A dominantly negative mutation in cardiac troponin I at the interface with troponin T causes early remodeling in ventricular cardiomyocytes

Hongguang Wei and J.-P. Jin

Department of Physiology, Wayne State University School of Medicine, Detroit, Michigan

Submitted 14 February 2014; accepted in final form 2 June 2014

Wei H, Jin JP. A dominantly negative mutation in cardiac troponin I at the interface with troponin T causes early remodeling in ventricular cardiomyocytes. Am J Physiol Cell Physiol 307:C338–C348, 2014. First published June 4, 2014; doi:10.1152/ajpcell.00053.2014.—We previously reported a point mutation substituting Cys for Arg111 in the highly conserved troponin T (TnT)-contacting helix of cardiac troponin I (cTnI) in wild turkey hearts (Biesiadecki et al. J Biol Chem 279: 13825–13832, 2004). This dominantly negative TnI-TnT interface mutation decreases the binding affinity of cTnI for TnT, impairs diastolic function, and blunts the β-adrenergic response of cardiac muscle (Wei et al. J Biol Chem 285: 27806–27816, 2010). Here we further investigate cellular phenotypes of transgenic mouse cardiomyocytes expressing the equivalent mutation cTnI-K118C. Functional studies were performed on single adult cardiomyocytes after recovery in short-term culture from isolation stress. The amplitude of contraction and the velocities of shortening and lengthening were lower in cTnI-K118C cardiomyocytes than wild-type controls. The intracellular Ca\(^{2+}\) transient was slower in cTnI-K118C cardiomyocytes than wild-type cells. cTnI-K118C cardiomyocytes also showed a weaker β-adrenergic response. The resting length of cTnI-K118C cardiomyocytes was significantly greater than that of age-matched wild-type cells, with no difference in cell width. The resting sarcomere was not longer, but slightly shorter, in cTnI-K118C cardiomyocytes than wild-type cells, indicating longitudinal addition of sarcomeres. More tri- and quadrinuclei cardiomyocytes were found in TnI-K118C than wild-type hearts, suggesting increased nuclear divisions. Whole-genome mRNA array and Western blots detected an increased expression of leukemia inhibitory factor receptor-β in the hearts of 2-mo-old cTnI-K118C mice, suggesting a signaling pathway responsible for the potent effect of cTnI-K118C mutation on early remodeling in cardiomyocytes. Cardiac troponin I; culture of adult cardiomyocyte; prolongation of cardiomyocytes; myocardial remodeling

TROPONIN I (TnI), the inhibitory subunit of the troponin complex, plays an essential role in the Ca\(^{2+}\) regulation of striated muscle contraction (8, 21, 22). We previously reported a point mutation in cardiac TnI (cTnI) in wild turkeys that generates a single-amino acid substitution of Cys for Arg\(^{111}\) (4). This residue is located in the a-helix interfacing with troponin T (TnT) in the I-T arm of the troponin complex (23, 26) and is highly conserved as Arg or Lys in the three muscle fiber type-specific (cardiac and slow and fast skeletal muscle) TnI isoforms in all vertebrate species examined (4, 12). Because of the variable length of the NH2-terminal segment, the residues in human and mouse cTnI corresponding to Arg\(^{111}\) in turkey cTnI are Lys\(^{117}\) and Lys\(^{118}\), respectively (12).

Our previous studies demonstrated that the Arg\(^{111}\)Cys substitution decreased binding affinity of turkey cTnI for cardiac TnT (4). Transgenic mouse hearts overexpressing cTnI-K118C showed decreased diastolic function and blunted contractile response to β-adrenergic stimulation (27). These dominantly negative effects of cTnI-K118C on myocardial function demonstrate the importance of the TnI-TnT interface in the regulation of muscle contraction.

In the past two decades, large numbers of cardiomyopathy-causing point mutations have been identified in cTnI and cardiac TnT (24). In sharp contrast, only one human mutation, cTnI-A116G (19), was found in helix 2 of cTnI or helix 2 of cardiac TnT, and it forms a coiled-coils structure at the TnI-TnT interface (23). This observation suggests two possibilities: 1) the TnI-TnT interface structure of troponin can tolerate point mutations without causing clinical presentation of cardiomyopathy, or 2) this coiled-coils structure is of highly critical functions, and, thus, mutations would be reproductively lethal, precluding their fixation in the population. Amino acid sequence alignments showed that the corresponding regions in TnI and TnT are highly conserved among muscle type isoforms and during vertebrate evolution (12, 28). Therefore, the latter hypothesis appears valid, and the presence of a cTnI-R111C mutation in wild turkeys at significant frequency is a rather unique case (4).

The cTnI-R111C mutation, together with aberrantly spliced cardiac TnT, which causes dilated cardiomyopathy, is present in wild turkey hearts (2, 3). We recently demonstrated that cTnI-K118C and the aberrantly spliced cardiac TnT mutually canceled each other’s dominantly negative effects when they were coexpressed in the hearts of double-transgenic mice (27). Therefore, the evolutionary fixation of cTnI-K118C in wild turkeys was probably based on the selection value of its mutualism with the abnormally spliced cardiac TnT (27). This observation further supports the importance of the TnI-TnT interface in the compound function of the troponin complex.

To further understand the function of the TnI-TnT interface, we used cardiomyocytes isolated from adult transgenic mouse hearts to study the cellular phenotypes of the cTnI-K118C mutation. In addition to revealing its dominantly negative effects on the amplitude of contraction and the velocities of shortening and lengthening while avoiding the influence of the extracellular matrix, the isolated adult cTnI-K118C mouse cardiomyocytes provided an accurately quantitative approach to characterization of the intracellular Ca\(^{2+}\) transient and cellular morphology. A novel finding was that ventricular cardiomyocytes of young cTnI-K118C transgenic mice were significantly longer than those isolated from age-matched wild-type mice. The results demonstrated an early remodeling in cardiomyocytes in the absence of clinical heart failure, indicating a potent effect of cTnI-K118C mutation in the TnI-TnT interface on myocardial adaptation.
MATERIALS AND METHODS

Genetically modified mice. A transgenic mouse line overexpressing cTnl-K118C in the adult heart driven by a cloned α-myosin heavy chain (MHC) promoter was described previously (27). By crossing the cTnl-K118C transgenic line with heterozygotes of cTnl gene (Tnni3)-deletion (Tnni3-knockout) mice (9), a double-transgenic mouse line with postnatal expression of cTnl-K118C in the absence of endogenous cTnl was confirmed using Western blot analysis (Fig. 1). Mice of both sexes were used in the present study, and the protocols were approved by the Institutional Animal Care and Use Committee.

SDS-PAGE and Western blotting. Cardiac muscle tissues were homogenized in SDS-PAGE sample buffer containing 2% SDS and 1% β-mercaptoethanol, pH 8.8, using a high-speed mechanical homogenizer to extract total proteins. Isolated cardiomyocytes were lysed in the same buffer without the use of a homogenizer. The SDS-PAGE samples were heated at 80°C for 5 min, centrifuged in a microcentrifuge at the top speed for 5 min to remove insoluble materials, and resolved on 14% SDS gel, with a 180:1 acrylamide-to-bis-acrylamide ratio, prepared in a modified Laemmli buffer system in which stacking and resolving gels were casted with pH 8.8 buffer. The gels were run using constant current, and the resolved protein bands were stained with Coomassie Blue R250. Total protein in each lane was quantified by densitometry using ImageJ software to normalize sample loading.

As described previously (27), duplicate gels were transferred to nitrocellulose membranes using a Bio-Rad semidry electrotransfer apparatus and probed with the anti-Tnl monoclonal antibody (MAb) Tnl-1 (11), the anti-cardiac TnT MAb CT3 (10), or the anti-tropomyosin MAB CH1 (30) under high-stringency conditions, including washes with Tris-buffered saline containing 0.5% Triton X-100 and 0.05% SDS. Western blots were also carried out using MAb c19 against leukemia inhibitory factor receptor (LIFR)-β or MAb M-20 against gp130 (Santa Cruz Biotechnology) following the manufacturer's instructions.

Isolation of adult mouse cardiomyocytes. A protocol modified from the method described previously (20, 31) was used to isolate cardiomyocytes from the hearts of wild-type and cTnl-K118C transgenic mice at 3 wk–8 mo of age. Each mouse was injected intraperitoneally with 100 U of heparin and 100 mg/kg pentobarbital. After 20 min, the heart was rapidly removed, cannulated through the aorta, and mounted on a modified Langendorff perfusion system. The heart was first perfused at constant flow of 3 ml/min for 3 min with a buffer containing (mM) 120 NaCl, 5.4 KCl, 1.2 MgSO4, 1.2 Na2HPO4, 5.6 glucose, 20 NaHCO3, and 5 taurine, with or without 10 mM 2,3-butanedione monoxime (BDM), in 95% O2-5% CO2. The perfusion was then switched to a circulating enzyme digestion solution consisting of 50 ml of perfusion buffer plus 12.5 μM CaCl2, 12.5 mg of Liberase Blendzyme 1 (Roche), and 0.278 ml of 2.5% trypsin (Invitrogen) at 37°C for 15–20 min until the heart became pale and flaccid.

The heart was removed from the perfusion apparatus and dissected, and the atria and large vessels were discarded. The ventricular tissue was disaggregated with forceps and gentle pipetting using a transfer pipette in a petri dish containing 10 ml of enzyme stopping buffer (perfusion buffer + 2.5% bovine serum albumin and 12.5 μM CaCl2). The cell suspension was filtered through a 100-μm nylon mesh and settled with gravity in a 15-ml conical tube for 15 min. After removal of the old medium, the isolated cells were resuspended in 12 ml of fresh stopping buffer. CaCl2 was slowly added from a 100 mM stock solution (in 4 steps over 20 min) to the final concentration of 1 mM. The isolated cardiomyocytes were used immediately for morphological and functional studies or for starting short-term cultures.

Morphological measurements. After restoration of Ca2+, resting cell length, cell width, and sarcomere length were measured from images of large numbers of unfixed isolated cTnl-K118C and age-matched wild-type cardiomyocytes obtained with an inverted phase-contrast microscope with a photographic attachment.

To investigate the number of nuclei per cell, the isolated cardiomyocytes were seeded in a culture dish. After adhesion for 1 h, the cells were fixed with 4% paraformaldehyde, pretreated with 10 μg/ml RNase for 30 min, and stained with 1.5 μM propidium iodide (added to the medium) for 20–30 min. Phase-contrast and epifluorescence microscopic images were photographed. Mono-, bi-, tri-, and quadrinucleated cells were counted to calculate their percentages in the cell population.

Short-term culture of adult mouse cardiomyocytes. Isolated adult mouse cardiomyocytes in Ca2+-restored medium were settled with gravity for 15 min to remove the medium. The cells were resuspended in MEM containing 5% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM l-glutamine, 20 mM HEPES, 4 mM NaHCO3, and 10 mM BDM and plated on laminin-coated 35-mm culture dishes or glass coverslips. After 2 h of cell adhesion, the plating medium was replaced with culture medium [MEM containing 20 mM HEPES, 4 mM NaHCO3, 0.1 mg/ml bovine serum albumin, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM l-glutamine, 1× insulin-transferrin-selenium supplement (Sigma), and 10 mM BDM].

Fig. 1. Expression of solely cTnl-K118C in the heart of cTnl-K118C/Tnni3-knockout (KO) double-transgenic mice. cTnl-K118C has a slower SDS gel mobility than wild-type (WT) cardiac troponin I (cTnl) (4, 27). MAb Tnl-1 Western blot demonstrates the presence of solely cTnl-K118C in the heart of cTnl-K118C/Tnni3-KO double-transgenic mice. MHC, molecular weight; MLC, myosin light chain.
The cells were cultured at 37°C in the presence of 2% CO₂ for 1–4 days.

**Glycerol-SDS-PAGE examination of cardiac MHC isoforms.** Total myocardial protein extract was resolved on 8% SDS gel, with a 50:1 acrylamide-to-bis-acrylamide ratio, containing 30% glycerol, 0.4% SDS, 200 mM Tris-HCl, and 100 mM glycine, pH 8.8. The upper running buffer contained 100 mM Tris base, 150 mM glycine, 0.1% SDS, and 10 mM β-mercaptoethanol. The lower running buffer was a 1:2 dilution of the upper running buffer without β-mercaptoethanol. The gel was run at 0°C in a box of ice water at 100 V for 24 h and stained with Coomassie Blue R250 to visualize the protein bands.

**Pro-Q Diamond phosphoprotein staining.** Total protein extracts from isolated cardiomyocytes before and after culture were resolved on 14% SDS-polyacrylamide gel as described above. The SDS gel was prefixed with 50% methanol and 10% acetic acid, washed with deionized water, and incubated with Pro-Q Diamond phosphoprotein staining reagent (Invitrogen) in a dark box for 90 min. The gel was then destained three times for 30 min each in 20% acetonitrile and 50 mM sodium acetate, pH 4.0, and washed twice with deionized water for 5 min each in a dark box. The destained gel was scanned on a fluorescence scanner (Typhoon 9410, GE Healthcare) with excitation at 532 nm and recording emission at 560 nm. A duplicate gel was stained with Coomassie Blue R250 to reveal the total protein contents. The relative amount of a phosphoprotein band was quantified using ImageJ software with normalization to the amount of total protein.

**Contractility analyses of isolated adult mouse cardiomyocytes.** Cardiomyocytes were loaded into a perfusion chamber mounted on the stage of an inverted microscope (Nikon Eclipse ST100) using a heating adapter with feedback temperature control. After settling at the bottom of the chamber, the cells were superfused at 1 ml/min with oxygenated buffer containing 132 mM NaCl, 4.8 mM KCl, 1.2 mM MgCl₂, 10 mM HEPES, 15 mM glucose, 2 mM sodium pyruvate, and 1.8 mM CaCl₂, pH 7.4 at 36.5–37°C.

Rod-shaped cardiomyocytes with sharp edges and clear sarcomeric striations in a stable quiescent state were selected for contractile analysis. Contractions were induced with field electrical pacing using a Myopacer stimulator (IonOptix, Milton, MA) with 10-μV 4-ms pulses at 0.5, 1, 2, 5, and 10 Hz in the absence or presence of 3 or 10 nM isoproterenol. Cell shortening and relengthening were recorded using a CCD video camera (IonOptix). Edge detection data were acquired at 0.5, 1, 2, 5, and 10 Hz in the absence or presence of 3 or 10 nM isoproterenol. Measurement of intracellular Ca²⁺ transient during the contractile cycle. As described previously (31), isolated cardiomyocytes in the superfusion buffer containing 1.8 mM CaCl₂ were incubated with 1.5 μM fura 2-AM (Invitrogen) in darkness at room temperature for 20 min. After the loading of fura, the cells were washed twice with the superfusion buffer containing 1.85 mM CaCl₂ and 500 μM probenecid and kept in the same medium at room temperature for 30 min. Loaded in the superfusion chamber as described above, the cardiomyocytes were paced as described for the contractility studies for examination of the intracellular Ca²⁺ transient through a photomultiplier tube (PMT-300, IonOptix). Intracellular free Ca²⁺ was measured as the ratio of fura 2 fluorescence excited at 360 nm to that at 380 nm with emission at 510 nm. The effect of isoproterenol was measured as the ratio of fura 2 fluorescence excited at 360 nm to that at 280 nm and absorbance at 260 nm to that at 230 nm. An aliquot of the RNA was assessed by microfluidics with the RNA 6000 Nano assay using a bioanalyzer (model 2100, Agilent). The electrophoretogram, RNA integrity number, and ratio of 28S to 18S RNA bands were collectively examined to determine the overall quality of the RNA.

**RESULTS**

Rod-shaped morphology and striation pattern were preserved in adult mouse cardiomyocytes after short-term culture. Enzymatic isolation of viable cardiomyocytes from adult mouse hearts is known to be highly dependent on the digestion conditions. Our refined protocol described in this report produced excellent reproducible results for mouse hearts at 3 wk to >1 yr of age. Approximately 2–4 × 10⁶ rod-shaped cells were obtained from each heart. The cells survived well after restoration of physiological Ca²⁺ concentration in the medium (data not shown), >90% of which were contractile upon pacing.

Short-term culture was applied to allow the adult mouse cardiomyocytes to recover from the stress of enzymatic isolation. The rod-shaped morphology and striation pattern of the cells did not show significant change after 1–3 days of culture and were similar in wild-type and cTnI-K118C transgenic mouse cardiomyocytes. The cardiomyocytes in 2-day cultures appeared identical to freshly isolated cells (data not shown); 70–80% of the rod-shaped cells were contractile upon pacing.

Preservation of myofilament protein expressions in short-term-cultured adult mouse cardiomyocytes. The SDS-polyacrylamide gel in Fig. 2A showed similar protein profiles in freshly isolated and 1- or 2-day serum-free-medium-cultured adult mouse cardiomyocytes. Western blots using specific MAb demonstrated normal expressions of troponymosin, cTnI, and cardiac TnT that were similar in freshly isolated and short-term-cultured adult mouse cardiomyocytes (Fig. 2A). The glycerol-SDS gel in Fig. 2C showed normal α-MHC expression in freshly isolated adult mouse cardiomyocytes and cells after 1–3 days of culture. The preserved expression of representative thin- and thick-filament proteins in the short-term-cultured adult mouse cardiomyocytes demonstrated maintenance of the differentiated state.

The perfusion-based enzymatic isolation of adult mouse cardiomyocytes unavoidably imposed an ischemia-reperfu-
sion-like stress on the heart. The MAb CT3 Western blot in Fig. 2A detected a fragment of cardiac TnT in the freshly isolated adult mouse cardiomyocytes. This ischemia-reperfusion stress-generated cardiac TnT fragment has been characterized previously in similarly isolated rat and mouse cardiomyocytes to be an NH2-terminal-truncated cardiac TnT (32) that remains in the myofibrils with effects on cardiac muscle function (6).

Although there was no detectable degradation of cTnI during the cardiomyocyte isolation, TnI and TnT are subunits of the troponin complex with directly related structure and function. The trace amount of NH2-terminal-truncated cardiac TnT in freshly isolated cardiomyocytes may complicate functional studies of cTnI-K118C. Therefore, the short-term culture approach is valuable, in that it allows the isolated adult mouse cardiomyocytes to recover from the isolation stress. The MAb CT3 Western blot in Fig. 2A and densitometry quantification in Fig. 2B showed that the NH2-terminal-truncated cardiac TnT (TnT-ND) was diminished after 2 days of culture. Values are means ± SE; n = 4. C: glycerol-SDS gel reveals normal α-MHC expression in isolated and cultured adult mouse cardiomyocytes. Neonatal mouse heart expressing α- and β-MHC was included as control.

Table 1. Preserved contractility of adult mouse cardiomyocytes after short-term culture

<table>
<thead>
<tr>
<th>Days in Culture</th>
<th>0 nM Iso</th>
<th>3 nM Iso</th>
<th>10 nM Iso</th>
<th>0 nM Iso</th>
<th>3 nM Iso</th>
<th>10 nM Iso</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell shortening, %</td>
<td>3.05 ± 0.53</td>
<td>8.49 ± 1.18*</td>
<td>11.88 ± 1.29*</td>
<td>3.79 ± 0.68</td>
<td>10.16 ± 0.96*</td>
<td>11.40 ± 0.59*</td>
</tr>
<tr>
<td>Maximum shortening velocity, μm/s</td>
<td>117.17 ± 19.76</td>
<td>295.81 ± 39.96*</td>
<td>389.16 ± 26.74*</td>
<td>93.25 ± 14.11</td>
<td>246.96 ± 30.62*</td>
<td>245.87 ± 18.82*</td>
</tr>
<tr>
<td>Maximum relengthening velocity, μm/s</td>
<td>87.67 ± 17.46</td>
<td>267.78 ± 39.06*</td>
<td>353.34 ± 25.74*</td>
<td>80.95 ± 16.17</td>
<td>241.71 ± 27.19*</td>
<td>228.10 ± 16.52*</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 4 mice in each group. Contractility of wild-type adult mouse cardiomyocytes was measured using edge detection on paced contractions at 2 Hz. Freshly isolated cells and cells cultured for 2 days showed no significant difference in shortening amplitudes and shortening and relengthening velocities. Two-day-cultured cells remained responsive to β-adrenergic stimulation. However, unlike freshly isolated cells, 2-day-cultured cells did not show additive increases in shortening and relengthening velocities when isoproterenol (Iso) was increased from 3 to 10 nM. *P < 0.01 vs. 0 nM Iso.
both groups. However, when isoproterenol concentration increased from 3 to 10 nM, the freshly isolated cells exhibited further responses in contractile amplitude and shortening and relengthening velocities, whereas the 2-day-cultured cells showed no further increase (Table 1). This trend of difference in responses to higher concentrations of isoproterenol suggested an alteration in the β-adrenergic response in the 2-day-cultured adult mouse cardiomyocytes. Overall, the cells have physiologically relevant contractility, at baseline or in the presence of a physiological level of isoproterenol.

Pro-Q Diamond phosphoprotein staining showed phosphorylation of cTnI and myosin-binding protein C in freshly isolated, as well as 2-day-cultured, cells, with increases after treatment with 1–10 nM isoproterenol (Fig. 3). Consistent with the preserved β-adrenergic responsiveness shown in the contractility studies (Table 1), the PKA-dependent phosphorylation of cTnI and myosin-binding protein C was preserved in 2-day-cultured adult mouse cardiomyocytes. Therefore, the loss of additive contractile response of adult cardiomyocytes preserved in 2-day-cultured adult mouse cardiomyocytes. Overall, the cells have physiologically relevant contractility, at baseline or in the presence of a physiological level of isoproterenol.

Pro-Q Diamond phosphoprotein staining showed phosphorylation of cTnI and myosin-binding protein C in freshly isolated, as well as 2-day-cultured, cells, with increases after treatment with 1–10 nM isoproterenol (Fig. 3). Consistent with the preserved β-adrenergic responsiveness shown in the contractility studies (Table 1), the PKA-dependent phosphorylation of cTnI and myosin-binding protein C was preserved in 2-day-cultured adult mouse cardiomyocytes. Therefore, the loss of additive contractile response of adult mouse cardiomyocytes to 10 nM isoproterenol after 2 days of culture (Table 1) may not be from the myofilaments but, rather, from changes in the Ca^{2+}-handling system (5). This hypothesis remains to be investigated.

The baseline and isoproterenol-stimulated phosphorylation of cTnI and myosin-binding protein C was similar in cTnI-K118C and wild-type cardiomyocytes.

cTnI-K118C cardiomyocytes exhibited decreased contractility. Paced contractions of 2-day-cultured adult cardiomyocytes showed significantly lower amplitude of cell shortening and contractile and relaxation velocities of cTnI-K118C cardiomyocytes than wild-type cells (Fig. 4). This difference was also seen in freshly isolated cardiomyocytes stimulated with 3 nM isoproterenol (Fig. 4).

Decreased function of cTnI-K118C hearts was demonstrated at the organ level in a previous study (27). Therefore, the diminished change in freshly isolated cardiomyocytes in the absence of isoproterenol was likely due to complications resulting from isolation stress.

Fresh and 2-day-cultured wild-type and cTnI-K118C cardiomyocytes responded to 3 and 10 nM isoproterenol with significantly increased contractile amplitude and velocities of shortening and relengthening (Fig. 4). In fresh cells, treatment with 3 nM isoproterenol generated less inotropic and lusitropic responses in cTnI-K118C than that in wild-type cardiomyocytes (Fig. 4). Inotropic function was significantly lower in cTnI-K118C cardiomyocytes after 2 days in culture than in wild-type control cells, but isoproterenol treatment corrected this weakness (Fig. 4).

Altered intracellular Ca^{2+} transient in cTnI-K118C cardiomyocytes. Simultaneous recording of the fura 2-AM fluorescence ratio of 360 nm vs. 380 nm excitations and contractile measurements indicated intracellular Ca^{2+} transients in cTnI-K118C and wild-type mouse cardiomyocytes (Fig. 5A). The results showed no difference between the baseline cytosolic Ca^{2+} in cTnI-K118C and wild-type cardiomyocytes (Fig. 5B). Treatment with 30 nM isoproterenol did not have a significant effect on the baseline Ca^{2+} in either group (Fig. 5B). The peak of the cytosolic Ca^{2+} transient following the electrical stimulation showed no significant difference between cTnI-K118C cardiomyocytes and the wild-type cells. Isoproterenol treatment increased contractility (Table 2), as well as the peak Ca^{2+} transient, similarly in both groups (Fig. 5B).

On the other hand, the time to develop 50% peak cytosolic Ca^{2+} (TP_{50}) was longer in cTnI-K118C cardiomyocytes than that in wild-type cells (Fig. 5C). The time from peak Ca^{2+} transient to 75% decay (TR_{75}) was also longer in cTnI-K118C cardiomyocytes than in wild-type cells at baseline (Fig. 5C). Isoproterenol treatment significantly shortened TP_{50} and TR_{75} in both groups, but the differences remained (Fig. 5C). The early phase of Ca^{2+} transient decay was further measured to

Fig. 3. PKA-dependent phosphorylation was preserved in 2-day-cultured adult mouse cardiomyocytes. A: Coomassie Brilliant Blue (CBB) and Pro-Q Diamond phosphoprotein staining of SDS gels show that isoproterenol (ISO) induced phosphorylation of cTnI and myosin binding protein C (MBP-C) in 2-day-cultured adult mouse cardiomyocytes similar to that in freshly isolated cells, indicating preservation of the PKA signaling pathway after short-term culture. Baseline (isolated in the absence of adrenergic stimuli) and isoproterenol-induced phosphorylation of cTnI and MBP-C in freshly isolated or 2-day-cultured cells did not differ between cTnI-K118C and wild-type cardiomyocytes. B: peppermint-stick phosphoprotein staining control validates conditions of Pro-Q staining. Results were summarized from 3 repeating experiments, and a representative set of data is shown.

AJP-Cell Physiol • doi:10.1152/ajpcell.00053.2014 • www.ajpcell.org

B
troponin. TR10 and TR25 showed no significant difference to evaluate the effect of cTnI-K118C on the release of Ca\textsuperscript{2+} from troponin. TR\textsubscript{10} and TR\textsubscript{25} showed no significant difference between cTnI-K118C and wild-type cardiomyocytes at baseline. However, 30 nM isoproterenol shortened TR\textsubscript{25} of wild-type, but not cTnI-K118C, cells (Fig. 5C).

cTnI-K118C mouse cardiomyocytes had significantly increased resting length. The isolated adult cardiomyocytes allow accurate measurements of cell length. This is a unique advantage over studies using myocardial tissue sections, in which it is rather difficult to accurately orient and measure the full length of individual cells. Therefore, we had the opportunity to readily and precisely measure the length, width, and sarcomere length in large numbers of the cTnI-K118C cardiomyocytes at rest, as well as in contracted states.

An intriguing finding in our study was a significantly longer resting length of cardiomyocytes isolated from cTnI-K118C mice than that of cells from strain- and age-matched wild-type mice (Fig. 6A). The data showed a clearly longer resting length of cTnI-K118C cardiomyocytes as early as 3 wk of age. The most significant difference was seen at 2–3 mo (Fig. 6A). At 5–8 mo, the difference became smaller but was still statistically significant (Fig. 6A). No significant difference was found between male and female mice in the each of the age groups (data not shown).

The cell width was not different between cTnI-K118C and wild-type cardiomyocytes (Fig. 6B). Cell width trended narrower in 2- to 3-mo-old TnI-K118C cardiomyocytes than wild-type control cells (Fig. 6B). Overall, the long and thin morphology of cTnI-K118C cardiomyocytes was clearly visible in microscopic images (Fig. 6C).

The resting sarcomere was not longer, but slightly shorter, in the cTnI-K118C cardiomyocytes than wild-type cells in age-matched groups (Fig. 6D). Therefore, the increased resting length of cTnI-K118C cardiomyocytes reflected a longitudinal addition of sarcomeres (Fig. 6E).

Prolongation of adult cTnI-K118C mouse cardiomyocytes was accompanied with nuclear division. Propidium iodide staining of nuclear DNA was applied to count the number of nuclei in the isolated cardiomyocytes. The results in Fig. 7A show more mono- and tri-/quadrinucleated cardiomyocytes in cTnI-K118C mouse hearts, together with a proportional decrease in the number of normal binucleated cells, than in wild-type cells. The tri- and quadrinucleated cardiomyocytes were significantly longer than the binucleated cells (Fig. 7B). Therefore, the increased numbers of tri- and quadrinucleated cells may have contributed to the elongated phenotype of cTnI-K118C cardiomyocytes.

Nonetheless, the binucleated normal cardiomyocytes remained in the majority in the hearts of adult cTnI-K118C mice and were also longer than in wild-type controls (Fig. 7B). Therefore, longitudinal addition of sarcomeres in the absence of nuclear division made important contributions to the prolonged phenotype of cTnI-K118C cardiomyocytes.

Upregulated LIFR in adult cTnI-K118C transgenic mouse heart. To explore the cellular signaling pathway related to the cTnI-K118C mutation–caused cardiomyocyte prolongation, we first examined mRNA expression profiles in ventricular muscle of cTnI-K118C transgenic and wild-type littermate mice at 2 mo of age, when the difference in resting cell length was most predominant (Fig. 6A).

In contrast to many gene expression profile studies, whole-genome microarray showed very few genes with statistically significant changes in the level of expression: only 62 genes with >30% increase or decrease in their level of expression in cTnI-K118C hearts compared with wild-type control hearts. Two internal controls, the overexpression of \(\alpha\)-MHC promoter-driven cTnI-K118C transgene (Tnnt3) and the decreased expression of slow skeletal muscle TnT gene (Tnnt1) due to partial deletion of its promoter when the linked endogenous cTnI gene was deleted (7), were clearly detected in the cTnI-K118C hearts, as expected, and nicely validated the results.

Among the proteins with altered levels of expression in the 2-mo-old cTnI-K118C mouse hearts, a search of genes with...
function in cell growth identified a 2.0-fold increase in the expression of LIFR encoded by the gene *Lifr*. A previous study demonstrated that LIFR plays a critical role in the cardiotrophin-1-induced longitudinal prolongation of cardiomyocytes during heart development (17). We verified the upregulation of LIFR, and the Western blot in Fig. 8A confirms the upregulation of LIFR protein in cTnI-K118C mouse hearts. The increase in LIFR expression in cTnI-K118C mouse cardiac muscle indicates a plausible cell signaling pathway responsible for the functional changes produced by cTnI-K118C mutation to cause significant remodeling in the cardiomyocytes.

In the mean time, no mRNA or protein level increase was found for gp130 (29), another signaling molecule downstream of cardiotrophin-1 signaling, in promoting myocardial remodeling (Fig. 8B). Therefore, the selective upregulation of LIFR in cTnI-K118C mouse hearts may be an early indicator of cardiac remodeling and may suggest a unique mechanism that is worth further investigation.
**DISCUSSION**

On the basis of previous studies at the organ level using ex vivo working heart preparations demonstrating the dominantly negative nature of the cTnI-K118C mutation (27), the present study investigated effects of this TnI-TnT interface mutation on the cellular phenotypes of cardiomyocytes isolated from adult transgenic mouse hearts. Excluding the influence of the extracellular matrix and connective tissues, the results provide several new insights into the experimental approach using isolated adult cardiomyocytes and the function of the TnI-TnT interface.

Adult mouse cardiomyocytes preserved phenotypes after recovery in short-term culture from isolation stress. Isolation of cardiomyocytes from adult mouse hearts is known to be much more technically demanding than isolation of adult rat cardiomyocytes (13). Using the protocol described here, we are able to reproducibly obtain sufficient numbers of viable cells for contractility and other functional studies (data not shown). However, the ex vivo perfusion-based enzymatic isolation of cardiomyocytes unavoidably imposes an ischemia-reperfusion-like stress on the cells. This was reflected by the significant amount of NH2-terminal-truncated cardiac TnT, a product of restrictive proteolysis previously shown to occur during myocardial ischemia-reperfusion, detected in the freshly isolated cardiomyocytes (Fig. 2) (31).

TnI and TnT are protein subunits of the troponin complex of striated muscle thin filament (21, 22). They function interactively in the Ca2+/H11001 regulation of contraction and relaxation (15). Table 2.

**Table 2. Contractility of cTnI-K118C and wild-type cardiomyocytes**

<table>
<thead>
<tr>
<th>Cell Shortening, %</th>
<th>Maximum Shortening Velocity, μm/s</th>
<th>Maximum Relengthening Velocity, μm/s</th>
<th>TP50, ms</th>
<th>TR75, ms</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>WT</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 nM Iso</td>
<td>4.82 ± 0.76</td>
<td>225.92 ± 36.65</td>
<td>173.60 ± 39.69</td>
<td>33.08 ± 2.75</td>
</tr>
<tr>
<td>30 nM Iso</td>
<td>9.86 ± 0.98*</td>
<td>431.72 ± 43.60</td>
<td>376.38 ± 41.36</td>
<td>28.00 ± 1.52</td>
</tr>
<tr>
<td><strong>K118C</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 nM Iso</td>
<td>3.65 ± 0.55</td>
<td>124.35 ± 17.90*</td>
<td>79.81 ± 15.05</td>
<td>43.23 ± 2.66*</td>
</tr>
<tr>
<td>30 nM Iso</td>
<td>11.98 ± 1.25§</td>
<td>414.27 ± 53.36</td>
<td>334.88 ± 55.30</td>
<td>40.85 ± 2.92</td>
</tr>
</tbody>
</table>

Values are means ± SE. Contractility of cTnI-K118C (K118C) and wild-type (WT) cardiomyocytes at baseline and under isoproterenol stimulation was analyzed. Day 0 cells isolated in the absence of 2,3-butanedione monoximine were loaded with fura 2 and stimulated at 2 Hz. cTnI-K118C cells showed lower shortening velocity and longer contractile and relengthening times than wild-type controls. Isoproterenol (Iso) increased contractility in cTnI-K118C and wild-type groups, whereas differences in time parameters [time to develop 50% peak shortening (TP50) and time from peak shortening to 75% relengthening (TR75)] remained. *P < 0.05; †P < 0.01 vs. wild-type control. ‡P < 0.05, §P < 0.01 vs. 0 nM Iso.

**Fig. 6.** Resting cardiomyocytes from hearts of cTnI-K118C mice were longer than age-matched wild-type cells. Cardiomyocytes isolated from hearts of 3-wk, 2- to 3-mo, and 5- to 8-mo-old cTnI-K118C transgenic mice were compared with age-matched wild-type control cells. A: cTnI-K118C cardiomyocytes were significantly longer than control cells in all age groups. B: cell width was not changed, although it trended narrower in cells from 2- to 3-mo-old cTnI-K118C hearts than age-matched wild-type cells. C: representative images of 2-mo-old wild-type and cTnI-K118C mouse cardiomyocytes demonstrate long and thin appearance of cTnI-K118C cardiomyocytes. D and E: in all age groups, resting sarcomeres were not longer, but slightly shorter, in cTnI-K118C cardiomyocytes than wild-type cells (D), with statistical significance; accordingly, there was a longitudinal increase in the number of sarcomeres per cell length in cTnI-K118C cardiomyocytes, more significantly so in the younger age groups (E). *P < 0.05; **P < 0.01. Values are means ± SE; n = 3–7 mice at each age point of cTnI-K118C and wild-type groups; ±100 cells from each mouse were measured.
The presence of the NH₂-terminal-truncated cardiac TnT in freshly isolated adult cardiomyocytes may interfere with characterization of the function of the cTnI-K118C mutation. To minimize this potential impact, we employed short-term culture in serum-free medium to allow the cardiomyocytes to recover from isolation stress. An effective recovery was evidenced by the elimination of the NH₂-terminal-truncated cardiac TnT after 2 days of culture (Fig. 2).

The timely replacement of posttranslationally modified cardiac TnT in cultured cardiomyocytes also reflected active synthesis of new cardiac TnT in healthy cardiomyocytes, consistent with cardiac troponin’s normal half-life of ∼3.5 days in vivo (18). The short-term culture approach provides an improved experimental system to study the function of the cTnI-K118C mutation using isolated adult mouse cardiomyocytes.

$cTnI-K118C$ decreased the contractility of ventricular cardiomyocytes. We previously showed at the organ level that $cTnI-K118C$ decreased left ventricular diastolic function and blunted the lusitropic effect of β-adrenergic stimulation (27). The contractility studies using adult cardiomyocytes isolated from $cTnI-K118C$ transgenic mice further demonstrated its dominantly negative effect on not only relaxation, but also contractile velocity, in the absence of external load (Fig. 4). This result may reflect the general consensus that organ- and tissue-level compensations could mask intrinsic functional differences. Therefore, the use of isolated adult cardiomyocytes as a reduced experimental system to avoid the influences of connective tissues, extracellular matrix, and potential paracrine compensations was effective in revealing occult phenotypes of the $cTnI-K118C$ mutation.

Previous working heart studies showed that the negative effect of $cTnI-K118C$ on systolic function was apparently compensated at the organ level, while its effect on diastolic function was detectable (27). This observation implies a more potent functional effect of this TnI-TnT interface mutation on diastolic function. On the other hand, the finding that $cTnI-K118C$ actually has a dominant-negative effect on systolic function supports the notion that dominant-negative mutations in TnI and cardiac TnT affecting the same step of the contractile cycle could cancel each other, as suggested by previous organ-level studies (27). Taken together, the data demonstrate that the highly conserved structure at the TnI-TnT interface is a critical site in the troponin complex and the thin-filament Ca²⁺ regulatory system of striated muscle. In addition to understanding the basic mechanism of cardiac muscle contraction, the TnI-TnT interface is also an attractive target for developing therapeutic interventions in the treatment of heart diseases.

Another advantage of isolated cardiomyocyte preparations is that they allow kinetic studies of the intracellular Ca²⁺ transient. The results in Fig. 5 showed that while there was no significant change in the peak Ca²⁺ between $cTnI-K118C$ and wild-type cardiomyocytes, the rise and decay of intracellular Ca²⁺ were slower in $cTnI-K118C$ cells. Isoproterenol shortened the time parameters but did not eliminate the difference between the two groups. The result suggests that the $cTnI-K118C$ mutation in the TnI-TnT interface of the troponin complex caused slower release of Ca²⁺ from troponin C, which could be directly responsible for the slower relaxation velocity. Although the sarcoplasmic reticulum Ca²⁺ reuptake or another Ca²⁺-handling and buffer system may also be affected indirectly, the impaired release of Ca²⁺ from troponin would have a negative impact on the restoration of Ca²⁺ in the sarcoplasmic reticulum and, in turn, limit release of Ca²⁺ from the sarcoplasmic reticulum during systole. A slower release of Ca²⁺ from troponin C in $cTnI-K118C$ cardiomyocytes would potentially affect the myofibril ATPase cycle and cross-bridge kinetics. A prolonged phase of the thin filaments in the activated state may delay detachment of the myosin head after the power stroke. The pathophysiological significance of this hypothesis requires further investigation.

The Ca²⁺ dynamic changes in the $cTnI-K118C$ cardiomyocytes may also explain how the alteration of troponin function
was able to override the potential increase in contractile velocity due to the prolonged cell length.

cTnI-K118C mutation caused early prolongation of cardiomyocytes in the absence of clinical heart failure. A novel finding in the present study was significantly greater resting length in ventricular cardiomyocytes isolated from cTnI-K118C transgenic mice than cells isolated from strain- and age-matched wild-type mice. Unlike compensative hypertrophy, the cell width was not increased. The increased resting length was not due to longer or more relaxed sarcomeres but, rather, to longitudinal addition of sarcomeres in the cTnI-K118C cardiomyocytes (Fig. 6).

During postnatal development and growth in early adulthood, the length of wild-type mouse cardiomyocytes increased from $\sim 100$ to $\sim 135 \mu m$ (Fig. 6A). In contrast, the cTnI-K118C cardiomyocytes reached a length of $>120 \mu m$ at 3 wk of age and $>140 \mu m$ at 2–3 mo. At 5–8 mo of age, the length difference between cTnI-K118C and wild-type cardiomyocytes became smaller but was still statistically significant (Fig. 6A). This pattern suggests an accelerated early growth in length of the cTnI-K118C cardiomyocytes. The reduced difference between cTnI-K118C and wild-type cardiomyocytes in mature adult hearts may reflect a restriction to the elongation phenotype by other structural and/or regulatory factors. While cTnI-K118C cells reach the limit earlier, they would not grow beyond this physiological limit, since cTnI-K118C hearts did not develop clinical failure and anatomic dilation during unstimulated cage life.

Nonetheless, the longitudinal addition of sarcomeres reflected a hypertrophic growth of cTnI-K118C cardiomyocytes (14). Although no anatomic level of chamber dilation was detected in the compensated young adult cTnI-K118C hearts, the prolongation of cardiomyocytes indicates an early sign of adaptive remodeling at the cellular level. The Ca$^{2+}$ transient data did not show significant change in the resting intracellular Ca$^{2+}$ (Fig. 5B); thus this early hypertrophic response at the cellular level may be secondary to changes in contractility other than to Ca$^{2+}$ overloading.

Since cTnI-K118C is a dominant-negative mutation that weakens the contractility of cardiomyocytes (Fig. 4), adaptive remodeling could logically occur in the ventricular muscle of the transgenic mice. However, the early remodeling in young cTnI-K118C transgenic mouse cardiomyocytes in the absence of an anatomic level of hypertrophy or clinical heart failure is an intriguing phenomenon. The early occurrence of cardiomyocyte prolongation in hearts with an occult failure condition, on one hand, suggested that cellular level remodeling is a very early response to changes in cardiac muscle contractility. This early change in cellular morphology provides a useful criterion for early detection of myocardial adaptation and maladaptation. On the other hand, this effect was apparently specific to the cTnI-K118C mutation and was not found in cardiomyocytes from other transgenic mouse models with various myopathic changes in cTnI or cardiac TnT (data not shown). Therefore, this specific phenotype of cTnI-K118C cardiomyocytes may indicate a unique consequence of abnormalities at the TnI-TnT interface.

The increased expression of LIFR in 2-mo-old cTnI-K118C mouse ventricular muscle linked the cTnI-K118C mutation to a cell signaling pathway that has been demonstrated to have an essential role in longitudinal prolongation of rat cardiomyocytes via the function of the cardiotrophin-1 signaling pathway (17, 29). The signals may originate from the altered contractility or through changes in Ca$^{2+}$ homeostasis due to altered tropinin function. This plausible observation merits future studies of the molecular mechanisms by which the structure and function of myofilament regulatory proteins trigger myocardial remodeling (1), especially dilatative prolongation of...
cardiomyocytes, which converts chronic heart diseases to irreversible terminal failure in human patients (16). The cTnI-K118C transgenic mouse cardiomyocytes provide an early adaptation model for investigations of this important question on the pathogenesis of dilated cardiomyopathy and heart failure.

ACKNOWLEDGMENTS

We thank Hui Wang for technical assistance, Dr. Han-Zhong Feng for participation in the microarray study and instrument operations, and Dr. Jim Lin (University of Iowa) for the CH1 MAb. The Tnnt3 gene deletion allele used in generating the double-transgenic mice was from a heterozygote mouse line provided by Dr. Xupei Huang (Florida Atlantic University).

GRANTS

This study was supported by National Institutes of Health Grants HL-098945 and AR-048816 and a Multidisciplinary Incubator Grant from the Office of the Vice President for Research, Wayne State University (to J.-P. Jin).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

H.W. performed the experiments; H.W. analyzed the data; H.W. and J.-P.J. interpreted the results of the experiments; H.W. and J.-P.J. prepared the figures; H.W. and J.-P.J. drafted the manuscript; H.W. and J.-P.J. edited and revised the manuscript; H.W. and J.-P.J. approved the final version of the manuscript; J.-P.J. is responsible for conception and design of the research.

REFERENCES


