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2451-Pos Board B437

N-Terminal Truncated Cardiac TnI Extends Frank-Starling Response of the Heart

Hanzhong Feng, Xupei Huang, J.-P. Jin.

Cardiac TnI (cTnI) has an N-terminal extension containing two protein kinase A (PKA) phosphorylation sites, Ser23/24, and its removal by restrictive proteolysis in cardiac adaptation improves myocardial relaxation. Through transgenic expression of the N-terminal truncated cTnI (cTnI-ND) in the heart of endogenous cTnI knockout mice, we studied the function of cardiac muscle containing only cTnI-ND. The pure cTnI-ND hearts showed no hypertrophy or dilation with normal baseline function as compared to wild type controls (WT), confirming the non-destructive nature of cTnI-ND. Echocardiography found increased left ventricular end diastolic dimension in cTnI-ND hearts as compared to WT, implying increased relaxation and compliance in vivo. A series of preloads was tested in ex vivo working hearts for the effects of cTnI-ND on Frank-Starling response. cTnI-ND hearts showed responses to 5-10 mmHg preloads similar to that of WT. When preload was increased from 10 to 20 mmHg, cTnI-ND hearts exhibited better maintained left ventricular relaxation velocity, lower left ventricular end diastolic pressure and larger stroke volume responses than that of WT hearts. 10 nM isoproterenol further increased the positive effects of cTnI-ND on the responses of cardiac function to preloads, indicating that cTnI-ND enhances Frank-Starling relationship in the absence of direct effect of PKA phosphorylation at Ser23/24. Slack sarcomere length in isolated cardiomyocytes and the optimal sarcomere length for maximum tension development in intact left ventricular papillary muscle from cTnI-ND mice were similar to that of WT, suggesting that cTnI-ND extends the range of Frank-Starling response to high preloads by enhancing contractility independent of further increases in sarcomere length. The results demonstrated that the removal of cTnI N-terminal extension by restrictive proteolysis provides a novel posttranslational modification to utilize Frank-Starling mechanism in cardiac adaptation to physiological and pathological stresses.

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Myopathic Splice-Out of the Exon 7 segment of Cardiac Troponin T Predominantly Impairs Systolic Function of the Heart

Hanzhong Feng, Guozhen Chen, Changlong Nan, J.-P. Jin.

The abnormal splice-out of the exon 7-encoded segment in the N-terminal variable region of cardiac troponin T (cTnT-DE7) found in turkeys and dogs with dilated cardiomyopathy significantly reduced the function of transgenic mouse hearts over-expressing cTnT-DE7 (Wei et al., JBC, 2010). Significantly decreased left ventricular peak pressure and contractile and relaxation velocities with elongated isovolumic contraction and relaxation time were seen in ex vivo working hearts from cTnT-DE7 mice in the absence of beta-adrenergic tone, which were correctable by physiological levels of isoproterenol. To further investigate the pathogenic mechanism of cTnT-DE7 under systemic neurohumoral regulation, echocardiography was employed to measure the function of cTnT-DE7 transgenic mouse hearts in vivo. No atrial enlargement, ventricular hypertrophy or dilation was detected in the hearts of 3 months old cTnT-DE7 mice, indicating a compensated state. However, left ventricular fractional shortening and ejection fraction were significantly decreased in cTnT-DE7 mice compared to wild type controls. Under both baseline and beta-adrenergic stimulated conditions, the left ventricular outflow tract velocity and gradient were both significantly decreased in the transgenic mouse hearts, indicating decreased systolic function. The impaired systolic heart function in vivo without changes in diastolic function suggests that cTnT-DE7 primarily reduces contractile function of the cardiac muscle. Interestingly, a restrictive proteolytic deletion of the N-terminal variable region of cTnT (Feng et al., J. Physiol. 2008) was increased in the cTnT-DE7 hearts in vivo. This adaptive response may compensate for the decreased systolic function from the N-terminal abnormality of cTnT-DE7. Our study demonstrated that the N-terminal variable region of cTnT is a regulatory element for cardiac systolic function and a therapeutic target in heart failure.

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Pathophysiologic Changes Induced by Mutations in the TNT1 Domain of cTnT that Cause FHC

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Familial Hypertrophic Cardiomyopathy (FHC) is a primary cardiac muscle disorder and one of the most common causes of sudden cardiac death in young people. A majority of Cardiac Troponin T (cTnT) mutations are located in the TNT1 domain and cluster at its N- and C-termini. We are investigating the cTnT deletion of glutamine 160 (delta-160E) that is known to be a severe mutation located in a predicted hinge region at the C-terminal end of TNT1. Previous *in vitro* motility studies in our laboratory showed that mutations in this region disrupt weak electrostatic interactions between the thin filament and myosin necessary for strong crossbridge formation. In the current study, we aim to examine the downstream pathophysioloic consequences of this mutation. Cardiac myocytes isolated from hearts of transgenic mice expressing delta-160E cTnT with 35% and 70% replacement and non-transgenic siblings were used to study mechanical function and calcium transients. Our study shows impairments in myocellular mechanics during contraction and relaxation and in the rise and decline of the calcium transient. Furthermore, the alterations in calcium kinetics were dose-dependent. These results support the progressive nature of delta-160E FHC suggested by electron micrographs that demonstrate ultrastructural sarcomeric disarray that increase with transgene expression. In addition, we determined downstream effects of the mutation on expression and function of calcium handling proteins in transgenic mouse hearts using functional assays and immunoblotting. We found that the delta-160E cTnT mutation causes secondary alterations in calcium handling, leading to decreased SR calcium uptake, increased NCX expression, and increased diastolic leak through RyR2. Collectively, these novel findings indicate a phenotype that is distinct from other cTnT mutations and support the need to establish genotype-phenotype links in order to better design molecular therapies to treat FHC.

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N-Terminal Truncated Cardiac TnI Improves Cardiac Function *In Vivo* and Rescues Restrictive Cardiomyopathy

Pierre-Yves Jean-Charles, Yuejin Li, Changlong Nan, Guozheng Chen, Han-Zhong Feng, J-P Jin, Xupei Huang.

Our previous studies have demonstrated that the phenotype of the transgenic mouse hearts expressing a restrictive cardiomyopathy (RCM) cardiac troponin I (cTnI) C-terminal mutation (R193H) is characterized by a diastolic dysfunction and sudden cardiac death (SCD) (Du et al, 2006, 2008). We have also observed that restrictive cleavage of the N-terminal extension of cTnI (cTnI-ND) that occurs in physiological and pathological adaptations (Yu et al., 2001; Feng et al, 2008; McConnell et al., 2009) desensitizes myofibril sensitivity for Ca^{2+} and enhances diastolic function in transgenic mice expressing cTnI-ND (Li et al, 2010). In the present study, we generated double transgenic mice (Double-TG) expressing different levels of mutant cTnI R193H and cTnI-ND to investigate the dose-dependent rescue effect of cTnI-ND and the mechanisms underlying the protective role of cTnI-ND in young and aged RCM mice. In 2-month-old Double-TG mice, cTnI-ND rescues RCM mice by correcting diastolic dysfunction caused by cTnI R193H mutation in the heart. The rescue effect of cTnI-ND shows a dose-dependent manner. In 8-10-month-old Double-TG mice, echocardiography and Doppler data indicate that cTnI-ND rescues RCM mice not only by reversing the diastolic dysfunction, but also by improving systolic function in the heart, since both diastolic and systolic functions are deteriorated in aged RCM mice. Cell-based assays measuring cardiac cell contractility further confirm the dose-dependent protective effect of cTnI-ND in correcting the impaired relaxation in isolated cardiac myocytes from various Double-TG mouse lines. Consistent with the beneficial effect of cTnI-ND on the function of non-myopathic aging hearts (Biesiadescki et al., 2010), these data demonstrate that cTnI-ND can rescue RCM phenotype not only by correcting diastolic dysfunction in young RCM mice but also by improving systolic function in aged RCM mice.

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Dose-Dependent Arrhythmia and Cardiac Dysfunction in Restrictive Cardiomyopathy Mice Due to Troponin Mutations

Yuejin Li, Pierre-Yves Jean-Charles, Changlong Nan, Guozhen Chen, Xupei Huang.

Restrictive cardiomyopathy (RCM) is associated with a cardiac troponin I (cTnI) C-terminal mutation (R192H) in human patients. The transgenic mice expressing this mutation have confirmed a phenotype of a diastolic dysfunction and sudden cardiac death (SCD) (Du et al, 2006, 2008). In the present study, we generated transgenic mice ($cTnI^{193His}$) expressing different levels of mutant cTnI R193H (mouse cTnI sequence) to investigate the dose-dependent cardiac dysfunction and to reveal the cause of the death in RCM mice. Our results indicated that the mice ($cTnI^{193His/KO}$ expressing only the mutant cTnI R193H at a wild type cTnI-null background had a dramatic early death at one-month old after birth. Telemetric ECG recording from these mice showed a significant bradycardia starting on day 22 or 23 after birth and a significant ischemia and arrhythmia 1-2 days before death. The diastolic function was deteriorated in these mice determined by echocardiography compared to wild type and the transgenic $cTnI^{193His}$ mice expressing 25% cTnI R193H and 75% wild type cTnI. Cell-based experiments indicated that myocardial contractility decreased significantly corresponding to the content of the mutant cTnI levels in cardiac myocytes and the alteration of Ca^{2+} dynamics in the mutant cTnI cardiac myocytes also showed a dose-dependent manner. Our study has demonstrated that cTnI R193H mutation-caused cardiac dysfunction is dose dependent. Bradycardia is likely an adaptive mechanism of RCM mice to compensate for the prolonged relaxation. The main cause of the death in RCM mice is associated with

fatal arrhythmia and ventricular ischemia due to the restricted ventricles and enlarged atria. The transgenic mouse model provides us with a good tool to study the mechanisms and the cause of the death of RCM, which will be useful for the prevention and treatment of the disease.

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Both PKA Treatment and Cardiac Troponin-I N-Terminal Phosphorylation Alone Decrease Ca-Sensitivity and Eliminate Length-Dependent Activation in Skinned Cardiac Muscle

Donald A. Martyn, Steven Korte, Maria Razumova, Erik Feest, Thomas Irving, Michael Regnier.

Protein kinase A (PKA) phosphorylation of myofibrillar proteins constitutes an important pathway for β-adrenergic modulation of cardiac contractility. PKA targets the cardiac troponin I (cTnI) N-terminus, cardiac myosin-binding protein C (cMyBP-C) and titin. To isolate cTnI and cMyBP-C /titin phosphorylation effects on force-[Ca^{2+}] relations, endogenous cardiac troponin (Tn) was exchanged in rat, skinned trabeculae with either WT Tn or Tn containing a non-phosphorylatable mutant cTnI(S23/24A) or phosphomimetic cTnI(S23/24D). PKA cannot phosphorylate either cTnI mutant, leaving cMyBP-C and titin as sole PKA targets. Force-[Ca^{2+}] relations and Ca^{2+}-sensitivity (pCa50) were measured at 2.3 and 2.0 μm SL. Decreasing steady SL reduced maximal force (Fmax) and pCa50 similarly with WT Tn and Tn containing cTnI(S23/24A). PKA treatment of native, WT and cTnI(S23/24A) exchanged trabeculae reduced pCa50 at 2.3, but not 2.0 um SL, eliminating SL-dependence of pCa50. Reconstitution with Tn containing cTnI(S23/24D) reduced pCa50 at both SL (compared to WT and cTnI(S23,24A) and eliminated pCa50 SL-dependence; PKA did not significantly alter pCa50 at either SL. At each SL Fmax was similar with WT and mutant troponins, and was unaffected by PKA. Low angle x-ray diffraction experiments were performed to determine whether shifts in pCa50 were associated with changes in myofilament spacing (D1,0) or interaction. D1,0 at 2.3 um SL was similar in native trabeculae, with WT Tn and Tn containing either cTnI(S23,24A) or cTnI(S23,24D); PKA increased D1,0 in all cases. The results suggest that PKA phosphorylation of either cTnI or cMyBP-C /titin reduced the Ca^{2+}-sensitivity of force and length-dependent activation. Supported by NIH HL067071-06.

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Reducing Thin Filament Ca^{2+} Affinity with a cTnC Variant (L57Q) Reduces Force but Enhances Cross-Bridge Dependence of Cooperative Activation in Demembranated Rat Trabeculae

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Activation of cardiac contraction is initiated by binding of Ca2+ to troponin C (cTnC) and regulated by cooperative strong cross-bridge binding. We previously showed that passive exchange with cTn containing a cTnC variant, L48Q, increased Ca2+ sensitivity of force development and eliminated sarcomere length (SL) dependence of Ca2+ sensitivity of force in rat cardiac trabeculae. This was shown to be due to decreased reliance on strong cross-bridge binding for full thin-filament activation, possibly due to stronger cTnC-cTnI interaction. We also showed that PKA phosphorylation decreased Ca2+ sensitivity of force and eliminated SL dependence of force-pCa relations, by unknown specific mechanisms. Here we test the hypothesis that incorporation of a cTnC variant with decreased Ca2+ binding affinity, L57Q cTnC, will result in increased reliance on cross-bridge binding for full activation, thus increasing SL-dependence of Ca2+ sensitivity. As expected, results indicate trabeculae passively exchanged with L57Q cTnC-cTn displayed decreased Ca2+ sensitivity and rate of force production compared to WT cTnC at a given [Ca2+]. Interestingly, preliminary results indicate L57Q cTnC-cTn mildly increased the SL-dependence of Ca2+ sensitivity of force and also significantly decreased maximal force. Both of these effects were not observed in PKA-treated trabeculae, which had a comparable reduction in Ca2+ sensitivity of force. These findings suggest that reducing cTnC Ca2+ affinity per se can reduce Ca2+ sensitivity of contractile activation to the point of limited overall force production, which may enhance cross-bridge dependence of cooperative thin filament activation. Current experiments aim to increase force in L57Q cTnC (to near WT cTnC) by increasing cross-bridge attachment using 2'-deoxy-ATP, which has previously been shown to increase force and Ca2+ sensitivity while maintaining SL dependence. NIH-HL65497(MR), AHA-2310117(FSK).

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Fluorescence Measurements Using Rhodamine-Labeled cTnC Mutants Indicate Little Cooperative Interaction Between Cardiac Thin Filament Regulatory Units

Don Martyn, **Vijay Rao**, Michael Regnier.

Exchange of mixtures of WT cTnC and mutant cTnC(D65A), which cannot bind Ca^{2+} at N-terminal site II (''dead'' cTnC), reduced maximal Ca^{2+} activated force (Fmax) with little effect on force-Ca^{2+} relations and force kinetics in skinned cardiac trabeculae (Gillis et al., J Physiol. 580:561-76, 2007), suggesting interaction between structural regulatory units (RUs; 7 actins, 1 troponin, 1 tropomyosin) along cardiac thin filaments is less than in skeletal muscle (Regnier et al., J Physiol. 540:485-97, 2002). To more directly test that this finding, we exchanged skinned cardiac trabeculae with mixtures of mutant cTnC(C35S) and cTnC(C35S,D65A), with one or the other labeled at Cys 84 with 5'tetramethyl rhodamine (IATR) for dichroism measurements. In trabeculae exchanged with 100% cTnC(C35S)-IATR, dichroism increased in response to both Ca^{2+} and rigor crossbridges, while trabeculae with 100% Tn containing (cTnC(C35S,D65A)-IATR) had no response to Ca^{2+}, but retained a strong response to rigor crossbridge binding. This response to strong crossbridges allows use of cTnC(C35S,D65A)-IATR to determine if isolated regulatory units containing cTnC(C35S,D65A)-IATR are perturbed by Ca^{2+}-induced active contraction in adjacent ''live'' RUs. To test this, trabeculae were exchanged with a mixture of 20% functional cTnC(C35S)-IATR and 80% unlabeled cTnC(C35S,D65A), to isolate functional RUs. Fmax decreased but there was little change in the Ca^{2+}-dependence of dichroism compared to trabeculae exchanged with 100% functional cTnC(C35S)-IATR. These data indicate minimal or no apparent spread of activation between adjacent RUs in cardiac muscle, indicating that the apparent cooperativity of force production in cardiac muscle results from interactions between myosin and thin filaments within a thin filament structural regulatory unit. Supported by NIH RO1-HL65497 (Regnier).

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Combination of Phosphomimetic Substitutions within Cardiac Troponin I Cause Functional Cross-Talk

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Protein kinase C (PKC) phosphorylates 3 clusters of residues within cardiac troponin I (cTnI) and yet, it is unclear whether phosphorylation at multiple sites produces additive and/or divergent functional modifications. Our goal was to evaluate the influence of cTnI with phosphomimetic substitutions on contractile performance under basal conditions and in response to PKC activation by endothelin. Endogenous cTnI was replaced with phosphomimetic substitutions using adenoviral-mediated gene transfer into adult rat cardiac myocytes. Phosphomimetics of Ser43/45, Ser43/45 plus Thr144, and Ser23/24 plus Ser43/45 were substituted with Asp to form AdcTnISer43/45Asp, $cTnIAsp_{Triple}$ (e.g. cTnISer43/45AspThrT144Asp), and $cTnIAsp_{Quad}$ (cTnISer23/24/43/45Asp). Isolated myocytes were electronically paced and studied 4 days after gene transfer. Gene transfer of epitope-tagged versions of each construct resulted in 30-40% replacement after 2 days and >65% replacement of endogenous cTnI 4 days after gene transfer without significant alterations in the expression of other myofilament proteins. In functional studies, peak shortening amplitude was significantly decreased in myocytes expressing cTnISer43/45Asp or $cTnIAsp_{Quad}$, while peak shortening in myocytes expressing $cTnIAsp_{Triple}$ was not significantly different from controls. Relaxation was accelerated in myocytes expressing $cTnIAsp_{Quad}$, but was not different from controls in myocytes expressing $cTnIAsp_{Triple}$ or cTnISer43/45Asp. Together, these results suggest the Ser23/24 and Ser43/45 sites have an additive influence on shortening, while substitution at Thr144 attenuates the influence of Ser43/45 on peak shortening. To further determine whether multiple phosphomimetic substitutions within cTnI influence myocyte shortening, we studied the change in peak shortening and relaxation produced by the PKC agonist, endothelin (10 nM). In preliminary studies, the increased peak amplitude and accelerated relaxation observed in control myocytes is not significantly different in myocytes expressing cTnISer43/45Asp. However, there is a trend for myocytes expressing $cTnIAsp_{Quad}$ to show an attenuated amplitude and relaxation response to ET.

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The Motif of Myosin Binding Protein-C is Mechanically Weak and Extensible

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Cardiac myosin binding protein-C (cMyBP-C) is a member of the immunoglobulin (Ig) superfamily of proteins and consists of 8 Ig- and 3 fibronectin (Fn)-like domains along with a unique regulatory sequence referred to as the MyBP-C ''motif'' or M-domain. The structure of the M-domain is not known, but small angle X-ray scattering experiments suggest that it adopts a compact shape in solution and that its overall dimensions are similar to other Ig-like domains. To investigate whether the M-domain behaves similarly to an Ig domain under mechanical stress or load, we used atomic force microscopy (AFM) to investigate single molecule elasticity and mechanical properties of recombinant full-length mouse cardiac cMyBP-C and smaller proteins containing just the M-domain and flanking Ig- sequences. Force-extension curves of full-length cMyBP-C showed unfolding of individual Ig or Fn-like domains at forces