supplemented with T3 (75 nmol/L) or T3+LY (50 μmol/L) for 2, 24, or 48 hours. Total titin transcripts remained unchanged. Bars show means±SEM (n=4 to 7).

rapidly reduced by the additional presence of 50 μmol/L LY for 2 hours (25% reduction), 24 hours (29%), or 48 hours (21%). We conclude that T3 regulates the developmental titin isoform transition via rapid PI3K/Akt-dependent signaling.

Mechanical Factors Can Play a Modulatory Role in the Titin Isoform Transition

To study a possible effect of external mechanical forces on titin expression, E18 CMs were plated on flexible rubber membranes and grown under standard culture conditions (20% FCS) for 4 days. Then, the CMs were exposed to radial stretch–release cycles (≈10% average maximum strain) at 0.02 Hz (Figure 7A and 7B) for another 4 days, and titin isoform composition was examined. Control CMs grown for 8 days on rubber membrane in the absence of stretch expressed on average 59% N2B titin; however, cyclic mechanical stretching did not significantly alter this value (56%) (Figure 7B). Furthermore, contractile arrest using 50 mmol/L KCl had no impact on these values; the N2B titin was now 59% in unstretched and 62% in stretched cells (Figure 7B). We also increased the stretch rate up to 1 Hz but again without affecting titin isoform composition (data not shown). These results suggest that neither cyclic stretching nor the combination of stretch and contractile arrest alter the developmental titin isoform transition.

Interestingly, in CMs grown on rubber membrane for 1 week, the N2B titin percentage was consistently lower by 15% to 20% compared with CMs cultured for the same period under standard conditions on a rigid plastic surface (Figure 7B, asterisk). We tested whether the T3 effect found in cells grown on plastic dishes was still present in CMs cultured on flexible membrane in chf-FCS medium (Figure 7C). Hormone reduction further decreased the mean N2B percentage measured on culture day 7% to 39%, ≈20% lower than in CMs cultured on rubber membrane in standard medium, but the presence of 75 nmol/L T3 restored the N2B proportion to 60% (Figure 7C).

Finally, the effect of different stretch regimes on titin expression was studied in 3D cultures of EHT generated from neonatal rat cardiac cells as described.[18] The mean N2B percentage amounted to 57% in EHTs after 12 days of culture, substantially higher than the N2B percentage in neonatal hearts (40%), where the cells were derived from (Figure 7D). However, the titin isoform composition was identical in EHTs exposed to either phasic (2 Hz) or static (up to 110% slack length) stretch between culture days 7 and 12 (Figure 7D). The values measured in the EHTs were identical to those found in the 2D cultures grown on flexible membrane for 1 week (Figure 7B). In conclusion, whereas various stretch modes imposed on 2D or 3D cultures did not affect the developmental titin isoform transition, increased substrate stiffness promoted this transition, hinting at a modulatory role played by rigidity sensing.

Discussion

We used primary cultures of late-embryonic rat CMs to study signaling events responsible for the dramatic titin isoform switching during perinatal heart development. The isoform transitions in CM culture were shown to not be correlated with myocyte hypertrophy per se and were unaltered by cell stretching, pacing, or contractile arrest. In contrast, the switching was found to be regulated predominantly by T3 and to be tunable also by Ang II and by altering substrate rigidity. The developmental titin switch was blocked by inhibiting Akt phosphorylation, suggesting that PI3K/Akt-dependent signaling is critical for setting the titin isoform pattern.

Increased plasma concentrations of T3 promote a hyperdynamic state of the heart particularly during development,[14] because T3 regulates the genes of important myocardial proteins. For instance, α-MHC and sarcoplasmic reticulum Ca^{2+}-ATPase are positively regulated, whereas β-MHC, phospholamban, and collagen are negatively regulated.[14] Before this study, the role of T3 in the developmental titin switch was unknown, although there were hints suggesting that thyroid gland maturation and titin isoform transition occur in the fetus at approximately the same time.[5,23] The classic pathway of T3 action requires complex formation of

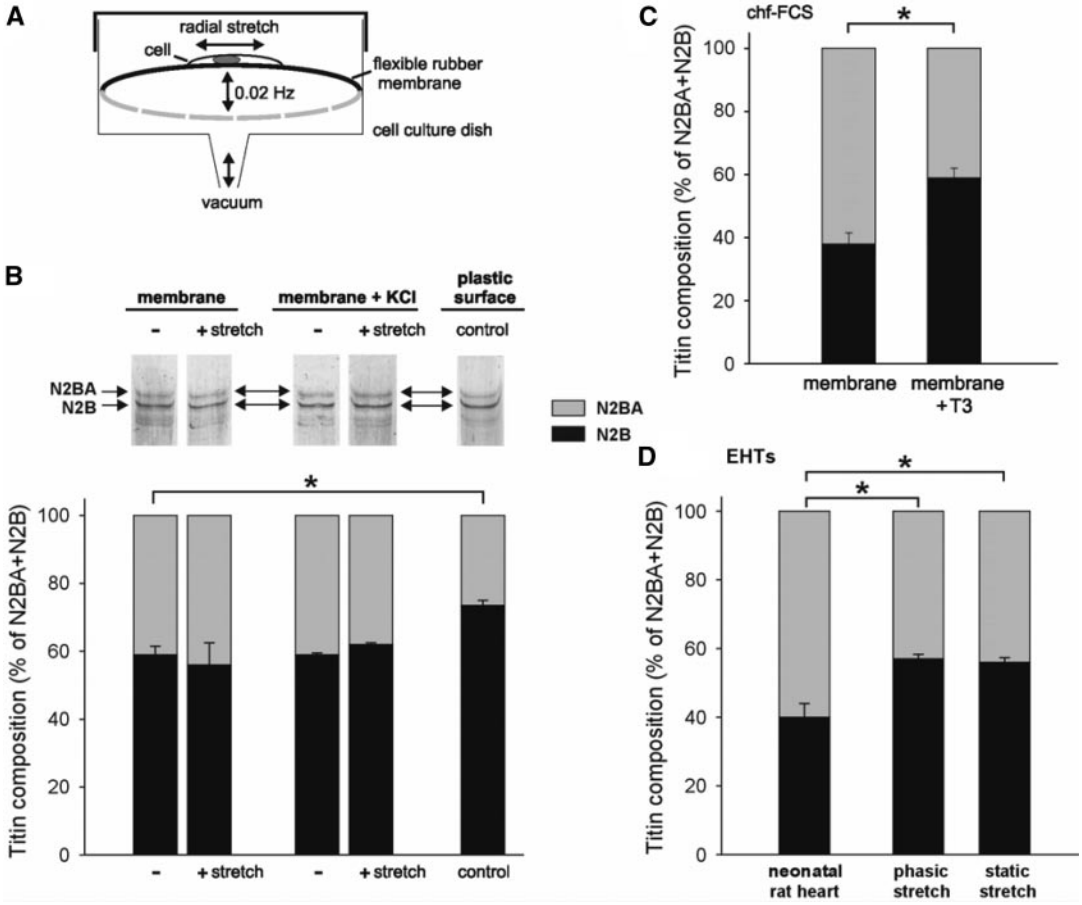

Figure 7. Mechanical strain and titin isoform expression. A, Scheme of experimental procedure for radial cell stretching. B, Titin isoform composition in CMs cultivated for 8 days (standard conditions) on rubber membrane and exposed to 0.02 Hz radial stretch–release cycles over the last 4 culture days (+stretch). Controls (-) were cultivated on rubber membrane without stretch. Some cultures were arrested using 50 mmol/L KCl. For comparison, CM cultures were also grown on rigid plastic surface. C, Titin isoform composition in CMs after cultivation for 7 days on rubber membranes in hormone-reduced medium (chf-FCS) in presence or absence of 75 nmol/L T3. D, Titin isoform composition in engineered heart tissues (EHTs) generated from neonatal rat heart cells and cultivated under phasic or static stretch from days 7 to 12 of culture. Data in all graphs are means±SEM (n=3 to 6).

T3 with nuclear thyroid hormone receptors; in the nucleus, this complex binds to thyroid hormone responsive elements in the promoter regions of target genes, thereby activating or repressing their transcription.[14,16,24] This genomic pathway is blocked by BPA, which displaces T3 from thyroid hormone receptors.[20,21] In our hands, however, BPA had no effect on the T3-induced titin isoform transition. This result is not unexpected, considering that the titin isoforms are generated by differential splicing from the copy of only a single titin gene, whereas the classic T3 action pathway requires the presence of differently regulated promoters on different genes.

We therefore hypothesized that T3 may act on titin through a novel pathway involving PI3K/Akt-dependent signaling, previously shown to mediate more rapid effects of T3 on CMs.[22,25,26] Indeed, the PI3K inhibitor LY effectively inhibited the titin isoform transition in culture in a concentration-dependent manner. At the mRNA level, the LY-induced decrease in N2B titin percentage occurred within a few hours, the same time frame within which we observed the effects of T3 and LY on Akt phosphorylation. These findings are consistent with previous reports showing that T3 specifically activates Akt signaling in neonatal rat CMs in <24 hours, causing altered expression of MHC genes[27] and hypertrophy[28]: effects that are reversed by LY.[22] Our data suggest that T3 regulates titin isoform composition via the novel, rapid action pathway by promoting Akt phosphorylation. Although the results do not prove that enhanced Akt signaling is really required for the titin transitions, we can conclude that PI3K is critically important; whether PI3K activation is the sole requirement remains to be determined.

Alterations in thyroid hormone metabolism also occur during heart disease. Heart failure is frequently associated with decreased serum T3 levels, referred to as low T3 syndrome.[14,15] A characteristic of this syndrome is a reactivation of the fetal gene program, a phenomenon thought to occur quite commonly in the progression to heart failure. A recent study demonstrated that experimentally induced hypothyroidism in adult rats causes reexpression of a large, compliant titin N2BA isoform normally expressed only in fetal rat hearts.[12] Moreover, human hearts in end-stage systolic failure express larger, more compliant N2BA titin isoforms than control donor hearts.[9–11] Hence it will be interesting to study, also in view of potential therapeutic

applications, whether alterations in thyroid hormone metabolism trigger (reversed) titin isoform transition in chronically diseased human hearts and whether T3 acts on titin expression through the PI3K/Akt pathway in failing myocardium as well. Analyses of animal models, in which the thyroid hormone metabolism or the PI3K/Akt pathway are pharmacologically or genetically disturbed, could be helpful in addressing these questions.

Addition of T3 to hormone-reduced medium restored the N2B proportion to nearly (cells on plastic surface) or exactly (cells on rubber membrane) the value found under standard culture conditions. However, factors other than T3 likely modulate the developmental titin isoform switching. Our focus on the peptide hormones ET-1 and Ang II, which promote cell hypertrophy and CM contractility and can induce MHC isoform transitions,[29–33] revealed that only Ang II has a small but significant effect on the titin isoform switching. Notably, Ang II, but not ET-1, increased Akt phosphorylation, consistent with previous work.[34,35] We therefore propose that also the Ang II–dependent effects on titin expression are mediated through the PI3K/Akt pathway.

One could speculate on a mechanism of how Akt phosphorylation affects the titin isoform pattern. To this end, serine/arginine-rich proteins are known regulators of mRNA splicing, which change their cellular localization and activity depending on Akt-mediated phosphorylation.[36] A member of the serine/arginine-rich protein family, alternative splicing factor/splicing factor-2, is involved in the regulation of tissue-specific alternative splicing of some muscle proteins, including cardiac troponin T.[37] By modifying the activity of such a splicing factor, Akt phosphorylation could alter titin isoform expression through effects on the spliceosome.

Finally, important triggers of cardiac hypertrophy and altered myocardial gene expression are external mechanical forces.[7,38–40] Because titin is a mechanical protein, we anticipated that application of various stretch regimes to 2D CM cultures or 3D cultures (EHTs) could modify the titin expression pattern. However, stretch did not alter the isoform transition. Nevertheless, mechanical forces remain a candidate for modulating the titin switching, because CMs cultured on flexible membrane showed less efficient switching than CMs grown on rigid plastic surface, suggesting a role for matrix stiffness and rigidity sensing. Substrate stiffness has been recognized as an important parameter during differentiation of cultured cells,[41,42] presumably by affecting mechanosensing events at the cell surface and altering downstream mechanochemical signaling pathways. Our evidence suggests that these mechanical factors are much less decisive in determining the developmental titin switching than changes in Akt signaling induced by hormones.

In summary, we demonstrate that developmental changes in the titin isoform pattern are mainly regulated through a PI3K/Akt-dependent signaling pathway. Via increasing Akt phosphorylation, T3 is a dominant trigger for the titin switch, whereas other hormones, particularly Ang II, can fine tune the switching. Mechanical factors such as matrix rigidity, but not stretch, further modulate the titin transition.

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Disclosures

None.

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Thyroid Hormone Regulates Developmental Titin Isoform Transitions via the Phosphatidylinositol-3-Kinase/ AKT Pathway

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THYROID HORMONE REGULATES DEVELOPMENTAL TITIN ISOFORM TRANSITIONS VIA THE PI3K/AKT PATHWAY

Heart tissue

Hearts obtained from adult pregnant Sprague Dawley rats and embryos after 18 days of gestation (E18) were dissected and stored in phosphate buffered saline (PBS; pH8) on ice until preparation of the cell culture. Adult and fetal tissue samples used for SDS-PAGE were excised, immediately frozen in liquid nitrogen, and stored at –80°C. All procedures were conducted in accordance with the guidelines of the university's Animal Care and Use Committee.

Primary cultures of embryonic rat cardiomyocytes

Single cardiomyocytes (CMs) were isolated from E18 hearts by enzymatic dissociation as described elsewhere,[1] plated in fibronectin-coated culture dishes (density, 8×10^5 cells/mm², and cultured at ambient conditions of 37°C and 5% CO_2. Once on culture-day 3 the cells were exposed to irradiation (15 Gy), which helped to enrich the CM fraction and prevent overgrowth of the CMs by the rapidly dividing fibroblast population. In one set of experiments, CMs were cultured in Iscove's modified Dulbecco medium (IMDM) supplemented with 20% FCS for two days to ensure cell adhesion and differentiation. From culture-day 3 on, the cells were serum-starved ("FCS-starved" medium) and the medium was supplemented with one of the following (or a mix of two of these components): angiotensin-II (Ang-II, 1 µmol/L); endothelin-1 (ET-1, 10 nmol/L); 3,5,3´-triiodo-L-thyronine (T3) (75 nmol/L); bisphenol-A (BPA, 100 µmol/L); or LY294002 (LY, 10 µmol/L). In some experiments, KCl (50 mmol/L) was added to arrest spontaneous contractions. In another set of experiments, CMs were isolated and cultured in medium containing charcoal-filtered serum (chf-FCS, Biochrom AG) for two days, before T3 (15-150 nmol/L) or T3 and either BPA (100 µmol/L) or LY294002 (50 µmol/L) were added to the culture medium to block signalling pathways of T3-action. Concentration of free T3 was 2.46×10^{-9} g/L in standard FCS and 1.54×10^{-9} g/L in charcoal-filtered FCS (tested by the Institute of Clinical Chemistry and Laboratory Medicine, University of Muenster). The media were renewed every other day.

Radial stretching and pacing of cultured cardiomyocytes

E18 CMs were isolated as described above and cultured in cell-culture dishes. On culture-day 2 the cells were detached by standard trypsin treatment (10× trypsin/EDTA, Gibco) and plated at a concentration of 8×10^5 cells/mm² onto 6-well Bioflex® culture dishes (Dunn Labortechnik GmbH) freshly coated with fibronectin. The cells were irradiated (15 Gy) on culture-day 3 to stop fibroblast proliferation. Usually from day 4 to day 8 of CM culture the cells were exercised by radial stretch-release cycles at a frequency of ~0.02 or 1.0 Hz using a StretchCo S-1 cell stretcher apparatus (Cell Lines Service, Eppelheim, Germany). The average stretch amplitude applied to the CMs was 10% of their resting length. The cells were either kept in standard culture medium (20% FCS) or were arrested using 50 mmol/L KCl. CMs cultivated under the same conditions but not exposed to stretch served as controls. After eight culture days the cells were harvested and solubilized for titin analysis by SDS-PAGE.

For the pacing experiments, E18 CMs were plated under standard culture conditions onto 4-well plates (Nunc) and paced with the C-pace system (IonOptix Europe, Wageningen, The Netherlands) between day 4 and day 8 of culture. Electrical stimulation consisted of 3-ms pulses of 27 V applied at a frequency of 2 Hz.

Histomorphometry

Digital images of the cultured cells were obtained with a Canon G7 (Power Shot A626) camera installed on an inverted light microscope (Olympus CKX41), using a 40× objective in phase-contrast mode. The contour of a cell was marked and the area of the cell calculated using AxioVision LE 4.4 software (Zeiss, Jena, Germany). Cell area (=size) following various pharmacological interventions was expressed relative to the average mean cell size of the respective control CM cultures.

Preparation and stretching of engineered heart tissue (EHT)

Circular EHTs were constructed as described previously.[2] Briefly, EHT rings (reconstitution volume, 0.9 ml) were prepared by mixing isolated heart cells from neonatal rats (2.5×10^6 cells/EHT) with collagen type-I from rat tails (0.8 mg/EHT), Engelbreth-Holm-Swarm tumor exudate (10% v/v; tebu) and concentrated serum-containing culture medium (2×DMEM, 20% horse serum, 4% chick embryo extract, 200 U/ml penicillin and 200 mg/ml streptomycin); pH was neutralized with 0.1 N NaOH. Within 3 to 7 days of culture, EHTs

coalesced to form spontaneously contracting circular structures. EHTs were transferred after 7 days onto custom-made stretch devices to facilitate static (110% of slack length) or phasic (from 100 to 110% of slack length at 2 Hz) loading. Culture medium was changed 12 hours after EHT casting and then every other day while the culture was performed in casting molds. After transfer into the stretch device, the culture medium was changed every day. EHTs were kept under either phasic or static strain from day-7 to day-12 of culture and were then frozen in liquid N_2 and stored at -80°C for analysis of titin-isoform composition.

Titin-isoform analysis by SDS-PAGE

Tissue strips from rat heart, as well as EHTs, were homogenized in a modified Laemmli buffer containing 8 mol/L urea, 2 mol/L thiourea, 3% SDS (w/v), 75 mmol/L DTT, 0.03% bromophenol blue, 10% glycerol, and 0.005 mol/L Tris-HCl, pH 6.8.[3] Samples of 2D CM cultures were analyzed for titin expression on different culture days up to day-9 (a few times up to day-12). The cells were first washed with ice-cold PBS, harvested with a cell scraper, and then centrifuged at 14,000 rpm and 4°C for 2 min. The pellets were solubilized in the modified Laemmli buffer, incubated for 30 minutes on ice and boiled for 3 minutes at 96°C while shaking at 1400 rpm.

Agarose-strengthened SDS-PAGE (2% polyacrylamide concentration) was performed using Laemmli buffer and a Biometra minigel apparatus. For details, see Refs 4 and 5. The Titin:MHC ratio was determined on 2-phase gels with 7% polyacrylamide in the lower, and 2% in the upper gel phase (2%/7% AA). Protein bands were visualized using Imperial™ protein stain (Pierce) and gels were digitized using a CanoScan 9900F scanner (Canon). Densitometric analyses were done using TotalLab software (Phoretix, Newcastle, UK). A minimum of three samples per experimental condition was analyzed and the average titin-isoform composition calculated.

Immunoblotting

Cardiomyocytes were isolated and cultured for 2 days in medium containing charcoal stripped FCS, before T3 (75 nmol/L) or a combination of T3 and LY294002 (LY, 50 µmol/L) was added. CMs were harvested 60 min and 24 hrs after addition of T3/LY.

For Western blot analysis, protein components were separated by 12.5% SDS-PAGE, transferred onto a PVDF membrane (Millipore, Schwalbach, Germany) by standard semi-dry

Western blotting, and probed by monoclonal antibodies against Akt, phospho-Akt (Ser-473), and phospho-Akt (Thr-308) (Cell Signaling Technology, Beverly, MA). Goat anti-rabbit IgG conjugated with horseradish peroxidase was used as secondary antibody (Phospho-Akt Pathway Sampler Kit, Cell Signaling Technology; Beverly, MA). Protein amount was normalized to beta-actin (monoclonal antibody, SigmaAldrich). Enzymatic activity was detected using an ECL-kit (Amersham Biosciences, Freiburg, Germany). All lanes were loaded with equal amounts of solubilized protein following protein-density determination using spectrophotometric analysis (Bradford method). Protein band intensity was quantified using TotalLab (Phoretix, Newcastle, UK) software.

Real-time reverse-transcriptase polymerase-chain reaction

For real-time quantitative RT-PCR measurements, E18 CMs were isolated and cultured in FCS-starved medium or in medium supplemented with chf-FCS. On culture-day 2 (chf-FCS medium) or culture-day 3 (FCS-starved medium, beginning with the serum-starvation), T3 (75 nmol/L) or a combination of T3 and LY294002 (10 or 50 µmol/L) were added, and samples were taken at several time-points, between 2 and 72 hours. Total RNA was isolated from the CM cultures using Trizol reagent (Invitrogen) according to standard protocols.[4] The cDNA was synthesized from 1 µg total RNA with a "First Strand cDNA Synthesis Kit" (Fermentas) using oligo$(dT)_{18}$ primer. Real-time RT-PCR was performed using 12.5 µl Brilliant SYBRGreen QPCR-Mastermix (Stratagene), 0.75 µl of 5 pmol/L forward and reverse primer each, 5 µl cDNA template, and 6 µl H_2O, and was conducted in an Mx3000P thermocycler (Stratagene). Primer sequences used to amplify the N2B (Exon 50-219) and N2BA (Exon 107-108) titin-mRNA transcripts were as follows:

Exons	Sense primer (5'-3')	Antisense primer (5'-3')	GeneBank Acc. No.
Exon 50-219 (amplifies N2B transcripts)	CCAACGAGTATGGCAGTGTCA	TGGGTTCAGGCAGTAATTTGC	AF525412
Exon 107-108 (amplifies N2BA transcripts)	CGGCAGAGCTCAGAATCGA	GTCAAAGGACACTTCACACTCAAAA	AF525411

All primers worked with 90-110% efficiency. The PCR reactions were checked by melting curves. The N2B and N2BA splice variants were directly compared to one another, as decribed.[4]

Statistics

To test for statistically significant differences, we used ANOVA and the unpaired Student's t-test. P-values <0.05 were taken as indicating significant differences.

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