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Altered myocyte contractility and calcium homeostasis in alpha-myosin heavy chain point mutations linked to familial dilated cardiomyopathy

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ABSTRACT

Mutations in the human cardiac motor protein beta-myosin heavy chain (βMHC) have been long recognized as a cause of familial hypertrophic cardiomyopathy. Recently, mutations (P830L and A1004S) in the less abundant but faster isoform alpha-myosin heavy chain (αMHC) have been linked to dilated cardiomyopathy (DCM). In this study, we sought to determine the cellular contractile phenotype associated with these point mutations. Ventricular myocytes were isolated from 2 month male Sprague Dawley rats. Cells were cultured in M199 media and infected with recombinant adenovirus containing the P830L or the A1004S mutant human αMHC at a MOI of 500 for 18 h. Uninfected cells (UI), human βMHC (MOI 500, 18 h), and human αMHC (MOI 500, 18 h) were used as controls. Cells were loaded with fura-2 (1 μM, 15 min) after 48 h. Sarcomere shortening and calcium transients were recorded in CO_2 buffered M199 media (36°±1 C) with and without 10 nM isoproterenol (Iso). The A1004S mutation resulted in decreased peak sarcomere shortening while P830L demonstrated near normal shortening kinetics at baseline. In the presence of Iso, the A1004S sarcomere shortening was identical to the βMHC shortening while the P830L was identical to the αMHC control. All experimental groups had identical calcium transients. Despite a shared association with DCM, the P830L and A1004S αMHC mutations alter myocyte contractility in completely different ways while at the same preserving peak intracellular calcium.

1. Introduction

Cardiomyopathy is a primary disease of heart muscle and is the most common cause of heart failure leading to cardiac transplantation [1]. Cardiomyopathies are usually classified based upon anatomic morphology and physiology, and the two most common forms are hypertrophic and dilated cardiomyopathy (HCM, DCM). DCM, which has a prevalence of 1 in 2500 worldwide, is characterized by left ventricular enlargement and systolic dysfunction leading to the clinical manifestations of heart failure, arrhythmias, and thromboembolic disease [2]. The pathogenesis of cardiomyopathy is poorly understood, but a genetic etiology has been firmly established in families with hereditary cardiomyopathies [3]. Familial DCM (representing 20–50% of all DCM) has been linked to mutations in over 40 genes and is usually transmitted in a dominant negative fashion [4]. Familial DCM usually manifests with adult age of onset and variable penetrance [4]. Recently mutations in the MYH6 gene encoding αMHC have been identified in familial cases of DCM [5–7].

Myosin is the molecular motor protein which drives muscle contraction in the heart. Myosin is a hexameric protein complex which is composed of two heavy chains and four light chains. An ATP-dependent motor domain is located in each heavy chain and the light chains perform regulatory roles. Myosin thick filaments interdigitate with actin thin filaments in a near-crystalline array in the cardiac sarcomere. The interaction between myosin and actin is regulated in a calcium-dependent fashion by the troponin-

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tropomyosin complex [8,9]. In the mammalian heart, there are two heavy chain isoforms, alpha-myosin heavy chain (αMHC) which has a fast ATPase activity and beta-myosin heavy chain (βMHC) which is 2–3 times slower [10,11]. Healthy humans express a predominance of the slow isoform βMHC in the cardiac ventricles, and αMHC represents only 10% of the total [11,12]. This small amount of α-MHC expression has a disproportionately large impact on cardiac myocyte contractile power production [13]. Thus, it is likely that mutations of α-MHC may have functional consequences on overall cardiac performance in patients.

We have focused on two such point mutations, A1004S and P830L which are associated with adult onset DCM. The phenotype of A1004S is severe progressive ventricular dilation and impaired systolic function, while P830L is associated with dilation and cardiac dysrhythmias [5]. Both are associated with a poor prognosis [6]. Because of the complex nature of their corresponding phenotypes, it is very difficult to sort out the primary effects of the mutations versus the compensatory and secondary effects that occur during the pathogenesis in vivo [14]. Therefore, we studied the primary cellular phenotype of these mutations in adult rat cardiac myocytes using highly efficient recombinant adenoviral gene delivery.

2. Methods

2.1. αMHC cloning and mutagenesis

The cDNA for human α-MHC measures 5820 bp. The gene was cloned from a human heart cDNA library and a C-terminal FLAG epitope tag was engineered using standard PCR techniques (Clontech, Mountain View, CA). Point mutations were generated using a QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). DNA sequencing of the entire constructs were used to validate the P830L and A1004S mutants.

2.2. Adenoviral vector production and expression analysis

All constructs were cloned into E1A/E3-deleted and replication-defective adenoviral vectors using the AdMax Ad5 serotype recombinant vector system. In order to ensure high level of protein expression, the mutant constructs were placed under the control of the strong cytomegalovirus (CMV) promoter. This approach has been used effectively to transduce ventricular myocytes [15,16]. Previously well characterized human wild-type αMHC and βMHC viruses were used as controls for all experiments [15,16].

2.3. Ventricular myocyte isolation, primary culture, and gene transfer

Rat ventricular cardiac myocytes were isolated and cultured as described previously [15,16]. All procedures were performed in strict adherence to the Institutional Animal Care and Use Committee policies under veterinary supervision. Cardiac myocytes were plated on laminin coated glass coverslips at a density of 2×10^4 per coverslip. Cardiac myocytes were first cultured in M199 culture media (M199 (Sigma) + 3.07 g Glutathione (Sigma) + 0.2 g Bovine Serum Albumin (Fisher Scientific) pH 7.4 at 37 °C) + P/S (Gibco™) + 5% FBS (Gibco™) for 2 h to facilitate attachment. Subsequently, the cells were cultured in M199 culture media + P/S and treated with AdMYH7, AdMYH6, AdMYH6 P830L, and AdMYH6 A1004S at a multiplicity of infection of 500 for 18 h. Then, the virus media was removed and cardiac myocytes were cultured for an additional 24 h before recording. All recordings took place 24–36 h after the virus media was removed.

2.4. Expression and localization of αMHC in the adult cardiac myocyte

The unique FLAG tag epitope was used to detect mutant myosin expression and incorporation into the sarcomere. Twenty-four hours after virus media removal, Laemmli sample buffer was used to collect whole cell homogenates from permeabilized and non-permeabilized cells which were subsequently separated by electrophoresis and transferred to nitrocellulose membranes for western blotting. Quantification was performed using Infrared, IRDye®-labeled secondary antibodies, Odyssey CLx scanner, and corresponding software (Li-Cor Corporate, Lincoln, NE).

Confocal Imaging was performed using a Nikon A1 confocal microscope system (Nikon Instruments Coporation, Melville, NY. Myocytes were first fixed in 4% paraformaldehyde 24 h after virus media was removed. Standard reagents and techniques were used to detect actin, the epitope tag, and the nucleus as previously described [15].

2.5. Western blotting for stoichiometric replacement of endogenous myosin

Laemmli sample buffer was used to collect whole cell homogenates from cardiac myocytes cultured on glass coverslips for 24 h, 48 h, and 72 h at 37 °C in a tissue culture incubator. Transduction times were the same for all groups and described in detail in Section 2.3. Proteins were subsequently separated by electrophoresis and transferred to nitrocellulose membranes for western blotting. All westerns were performed as previously described using antibodies against β-MyHC (ATCC, 1:1000), Total Myosin (MF 20, Developmental Studies Hybridoma Bank, 1:1000), α-actinin (Sigma, 1:2500), and M2 FLAG (Sigma 1:1000) [15].

2.6. Contractility and calcium measurements in single cardiac myocytes

Sarcomere shortening and intracellular calcium transients were measured using an IonOptix recording system 24–36 h after the virus media was removed.

Before recording, cells were loaded with 1 μM Fura-2 in M199 culture media + P/S for 15 min in a 37 °C tissue culture incubator. Afterwards, the cells were washed with M199 culture media + P/S and allowed a 20-min de-esterification period at room temperature before recordings were started. All cover slips were used within 60 min of loading.

After the de-esterification period, coverslips were placed in a FHD rapid change stimulation chamber and perfused with CO2 buffered M199 culture media + P/S at 36 ± 1 °C. Temperature was maintained using a mTCII micro-Temperature Controller. An inverted Motic Fluorescence Microscope and a 40× Olympus UApo/340 objective was used to collect single cell data.

Fura-2 excitation wavelengths, 360-nm and 380 nm, were generated using an IonOptix HyperSwitch. An Ion Optix Photo Multiplier Tube 400 sub system, (180–600 nm, 400 nm peak sensitivity) was used to record emitted fluorescence (510 nm).

A high speed digital camera (MyoCam-S, 240 Hz) was used to record sarcomere shortening. Units/pixels were determined by calibrating the system with a micrometer. IonOptix's sarcomere spacing algorithm was used to record sarcomere shortening. Because the sarcomere is highly periodic, the algorithm uses the Fast Fourier Transform (FFT) to determine the average sarcomere spacing of the region of interest (ROI) in one or more lines of a video image. Myocytes with a resting sarcomere length less than 1.75 μM were excluded.

Cells were stimulated by applying field stimulation (40 V, 1 Hz)