Titin isoform expression in normal and hypertensive myocardium

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Abstract

\textbf{Objective:} Titin isoform expression patterns were examined to explain previously observed genetic differences in rat cardiac passive tension. \textbf{Methods:} Rat ventricles from male spontaneously hypertensive (SHR) and Wistar–Kyoto (WKY) rats (normotensive) were used to analyze the titin isoform patterns. The hypertensive status was verified by blood pressure measurements and heart weight to body weight ratios. Gel electrophoresis and scanning densitometry were performed to determine ratios of myosin heavy chain and titin isoforms expressed. In situ hybridization using a cRNA probe specific for N2BA titin and a positive control in the N2B unique region was used to demonstrate tissue location of the titin message. \textbf{Results:} Regression analysis of titin isoform ratios, myosin heavy chain isoform ratios, and heart weight to body weight ratios all suggest a smaller proportion of N2BA titin (longer isoform) was expressed in rat left ventricles with increased hypertrophy. In situ hybridization showed that the N2BA and N2B isoforms were co-expressed within most of the cardiomyocytes. Agarose gel electrophoresis demonstrated two different N2BA titin isoforms in all rat ventricles. \textbf{Conclusions:} Expression of less N2BA and more N2B titin in response to pressure overload will result in higher passive tension upon stretch at a given sarcomere length and thus affect cardiac performance.

\section{1. Introduction}

Increased arterial blood pressure of spontaneously hypertensive rats (SHR) has been shown to elicit a hypertrophic response as the heart compensates for the additional work required [1]. Normal adult rat cardiac tissue expresses \(\alpha\)-MHC (myosin heavy chain) predominantly, but hypertrophic hearts express more \(\beta\)-MHC than \(\alpha\)-MHC [2]. In a previous study, detergent-skinned cardiomyocytes from SHR rats were found to have a higher resting stiffness than the age-matched Wistar–Kyoto (WKY) controls [3]. Since titin is believed to be responsible for the development of passive force or resting tension in both cardiac and skeletal muscles [4,5], it was hypothesized that some alteration in titin might be responsible for the resting stiffness changes. Titin isoform expression patterns may help to explain the differences in passive tension between SHR and WKY rats.

Titin has different sizes among different isoforms [6]. A single titin molecule in situ spans from the Z-line to the M-line in the sarcomere [7]. Passive force is generated from the I-band region of titin [8]. Two major classes of titin isoforms have been found in cardiac muscle and are referred to as N2B and N2BA (Fig. 1) [9,10]. Within the I-band, all the isoforms contain a PEVK region (a sequence rich in proline (P), glutamine (E), valine (V), and lysine (K) residues), a N2B unique sequence, and a series of Ig domains that function as a three element spring [11,12]. Greater passive tension would be expected at a given sarcomere length with the N2B than with the N2BA.

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isoform since the N2B has both fewer Ig domains and a shorter PEVK [13] (185 residues for N2B; 525 for N2BA isoform (N2B) in hypertrophic cardiomyocytes. Hence, altered titin isoform expression may be a contributing factor to increased cardiac passive tension in SHR rats.

The N2B and N2BA isoforms may co-exist in the same sarcomere and may tune the passive tension of the sarcomere without impacting other functions of titin [15]. The passive tension–SL relationship between different species shows a large difference between animals that express different ratios of N2B and N2BA titin [9,16]. Animals that express more N2BA generate less tension at a given sarcomere length (SL) than animals that express mainly N2B. Thus, the co-expression of titin isoforms gives a structural basis for myofibrillar elastic diversity in different vertebrate species.

This is the first report to examine titin isoform ratios as an explanation for the differences observed in the passive tension of WKY and SHR rats. The decreased N2BA to N2B ratio in relation to the heart weight to body weight ratios (HW/BW) of SHR left ventricle means the cardiac cells are expressing a larger proportion of the shorter titin isoform (N2B) in hypertrophic cardiomyocytes. Hence, altered titin isoform expression may be a contributing factor to increased cardiac passive tension in SHR rats.

2. Methods

2.1. Determination of arterial pressures

This investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Seven SHR and seven WKY (20–27 weeks old) male rats (Charles Rivers Laboratory) were used in this study. Peak systolic arterial pressure was determined non-invasively via a tail cuff system (Harvard Apparatus, South Natick, MA, USA). Each rat was measured two or three times over a period of days to insure consistent tail
cuff pressure data and eliminate any contribution from non-habituation to the environment. To confirm the tail cuff measurements, arterial pressures were obtained just prior to tissue harvesting and morphometric evaluation. Rats were anesthetized with Pentobarbital (100 mg/kg body mass) and intubated with a 16 Ga tubing adaptor inserted into the airway. Core body temperature was maintained at 37 °C. The systolic, diastolic and mean arterial pressures were determined from the cannulation of the right carotid artery. Pressure data calibrated to a mercury manometer (Baumaster, FL, USA) were recorded continuously for a minimum of 30 min and analyzed with HEM V3.3 software (Notocord Systems, Croissy sur Seine, France). During this period, physiological levels of pressure and heart rates returned as the acute depressive effect of the anesthesia passed.

2.2. Morphometry and tissue freezing

Total body mass was determined prior to acute pressure measurement. After the pressure measurements, the rat was given an overdose of pentobarbital, the chest was opened and the heart dissected free from other tissue and weighed (dry). Immediately after weighing, the heart tissue was separated into RV, LV and septal segments or base and apex segments as dictated by the experiments. The tissue was flash frozen in an aluminum vessel filled with liquid nitrogen cooled isopentane (Sigma–Aldrich). The heart was cut cross sectionally to show endocardium to epicardium and base to apex differences. The systolic, diastolic and mean transferred for 2 h and 20 min at 40 V constant voltage. The blotting procedure was adapted from one used previously [24]. The titin primary antibody along with the respective secondary antibody was diluted in 50 mM Tris–HCl (pH 7.5), 200 mM NaCl, and 0.05% Tween 20 (TBST). The transferred titin membranes were blocked with 5% (w/v) non-fat dry milk in TBST overnight. The primary antibody H4 (anti-titin monoclonal that binds to titin in the A-band [25]) was diluted to 4 μg/ml and was incubated overnight. Blots were then washed and incubated with secondary antibody [anti mouse IgG (H+L) peroxidase-linked (Amersham Pharmacia Biotech, Piscataway, NJ, USA)] diluted to 1:10 000 and incubated for 2 h. After the secondary antibody incubation, the membranes were washed and developed by using the ECL plus kit following the manufacturer’s recommendations (Amersham Pharmacia Biotech). The membrane was then exposed to X-OMAT film from Kodak and developed as previously described [24].

2.4. Probe preparation and in situ hybridization

Complementary DNA (cDNA) clones I24 and J26B, which are rat cardiac specific clones [9], were used to make the titin probes. The titin clones were subcloned into the pGEM-T EASY vector (Promega, Madison, WI, USA). The cDNA was in vitro transcribed using Ambion’s MAXIscript kit according to the manufacturer’s specifications. All probes were labeled with Biotin-16-UTP (Roche Applied Science, Indianapolis, IN, USA) diluted to 1:10 000 and incubated for 2 h. The running buffer contained 50 mM Tris, 0.384 M glycine, 0.1% (w/v) SDS for myosin gels as previously described [20]. After the electrophoresis was complete the MHC gels were stained with Coomassie blue R-250 [21]. A 1% agarose gel (no stacking layer) was used to visualize titin isoforms. The same buffer system was used as with the myosin gels. The gel was 1.5 mm thick and poured in the same vertical apparatus as the myosin gels (SE600), and run for 5 h at 15 mA constant current with constant cooling to 8 °C. After the gel had finished running, it was fixed and silver stained as described previously [22].

For Western blots, the agarose gels were placed in transfer buffer as described [23] except a TE series Transphor unit (Hoefer) was used and the gels were transferred for 2 h and 20 min at 40 V constant voltage. The blotting procedure was adapted from one used previously [24]. The titin primary antibody along with the respective secondary antibody was diluted in 50 mM Tris–HCl (pH 7.5), 200 mM NaCl, and 0.05% Tween 20 (TBST). The transferred titin membranes were blocked with 5% (w/v) non-fat dry milk in TBST overnight. The primary antibody H4 (anti-titin monoclonal that binds to titin in the A-band [25]) was diluted to 4 μg/ml and was incubated overnight. Blots were then washed and incubated with secondary antibody [anti mouse IgG (H+L) peroxidase-linked (Amersham Pharmacia Biotech, Piscataway, NJ, USA)] diluted to 1:10 000 and incubated for 2 h. After the secondary antibody incubation, the membranes were washed and developed by using the ECL plus kit following the manufacturer’s recommendations (Amersham Pharmacia Biotech). The membrane was then exposed to X-OMAT film from Kodak and developed as previously described [24].
50% deionized formamide, 0.3 M NaCl, 20 mM Tris–HCl pH 7.4, 5 mM EDTA, 10 mM NaPO₄, 10% dextran sulfate, 1× Denhardt’s, 50 µg/ml total yeast RNA, and 140 ng/µl of Biotin-16-UTP-labeled cRNA probe. Slides were washed in 5× SSC for 30 min at 50 °C. A high stringency wash in 2× SSC, 50% formamide at 65 °C for 20 min was performed twice. Slides were washed in 0.1 M Tris–HCl, pH 7.5, 5 mM EDTA, 0.4 M NaCl at room temperature for 10 min and repeated once. This protocol was adapted from those previously described [26,27]. Slides were placed in 1× ELF wash solution for 5 min and repeated once (Molecular Probes, Eugene, OR, USA). The ELF-97 mRNA in situ hybridization kit (Molecular Probes) was used for signal development of the probe and used based on the manufacturer’s recommendations. Slides were photographed using a Nikon Diaphot epifluorescence microscope attached to a Roper Scientific NTE/CCD-1024-EB camera. After background subtraction, intensity values were determined using IPLabs 3.6 software (Scanalytics, Fairfax, VA, USA). The antisense and sense slides were normalized for visual comparisons. The slides were also pseudocolored and merged to indicate where the two-titin probes hybridize. Hematoxylin and eosin slides were stained as described [28].

2.5. Densitometry

The agarose and myosin gels were scanned using a BioRad GS-700 imaging densitometer. The image analysis was performed using BioRad Molecular Analyst version 1.4.1. The gel lanes were profile analyzed and the peak areas were integrated for both the titin and myosin gels. For the titin isoform comparisons, an external control was loaded on every gel, which was then compared to the unknowns to determine quantitative amounts of the isoforms expressed relative to the control. The external control was loaded in a linear fashion to form a standard linear line to which the unknowns could be compared. The external control was prepared the same way as the unknowns and from the same species of rat left ventricle. The unknowns were loaded in at least triplicate and averaged to minimize lane-to-lane variability. The optical densities of the two N2BA bands were pooled.

2.6. Statistical methods

Data were compiled and shown as means and standard errors. Data were statistically evaluated using two-tailed t-tests and correlation coefficients were determined with Microsoft Excel. A P-value of 0.05 was chosen as the limit of statistical significance. A one-way analysis of variance (ANOVA) was performed on the correlation of titin to HW/BW ratios using SAS. The correlation data of titin to myosin ratios were fit to a parallel and nonparallel slope model, and the slope interaction was checked for significance by an F-test using SAS.

3. Results

3.1. Physiological data

The tail cuff and carotid measurements all showed significantly higher arterial blood pressures in SHR rats as compared to WKY controls (Fig. 2a). The mean arterial pressure increased from 122±7 in the WKY to 181±11 in the SHR (mean±S.E.M. in mmHg, n=7 ea). All of the SHR rats had systolic blood pressures exceeding 160 mmHg, a level considered hypertensive [29]. An indicator of hypertrophy is the heart weight to body weight ratio, and, in the SHR group, the ratio was 10.4% (P<0.05) greater than the WKY group, suggesting a larger heart in

![Graph](image-url)
proportion to the body (Fig. 2b). Thus the SHR animals used in the current study had elevated blood pressures and the resultant cardiac hypertrophy demonstrated previously with this strain.

3.2. Gel electrophoresis comparisons

A significant decrease of the α/β-myosin ratios in the SHR left ventricles compared to the right ventricles was demonstrated by gel electrophoresis (Fig. 3a). In the WKY group, there was no significant difference between the left and right ventricle (data not shown). Since there were differences in MHC isoenzymes in the SHR left ventricle, which has the greatest amount of pressure overload, and not in the WKY group, this further suggests the left ventricle of the SHR group had a hypertrophic response. Densitometric analyses of silver-stained agarose gels show no significant difference between the N2BA to N2B ratio means for the SHR and WKY groups (Fig. 3b).

Titin isoform ratios compared to HW/BW ratios of the SHR left ventricle showed a significant negative correlation, suggesting that the N2BA/N2B ratio decreases as the cardiomyocytes become increasingly hypertrophic (Fig. 4a). Linear regression analysis of titin to myosin isoform ratios (Fig. 4b) showed correlation coefficients of 0.628 and −0.512 for the SHR and WKY left ventricles, respectively, with \( P=0.053 \), approaching significance. Thus the SHR rats with a higher proportion of β-myosin

Fig. 3. Gel electrophoresis analysis. (a) Gel electrophoresis densitometric comparisons between the ratios of α/β-MHC isoforms in the left and right ventricle of SHR were significantly different (*\( P<0.02 \)). The ratios of α/β-MHC in left and right ventricles of WKY were not significantly different. (b) Gel electrophoresis densitometric comparisons of N2BA/N2B titin isoform ratios were not significantly different.

Fig. 4. Regression analysis. (a) Gel electrophoresis of titin isoform ratios compared to HW/BW ratios in SHR left ventricle. Regression analysis of the SHR left ventricles shows a negative correlation coefficient of −0.772 and \( P<0.05 \). Regression relationships of titin isoform ratios to HW/BW ratios in the SHR right ventricle, WKY left ventricles, and WKY right ventricles were significantly different. (b) Gel electrophoresis comparisons of myosin and titin isoforms in the left ventricles. Regression analysis between WKY and SHR animals showed negative and positive correlations with values of −0.512 and 0.628, respectively, with \( P=0.053 \).
(an indicator of hypertrophy) had a lower proportion of the N2BA with the opposite happening in the WKY rats (Fig. 4b).

Fig. 5 demonstrates that there are two N2BA isoforms expressed in the rat cardiac ventricles. A dog cardiac sample was included with the gel as a standard to show the titin N2B and N2BA isoform positions. In addition, the titin degradation product T2 has been resolved into three separate bands (Fig. 5). Neither the relative ratios of these two N2BA bands nor the amounts of the T2 bands appeared to vary between the SHR and WKY animals (data not shown).

A Western blot was performed after agarose gel electrophoresis using a specific titin monoclonal antibody (H4), and it demonstrated that the bands identified by silver staining are titin (Fig. 5b). A dog cardiac sample was included with the blot as a standard to show that there are only single titin N2B and N2BA bands in this species.

3.3. In situ hybridization

Sections from different regions of the hearts were examined by in situ hybridization using titin I82 (N2BA specific) and N2B unique cRNA probes, the latter recognizing both N2BA and N2B isoforms. Both titin probes appear to hybridize within most individual cardiomyocytes (Fig. 6). The signal intensity was much greater for the N2BA probe than with the N2B probe (Fig. 6). The larger N2BA signal intensity probably indicated that this particular probe hybridized more efficiently and not that there was more N2BA message. Interestingly, there were no distinctions in titin isoform ratios, in either group, that could be made from cross sections of the heart (as described in the Methods) between layers near the endocardium and epicardium from the in situ experiment (data not shown). Also, no differences were found in the locations or intensity ratios of the mRNA between the apex and base of the ventricles using the titin cRNA probes (data not shown). There was significant variability in the intensities and no consistent patterns of expression visually or quantitatively using intensity values.

4. Discussion

The SHR rat model has been an extensively used model system for studying the physiological effects of hypertension. The SHR rats were derived from selectively inbreeding Wistar–Kyoto rats with high blood pressures [29]. At about 12 weeks of age, the SHR rats show significantly higher blood pressures vs. the WKY rats, and the condition is fully manifested by 15–18 weeks [30]. With increased blood pressure, the heart responds by becoming hypertrophic [1]. In this study, all the rats were sacrificed between 20 and 27 weeks from birth, a sufficient time to allow a hypertrophic response to occur. In a previous study, SHR rats of a similar age were found to have a higher resting stiffness than the age-matched WKY controls [3].

Several recent studies have demonstrated changes in titin that may be related to the physiological function of
β-Adrenergic stimulation of cardiac muscle activates protein kinase A (PKA), and has been shown to phosphorylate titin and cause a decrease in passive tension in skinned rat cardiac myocytes [31]. Reduced PKA activity could increase the passive tension in the SHR rats, however the amount of passive tension differences observed due to the PKA could not explain the differences observed in previous passive tension studies with the WKY and SHR rats. There is also a significant increase in the myocardium cell volume of desmin null mice due to an increase in transverse section area, suggesting that mice lacking desmin develop concentric cardiomyocyte hypertrophy [32]. Desmin null mice have myocyte ultrastructure defects and myocyte cell death results, leading to fibrosis and calcification of the myocardium [32]. The calcification and fibrosis could conceivably lead to increased passive tension in SHR rats. Therefore, protein expression of desmin in our rat cardiac tissue was determined by Western blot analysis, but there was no statistically significant difference ($P = 0.18$, two-tailed $t$-test, unpublished results) in the expression of desmin between the WKY and SHR rats. Our results agree with previous work showing that desmin does not appear to change in rats with induced hypertrophy [33]. Another contributing factor could be microtubules, which were shown to change in hypertrophic rats [33]. However, in hypertrophic SHR rats there was no evidence that colchicine-induced cardiac microtubular depolymerization affects the passive properties of left ventricles [34,35]. If microtubules were contributing to passive tension then the treatment with colchicine should in part make the myocardium more compliant.

A more likely mechanism is titin isoform ratio differences. Several model systems have been used to determine the role of titin in various cardiomyopathies. A tachycardia-induced dilated cardiomyopathy canine model has shown a decrease of N2BA vs. N2B titin [36]. In contrast, severely diseased human patients with coronary artery disease (CAD) and Sprague–Dawley rats that had induced myocardial infarction via a left anterior descending coronary artery (LAD) ligation showed an increase in the N2BA vs. N2B [37]. In the current study we used the SHR rat model, which mimics the natural progression of hypertension and not induced hypertension. Since these models differ mechanistically, it is not surprising that the isoform changes are not all consistent.

Titin N2BA to N2B isoform ratios were significantly correlated with HW/BW ratios in the SHR left ventricle (Fig. 4a). The decreased titin ratio in relation to the HW/BW ratios of SHR left ventricle implies that the cardiac cells are expressing more of the shorter titin isoform relative to the WKY animals. Myosin isoform changes are also an indicator of hypertension-induced remodeling in the rodent heart. Myosin isoform switches from mainly α-MHC in normally functioning rodent hearts to β-MHC in pressure overloaded hearts [2]. The correlation between titin and myosin isoform ratios approached significance ($P = 0.053$) (Fig. 4b). Regression analysis comparing titin and myosin isoform ratios demonstrated that SHR animals with a greater proportion of β-MHC (an indicator of hypertrophy) had smaller N2BA/N2B ratios. This suggests that animals have compensated for the pressure overload by expressing both a higher proportion of β-MHC and N2B titin in relation to the control WKY group.

As expected with increased hypertrophy (normalized by the HW/BW ratio), more of the N2B titin isoform is expressed in relation to the N2BA isoform. As has been suggested previously, different species with different passive forces at a given sarcomere length express different ratios of the titin isoforms in cardiac tissue as a possible way to modulate resting tension [9,12]. The N2B isoform has a considerably higher fractional extension of the Ig and PEVK segments at a given SL vs. the N2BA [38]. A higher fractional extension in the N2B produces higher...
passive forces in relation to the N2BA [38]. The shift in isoform expression may act as a modulator for the increased demand placed on the left ventricle during early stages of hypertrophic response to pressure overload. The increased passive force previously observed in SHR rats compared to the age-matched WKY controls [3] can be explained by the increase in N2B expression of the SHR group.

There was no statistically significant difference in α/β-MHC or N2BA to N2B expression between the SHR and WKY groups due to animal variability in both groups. The variability between SHR and WKY rats could be due to a premature release of the strains and a 10 year delay in moving to brother and sister inbreeding of the WKY [39]. Therefore, the SHR and WKY groups we studied could reasonably show genetic heterogeneity [40]. Most of the animals in their respective groups, however, showed the expected hypertrophy, and myosin isoform transitions.

In situ hybridization with titin isoform specific cRNA probes to the rat heart showed the two titin probes appeared to hybridize in most cardiomyocytes (Fig. 6). Hybridization of both probes to most of the cells suggests that both isoforms were being expressed regionally throughout the ventricles. If both titin isoforms are within the same cell it could be that the N2BA/N2B ratio differences regulate the elasticity of the heart cell as previously suggested [15]. In addition, there were no differences in the type of mRNA expressed near the endocardium or epicardium. This was true for both left and right ventricles and for both SHR and WKY animals. These results differ from those observed previously in the pig where it has been shown that there is a larger proportion of N2BA expressed near the endocardium vs. near the epicardium [9]. The much smaller wall thickness in the rat may not require the apparent gradient of titin isoform expression seen in the larger species. Alternatively, the message levels for the major isoform types may not fully correspond to the protein ratios in the different areas of the heart.

Surprisingly, two N2BA bands were resolved on the silver-stained agarose gels using rat cardiac tissue (Fig. 5a). In contrast, only a single N2BA band is observed with dog ventricle. The two rat bands are present in samples from all regions of ventricular tissue, and have been observed in ventricles from Sprague-Dawley rats as well (Warren, Krzesinski, and Greaser, unpublished observations). Since the N2B to N2BA ratio in rats is much higher than in the larger species, it may be advantageous for the rat to express more than one size of N2BA isoform for modulating the passive tension. It is unlikely that the lower N2BA band is a breakdown product of the upper one because the upper and lower band ratios remain fairly constant between samples. It is important to note that silver staining must be used instead of Coomassie blue in order to clearly visualize the two N2BA bands in rat cardiac tissue. The H4 titin specific monoclonal antibody reacted with both N2BA bands of the rat cardiac tissue (Fig. 5b). The T2 degradation products were resolved into three distinct bands (Fig. 5); it is not clear whether these bands are isoform specific or arise from cleavage at different positions in the N2B titin.

In conclusion, regression analysis indicates SHR animals with greater hypertrophy had lower N2BA to N2B isoform ratios. Thus, animals that express a greater proportion of N2B (short isoform) titin would have a higher passive tension upon stretch at a given sarcomere length. Hence, titin isoform expression may be a contributing factor for the increased passive tension seen previously in SHR rats.

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