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Sexually dimorphic myofilament function and cardiac troponin I phosphospecies distribution in hypertrophic cardiomyopathy mice

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ABSTRACT

The pathological progression of hypertrophic cardiomyopathy (HCM) is sexually dimorphic such that male HCM mice develop phenotypic indicators of cardiac disease well before female HCM mice. Here, we hypothesized that alterations in myofilament function underlies, in part, this sex dimorphism in HCM disease development. Firstly, 10–12 month female HCM (harboring a mutant [R403Q] myosin heavy chain) mice presented with proportionately larger hearts than male HCM mice. Next, we determined Ca^{2+}-sensitive tension development in demembranated cardiac trabeculae excised from 10–12 month female and male HCM mice. Whereas HCM did not impact Ca^{2+}-sensitive tension development in male trabeculae, female HCM trabeculae were more sensitive to Ca^{2+} than wild-type (WT) counterparts and both WT and HCM males. We hypothesized that the underlying cause of this sex difference in Ca^{2+}-sensitive tension development was due to changes in Ca^{2+} handling and sarcomeric proteins, including expression of SR Ca^{2+} ATPase (2a) (SERCA2a), β-myosin heavy chain (β-MyHC) and post-translational modifications of myofilament proteins. Female HCM hearts showed an elevation of SERCA2a and β-MyHC protein whereas male HCM hearts showed a similar elevation of β-MyHC protein but a reduced level of cardiac troponin T (cTnT) phosphorylation. We also measured the distribution of cardiac troponin I (cTnI) phosphospecies using phosphate-affinity SDS–PAGE. The distribution of cTnI phosphospecies depended on sex and HCM. In conclusion, female and male HCM mice display sex dimorphic myofilament function that is accompanied by a sex- and HCM-dependent distribution of sarcomeric proteins and cTnI phosphospecies.

Introduction

The identification that familial hypertrophic cardiomyopathy (HCM)[1] has a genetic and molecular basis has prompted many investigations into the precise functional impairment in the sarcomere as it relates to the clinical manifestation [1–4]. As the first identified mutation genetically linked to HCM, the myosin 403 (R403Q) mutation typifies this approach. The R403Q model has significantly contributed to our understanding of HCM largely because this particular murine model of human HCM possesses multiple phenotypic similarities with their human counterparts including, (1) histologic and physiological characteristics, (2) course of disease progression, (3) pathological spectrum of disease phenotype, and (4) phenotypic differences between the sexes [5,6]. The R403Q mutation resides in the actin-binding domain and previous studies examining how the R403Q mutation impacts protein kinetics give inconsistent results such as either reduced [7] or enhanced [8] actin filament velocity and either reduced [9] or enhanced [10] actin-activated ATPase. On the other hand, myosin isolated from mouse hearts with the R403Q mutation consistently demonstrates increased actin velocity, actin-activated ATPase, and shorter crossbridge time-on measured by laser trap [8,10,11].

Yet, the role that Ca^{2+}-sensitive tension development as an index of cardiac contractility plays in the progression of HCM is unclear. Studies consistently show that Ca^{2+}-sensitive tension development of cardiac fibers is not different between young (6–20 weeks) WT and R403Q hearts [11–13] even though previous studies show that intact hearts from male mice expressing the R403Q mutation show greater contractility compared to controls [14,15]. The suggestion is that inherent myofilament tension

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[1] *Abbreviations used:* HCM, hypertrophic cardiomyopathy; β-MyHC, β-myosin heavy chain; cTnT, cardiac troponin T; cTnI, cardiac troponin I; PMSF, phenylmethylsulfonyl fluoride; MyHC, myosin heavy chain; PVDF, polyvinylidene fluoride; PKA, protein kinase A; VM, ventricular weight; TL, tibial length; PLB, phospholamban; cTm, cardiac tropomyosin; MLC2, myosin light chain 2; MBP-C, myosin binding protein C; PKC, protein kinase C.

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development is not altered by the R403Q mutation and R403Q HCM disease progression is a complex integration of myofilament function, crossbridge kinetics, and cellular signaling.

Another confounding observation with the R403Q mutation is that R403Q HCM mice display sex dimorphisms despite similar expression levels of the mutation [5,6,16]. Male HCM mice develop progressive left-ventricular dilation and impaired cardiac function whereas female counterparts show increasing hypertrophy without dilation and maintain adequate ventricular function well beyond that of males. Some of the cellular and molecular mechanisms behind the sex differences in the R403Q mice include a number of pathologic indicators such as fibrosis, induction of β-myosin heavy chain, inactivation of glycogen synthase kinase-3β, and activation of pro-apoptotic pathways in males but not females [5,16,17].

In a recent report identifying research priorities for HCM [18], there is a clear consensus that HCM mutations initiate pathological phenotypes by activating specific signaling pathways, some of which have already been studied using the R403Q mice [5,6,16,17,19]. However, the impact of post-translational modification of myofilament proteins in R403Q hearts as it relates to HCM disease progression and sex dimorphisms has not been adequately studied and may provide unique insight as to differences between the sexes. Post-translational modifications of myofilament proteins and the subsequent impact on myofilament function such as Ca^{2+}-dependent tension development (Ca^{2+}-sensitivity) becomes a key mechanism of short- and long-term remodeling within the cardiac cell [20,21]. However, several studies that have investigated phosphorylation status in diseased hearts do not present universal findings; some report a significant reduction in basal cardiac troponin I (cTnI) phosphorylation at PKA sites (Ser23/24) in human heart failure [22,23] whereas others show increased cTnI phosphorylation [24].

In this study, we tested the hypothesis that the sexually dimorphic progression of HCM was, in part, due to underlying differences in Ca^{2+}-sensitive tension development and a differential pattern of post-translational modifications. To do this, hearts of female and male mice harboring an HCM (R403Q) mutation at 10–12 months of age, when males exhibit established HCM disease [5,6], were assessed for Ca^{2+}-sensitive tension development, hypertrophic markers, and post-translational changes in myofilament proteins. Here, we report that male and female HCM hearts display a unique pattern of Ca^{2+}-sensitivity and myofilament protein phosphorylation.

Material and methods

Animal models

The experimental murine model has been detailed previously and consisted of male and female mice heterozygous for the mutant α-myosin transgene [5]. Wild-type (WT) littermates were used as controls for the HCM mice. All experiments were performed using protocols that adhered to guidelines and approved by the Institutional Animal Care and Use Committee at the University of Arizona and to 2012 NIH guidelines for care and use of laboratory animals.

Skinned cardiac trabeculae for calcium-sensitive force measurement

Wild-Type and HCM male and female mice (10–12 months of age) were anesthetized with inhaled anesthesia (isoflurane) and the hearts were rapidly excised and retrogradely perfused with a modified Krebs–Henseleit (K–H) solution (NaCl 118.5 mmol/L, KCl 5 mmol/L, $MgSO_4$ 1.2 mmol/L, NaH_2PO_4 2 mmol/L, D-(+)-glucose 10 mmol/L, $NaHCO_3$ 25 mmol/L, $CaCl_2$ 0.2 mmol/L) [25]. 2,3-Butanedione monoxime (20 mmol/L) was used to inhibit contraction presumably through the energetic stabilization of the unattached state of the myosin molecule [26]. Thin, uniform, and unbranched trabeculae were dissected from the RV free wall and transferred to an ice-cold standard relaxing solution (EGTA = 10 mmol/L; ionic strength = 180 mmol/L) containing 1% Triton X-100 for a minimum of 2 h to allow solubilization of all membranous structures. Following excision of cardiac trabeculae, atria were removed and right and left ventricles separated. All tissue was then flash frozen in liquid nitrogen, and stored at −80 °C for sample analysis at a later time.

Ca^{2+}-sensitivity of force development

The experimental apparatus for mechanical measurements of skinned cardiac trabeculae was similar to that described previously [27,28]. The fiber was attached to the apparatus via aluminum T-clips to stainless steel hooks that extended from a high-speed servomotor and a modified strain gage force transducer (Permeabilized Fiber Test System, Aurora Scientific), both of which were attached to X–Y–Z manipulators mounted on a movable microscope stage. This allowed the suspended muscle to be lowered into a muscle trough that was temperature controlled (15 ± 0.1 °C). Sarcomere length was measured in a central segment of the muscle preparation by video micrometry and set at 2.2 μm for all experiments.

The Ca^{2+}-sensitivity of force as a function of sarcomere length was determined as described previously [28] with slight modifications. Prior to activation, the relaxing solution in the muscle bath was exchanged with a pre-activating solution (Table 1). Following the determination of force, a quick release prior to fiber relaxation was used to identify the zero force level. The step size of the quick release was the minimum step that allowed accurate determination of both steady-state tension and the zero force level. Subsequently, the amount of active force generated at each [Ca^{2+}] was calculated as the difference between total force and relaxed, passive force that was assessed by slackening the fiber while it was in the relaxed state. To determine any decline in force-generating capability, the fiber was maximally activated at the beginning and at the end of the protocol. If the fibers did not maintain 90% of initial maximal force then the fiber data was discarded. Because of the experimental criteria, approximately 90% of the muscle preparations were discarded.

Force in submaximally activating solutions was expressed as a fraction (F_{rel}) of the maximum force (F_0) at the same sarcomere length. The F_0 value used to normalize submaximal force was obtained by linear interpolation between successive maximal activations. Each individual Ca^{2+}-force relationship was fit to a modified Hill equation where $F_{rel} = [Ca^{2+}]^n/(EC_{50}^n + [Ca^{2+}]^n)$, F_{rel} = relative force, EC_{50} = [Ca^{2+}] at which force is half-maximal, n = slope of

Table 1
Ca-EGTA is made by mixing equimolar amounts of $CaCl_2$ and EGTA. In addition, all solutions contained the following (in mmol/L): 10 phosphocreatine, 100 N,N-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid (BES), 0.1 leupeptin, 0.1 phenylmethylsulfonyl fluoride (PMSF), 1 dithiothreitol (DTT), and 4 U/ml creatine phosphokinase. Free Mg^{2+} and Mg-ATP concentration was 1 and 5 mmol/L, respectively. Relaxing and activating solutions was mixed to obtain the desired range of free [Ca^{2+}] assuming an apparent stability constant of the Ca^{2+}-EGTA complex of $10^{6.39}$ at 15 °C. The preactivating solution with low Ca^{2+} buffering capacity containing 1,6-diaminohexane-N,N,N′,N′-tetraacetic acid (HDTA) was used prior to the activating solution.

Solution	$MgCl_2$	Na^2ATP	EGTA	HDTA	Ca-EGTA	KProp
Relaxing	6.41	5.95	10			50.25
Preactivating	6.25	5.95	0.25	9.75		50.51
Activating	6.20	6.08			10	29.98