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Myofilament incorporation determines the stoichiometry of troponin I in transgenic expression and the rescue of a null mutation

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ABSTRACT

The highly organized contractile machinery in skeletal and cardiac muscles requires an assembly of myofilament proteins with stringent stoichiometry. To understand the maintenance of myofilament protein stoichiometry under dynamic protein synthesis and catabolism in muscle cells, we investigated the equilibrium of troponin I (TnI) in mouse cardiac muscle during developmental isoform switching and in under- and over-expression models. Compared with the course of developmental TnI isoform switching in normal hearts, the postnatal presence of slow skeletal muscle TnI lasted significantly longer in the hearts of cardiac TnI (cTnI) knockout (cTnI-KO) mice, in which the diminished synthesis was compensated by prolonging the life of myofilamental TnI. Transgenic postnatal expression of an N-terminal truncated cTnI (cTnI-ND) using α-myosin heavy chain promoter effectively rescued the lethality of cTnI-KO mice and shortened the postnatal presence of slow TnI in cardiac muscle. cTnI-KO mice rescued with different levels of cTnI-ND over-expression exhibited similar levels of myocardial TnI comparable to that in wild type hearts, demonstrating that excessive synthesis would not increase TnI stoichiometry in the myofilaments. Consistently, haploid under-expression of cTnI in heterozygote cTnI-KO mice was sufficient to sustain the normal level of myocardial cTnI, indicating that cTnI is synthesized in excess in wild type cardiomyocytes. Altogether, these observations suggest that under wide ranges of protein synthesis and turnover, myofilament incorporation determines the stoichiometry of troponin subunits in muscle cells.

Skeletal muscle fibers and cardiac myocytes contain abundant myofibrils consisting of myosin and actin filaments that are precisely assembled in the form of sarcomeres [1]. Muscle cells have high capacities of protein synthesis and catabolism [2]. On the other hand, the highly organized contractile machinery in skeletal and cardiac muscles requires the assembly of myofilament proteins with a stringent stoichiometry. The maintenance of myofilament protein stoichiometry is important during muscle and heart development or remodeling in aging, disuse, malnutrition, cachexia, and myocardial hypertrophy or dilated cardiomyopathy.

Muscle contraction is regulated by Ca^{2+} through the troponin complex in the actin thin filaments. Troponin I (TnI)[1] is a subunit of the troponin complex [3]. Amphibian, avian and mammalian species have evolved with three TnI isoforms in cardiac, fast and slow skeletal muscle fibers [4]. Physiological and pathophysiological switches of troponin isoforms occur during muscle and heart development and adaptation as rapid processes [5–8], consistent with the short half life of troponin subunits in muscle cells (3–4 days [9]). Embryonic hearts express exclusively slow skeletal muscle TnI (ssTnI) that is down-regulated during postnatal development while cardiac TnI (cTnI) is up-regulated [6,10]. Deletion of the cTnI gene (*Tnni3*) from mouse genome resulted in postnatal lethality when ssTnI in the cardiac muscle of homozygote mice decreases to below a critical level [11].

Interestingly, over-expression of myofilament proteins, including TnI, in transgenic mouse hearts using the strong α-myosin heavy chain (MHC) promoter resulted in competitive replacement of endogenous protein with no increase in stoichiometry [12]. Although this phenomenon has been observed in numerous studies, the cellular mechanism has yet to be established. The high tolerance of muscle cells to the over-expression of TnI [13] and troponin T (TnT) [14] indicates an effective removal of excessive troponin subunit proteins. On the other hand, a single copy of cTnI gene in heterozygotes of *Tnni3*-deleted mice could sustain cardiac

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[1] *Abbreviations used:* TnI, troponin I; cTnI, cardiac TnI; cTnI-ND, N-terminal truncated cTnI; cTnI-KO, cTnI gene deletion; mAb, monoclonal antibody; MHC, myosin heavy chain; PAGE, polyacrylamide gel electrophoresis; ssTnI, slow skeletal muscle TnI; TnT, troponin T.

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function [11]. Similarly, a single copy of slow skeletal muscle TnT gene can sustain skeletal muscle function in the heterozygotes of a recessive nemaline myopathy found in the Amish [15,16]. Therefore, the syntheses of TnI and TnT might already be in excess in normal diploid muscle cells.

To understand the regulation of troponin stoichiometry in muscle cells under dynamic protein synthesis and turnover, we investigated in the present study the equilibrium of ssTnI and cTnI in cardiac muscle during developmental isoform switching and the slower turnover rate of ssTnI in cardiac muscle of cTnI knockout (cTnI-KO) mice in the absence of cTnI synthesis. Rescuing the postnatal lethality of the cTnI-KO mice with transgenic expression of an N-terminal truncated cTnI (cTnI-ND) accelerated the turnover of ssTnI and cTnI-ND alleles with different levels of over-expression produced similar normal levels of TnI in the cTnI-KO cardiac muscle. These experiments suggest that myofilament incorporation determines the stoichiometry of troponin subunits in muscle cells.

Materials and methods

Genetically modified mouse lines

A cTnI-KO mouse line was generated as described previously by homologous recombination-mediated deletion of the entire *Tnni3* gene in embryonic stem cells [11]. Due to postnatal lethality of homozygous *Tnni3* deletion, the *Tnni3* knockout allele was maintained in heterozygous lines.

Two lines of transgenic mice (line #7 and line #27) over-expressing an N-terminal truncated cTnI (cTnI-ND) under an α-MHC promoter [17] were generated previously [18]. cTnI-ND was originally found as a product of restricted proteolytic modification that selectively removes the cTnI-specific N-terminal extension from the conserved core structure [19]. cTnI-ND is present at low levels in normal adult ventricular muscle and is up-regulated in myocardial adaptation to haemodynamic changes [19] and in β-adrenergic deficient hearts [20]. Transgenically expressed cTnI-ND effectively incorporates into the cardiac myofilaments to produce functional effects [18].

Production of double transgenic mouse Lines

cTnI-KO/cTnI-ND double transgenic mouse lines were generated by crossing single transgenic mouse lines cTnI-ND #7 and cTnI-ND #27 with cTnI-KO heterozygous mice (cTnI-KO$^{+/-}$). Genotype screening of the offspring was done by polymerase chain reaction (PCR) on genomic DNA isolated from tail biopsies as described previously [11,18]. The lack of intact cTnI and the expression of cTnI-ND were confirmed by Western blotting of total cardiac muscle protein extracts as described below.

All animal procedures were approved by the Institutional Animal Care and Use Committee and were conducted in accordance with the Guiding Principles in the Care and Use of Animals, as approved by the Council of the American Physiological Society.

SDS–polyacrylamide gel electrophoresis and Western blotting

Immediately after isolation, cardiac muscle samples from ventricular apex were homogenized in SDS–polyacrylamide gel electrophoresis (SDS–PAGE) sample buffer containing 2% SDS using a high speed mechanical tissue homogenizer to extract total muscle proteins. The protein extracts were immediately heated at 80 °C for 5 min to inactivate tissue proteases. The samples were resolved using 14% SDS–PAGE in Laemmli buffer system with an acrylamide:bisacrylamide ratio of 180:1 using a Bio-Rad mini-gel system. The protein bands resolved on the gel were visualized using Coomassie Blue R250 staining.

The protein bands from duplicate gels were transferred to nitrocellulose membranes using a Bio-Rad Lab semidry electrotransfer apparatus. The blotted nitrocellulose membranes were blocked with 1% bovine serum albumin in Tris-buffered saline (150 mM NaCl, 50 mM Tris–HCl, pH 7.5) and incubated with an anti-TnI C-terminus monoclonal antibody (mAb) TnI-1 [21] or an anti-cardiac TnT mAb CT3 [14] diluted in Tris-buffered saline containing 0.1% bovine serum albumin. The subsequent washes, incubation with alkaline phosphatase-labeled anti-mouse IgG second antibody (Sigma), and 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium substrate reaction were carried out as described previously [8].

Data analysis

Densitometry analysis of SDS-gels and Western blots was performed on images scanned at 600 dots/inch using the NIH Image software version 1.61. Quantitative data were documented as mean ± SEM. The linear range of Western blot quantification of TnI and TnT was determined on serial dilutions of mouse cardiac muscle samples (Fig. 1). Statistical significance of differences between the mean values was analyzed by unpaired two-tail Student's *t* test.

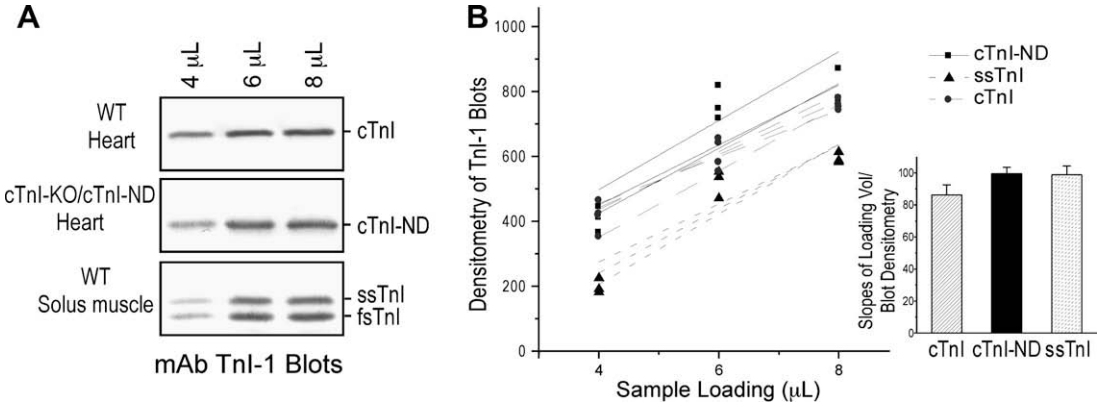

Fig. 1. Quantification of TnI variants using mAb TnI-1 Western blot densitometry. (A) Serially increased amounts of ssTnI in soleus muscle (n = 3) and cTnI (n = 4) or cTnI-ND (n = 3) in cardiac muscles of wild type or cTnI-KO/cTnI-ND mice were examined using mAb TnI-1 Western blot. (B) Two-dimensional densitometry data of the mAb TnI-1 Western blots were analyzed by linear fitting against the amounts of loading. The bar figure showed similar quantitative slopes for the three TnI variants. This range of loading was used in subsequent Western blot quantifications in the present study. Values were presented as mean ± SEM by Student's *t* test.