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Ablation of cardiac myosin binding protein-C disrupts the super-relaxed state of myosin in murine cardiomyocytes

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ABSTRACT

Cardiac myosin binding protein-C (cMyBP-C) is a structural and regulatory component of cardiac thick filaments. It is observed in electron micrographs as seven to nine transverse stripes in the central portion of each half of the A band. Its C-terminus binds tightly to the myosin rod and contributes to thick filament structure, while the N-terminus can bind both myosin S2 and actin, influencing their structure and function. Mutations in the *MYBPC3* gene (encoding cMyBP-C) are commonly associated with hypertrophic cardiomyopathy (HCM). In cardiac cells there exists a population of myosin heads in the super-relaxed (SRX) state, which are bound to the thick filament core with a highly inhibited ATPase activity. This report examines the role cMyBP-C plays in regulating the population of the SRX state of cardiac myosin by using an assay that measures single ATP turnover of myosin. We report a significant decrease in the proportion of myosin heads in the SRX state in homozygous cMyBP-C knockout mice, however heterozygous cMyBP-C knockout mice do not significantly differ from the wild type. A smaller, non-significant decrease is observed when thoracic aortic constriction is used to induce cardiac hypertrophy in mutation negative mice. These results support the proposal that cMyBP-C stabilises the thick filament and that the loss of cMyBP-C results in an untethering of myosin heads. This results in an increased myosin ATP turnover, further consolidating the relationship between thick filament structure and the myosin ATPase.

1. Introduction

Hypertrophic cardiomyopathy (HCM) is a disease of the heart muscle with a prevalence of at least 1 in 500 [1]. It is one of the most commonly inherited cardiac disorders [2], and the most common cause of sudden cardiac death in young, apparently healthy individuals [3]. Clinically, it is usually characterised by asymmetric thickening of the left ventricle (LV) [4] and while systolic function is normally preserved, it is often associated with significant diastolic dysfunction [4]. Mutations in sarcomeric genes are the most common cause of HCM [5] and mutations in the genes encoding myosin heavy chain (*MYH7*) and cardiac myosin binding protein-C (cMyBP-C encoded by *MYBPC3*) are the most frequent.

The discovery that mutations in *MYBPC3* cause HCM [6,7] led to a wave of research focused on understanding how these mutations result in the pathology observed in patients with HCM (for a review, see [8]). Homozygous *Mybpc3* knockout (cMyBP-C$^{(-/-)}$) mice do not express cMyBP-C and exhibit significant cardiac hypertrophy compared to both wild type (WT) and heterozygous knockout (cMyBP-C$^{(+/-)}$) littermates [9] and together they have provided valuable insights into the mechanism that cMyBP-C plays in cardiac muscle regulation and disease. Although the cMyBP-C$^{(-/-)}$ phenotype recapitulates aspects of disease in patients with *MYBPC3* mutations (which are most commonly heterozygous), the cMyBP-C$^{(+/-)}$ mice do not display the disease phenotype. It remains unclear whether *MYBPC3* mutations in human patients result in a "poison peptide" effect or rather display haploinsufficiency, with reports supporting both possibilities [10–12]. It is possible that both are relevant and that the phenotype depends on the specific mutation of *MYBPC3*.

The super-relaxed (SRX) state of myosin is a relatively new discovery in the field of muscle biochemistry (for reviews see [13,14]). It is characterised by a strong inhibition of myosin ATPase activity and has

Abbreviations: SRX, super-relaxed state; DRX, disordered-relaxed state; cMyBP-C, cardiac myosin binding protein-C; *MYBPC3*/*Mybpc3*, cardiac myosin binding protein-C gene in human/mouse; HCM, hypertrophic cardiomyopathy; MOPS, 3-(*N*-morpholino) propanesulfonic acid; DTT, dithiothreitol; BDM, 2,3-butanedione monoxime; mATP, 2′-(or-3′)–O–(*N*-methylanthraniloyl) adenosine 5-triphosphate.

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been observed in rabbit, mouse, and tarantula skeletal muscle as well as rabbit cardiac muscle [15–18].

It is believed that myosin heads in the SRX state are aligned along the core of the thick filament, as seen in electron micrographs of isolated thick filaments from a range of striated muscles [19–24]. Three-dimensional reconstructions have revealed multiple specific interactions between neighbouring myosin heads, termed the "interacting heads motif" (for a detailed review, see [25]). Briefly, this motif inhibits the ATPase activity of myosin heads by blocking the ATP-binding site of an adjacent head (free head) and inhibiting the other head from interaction with actin (blocked head). This contrasts to the other state of relaxed myosin, which we refer to as the disordered relaxed (DRX) state. In the DRX state, myosin heads project azimuthally from the thick filament and have an ATPase activity up to ten times faster than those in the SRX state [26].

Our recent review [14] suggested that the SRX state of cardiac myosin may be reduced in mice lacking cMyBP-C because cMyBP-C binds to myosin near the S1/S2 junction and is thus in a position that could influence the interacting heads motif. This hypothesis is supported by published structural data using these mice that shows a disordering of myosin heads in the thick filament [22,27,28]. In this report we directly test the hypothesis that cMyBP-C affects the SRX state using cMyBP-C$^{(-/-)}$ and cMyBP-C$^{(+/-)}$ mouse models as well as wild type mice and a mutation negative mouse model of cardiac hypertrophy. Results show that the loss of cMyBP-C reduces the SRX state of cardiac myosin. Recently, it was shown that estradiol deficiency in ovariectomised mice disrupts the SRX state of skeletal muscle [15], however this is the first known report of alterations to the SRX state in a model of cardiac disease.

2. Methods

2.1. Animal models

Care and handling of all cMyBP-C knockout and littermate control mice was performed according to protocols approved by the Institutional Animal Care and Use Committee (IACUC) of the University of California, Davis, Davis, CA. Heterozygous (cMyBP-C$^{(+/-)}$) and homozygous (cMyBP-C$^{(-/-)}$) knockout mice were generated by targeted replacement of exons 3 to 10 encoding for *Mybpc3* as previously described [9]. These mice, as well as wild type (WT) littermates were anesthetized using isoflurane and euthanased by cervical dislocation. Hearts were excised and weighed as previously described [29]. LV samples were dissected and frozen immediately in liquid nitrogen and shipped to The University of Sydney on dry ice. LV samples were stored in liquid nitrogen at −196 °C until used.

Experimental hypertension was induced by transverse aortic constriction (TAC) in mice under AERC approval (#11/25) at the Victor Chang Cardiac Research Institute. Briefly, LV hypertrophy was induced in 12 week old male C57BL/6J mice by permanent narrowing of the transverse aorta to the diameter of a 27 G needle, as described previously [30], except a 27 G needle was used to create a narrower lumen. Mice displayed significant cardiac hypertrophy after seven days, without decompensation, and were euthanased by cervical dislocation prior to the rapid excision of their hearts, which were frozen for later use. In this report we used six mice each from the WT, cMyBP-C$^{(+/-)}$, and cMyBP-C$^{(-/-)}$ groups, and three mice from the TAC group, with three to five technical repeats per heart.

2.2. SRX solutions

Skinning buffer: NaCl, 100 mM; $MgCl_2$, 8 mM; EGTA, 5 mM; K_2HPO_4, 5 mM; KH_2PO_4, 5 mM; NaN_3, 3 mM; ATP, 5 mM; DTT, 1 mM; BDM, 20 mM; Triton-X 100, 0.1%, pH 7.0.

Glycerinating solution: K acetate, 120 mM; Mg acetate, 5 mM; K_2HPO_4, 2.5 mM; KH_2PO_4, 2.5 mM; MOPS, 50 mM; ATP, 5 mM; BDM, 20 mM; DTT, 2 mM; glycerol, 50% (v/v), pH 6.8.

Rigor buffer: K acetate, 120 mM; Mg acetate, 5 mM; K_2HPO_4, 2.5 mM; KH_2PO_4, 2.5; MOPS, 50; DTT, 2 mM (added fresh), pH 6.8.

mATP buffer: Rigor buffer + 250 μM mATP.

ATP chase buffer: K acetate, 120 mM; Mg acetate, 5 mM; K_2HPO_4, 2.5 mM; KH_2PO_4, 2.5 mM; ATP, 4 mM; MOPS, 50; DTT, 2 mM (added fresh), pH 6.8.

2.3. Preparation of glycerinated cardiac muscle fibres

Glycerinated muscle fibres were prepared as reported previously, with minor modifications [16]. Cryopreserved LV was fractured under liquid nitrogen in a mortar and pestle. A ~10 mg piece was immersed in skinning solution on ice. This was agitated on ice for 6 h on an orbital shaker with 3 changes of solution. Skinning solution was then exchanged for glycerinating solution and left overnight (~15 h) on ice to equilibrate. The glycerinating solution was then refreshed and the glycerinated muscle stored at −20 °C for use within 1 week.

2.4. SRX experiments

Small bundles of skinned cardiac muscle fibres (<90 μm in diameter) were dissected in glycerinating solution at 4 °C and immobilised on a glass coverslip using two layers of double-sided tape. These coverslips were then placed fibre side down onto a pre-cooled glass slide, creating a flow chamber that allowed rapid flow of buffers around the fibre. Fibres were stored in glycerinating solution on ice and imaged within a few hours. Prior to imaging, fibres were rinsed in rigor buffer to remove ATP, BDM, and glycerol. Rigor buffer was refreshed several times over 5 min to ensure complete exchange of rigor buffer. Fibres were then incubated in the fluorescent mATP buffer for 5 min prior to imaging. Fibres were imaged at 22 °C using a Nikon Ni-E upright epifluorescence microscope with a 20X Plan Apo objective lens (0.75 NA). Fibres were located using bright-field differential interference contrast (DIC) to avoid photobleaching of the mATP probe. Fibres were then excited at 395 nm (DAPI setting) at 10% laser power and 1.5× gain and an exposure time of 20 ms. Images were acquired on a Nikon DS-Qi2 camera at 5 s intervals for 600 s for a total exposure time of 2.42 s. After 60 s, the mATP buffer was flushed with ATP buffer and the decay in fluorescence intensity was recorded. Twenty seconds continuous exposure to the fibre resulted in ~3% decrease in fluorescence intensity, demonstrating that photobleaching of the mATP probe was negligible over the course of the experiment (data not shown).

2.5. SRX analysis

During DIC imaging, two to three rectangular regions of interest (roughly 15 μm × 40 μm) were drawn over randomly selected areas of the muscle fibre as well as over two areas next to the fibre to measure the fluorescence intensity of the fibre and background, respectively. The mean background fluorescence intensity was subtracted from the average of the fibre fluorescence intensity. Each time point was divided by the fluorescence intensity of the final mATP image before washout (t = 0). The resulting data were exported to Prism 6.0 (GraphPad Software, Inc.). The decay in fluorescence intensity was then fit using a user-defined two-state exponential:

$$I = 1 - P_1\left(1 - \exp\left(-t/T_1\right)\right) - P_2\left(1 - \exp\left(-t/T_2\right)\right)$$

where: I is fluorescence intensity, P_1 and P_2 are the initial proportion of fluorescence for the two states, and T_1 and T_2 are the time constants for the lifetime of these states. P1 and T1 represent the initial rapid decay in fluorescence intensity, which comprises myosin in the DRX state and the release of non-specifically bound mATP. P2 and T2 are representative of the subsequent the slow decrease in fluorescence intensity due to myosin in the SRX state.