Journal of Molecular and Cellular Cardiology 72 (2014) 186–195

Contents lists available at ScienceDirect

Journal of Molecular and Cellular Cardiology

journal homepage: www.elsevier.com/locate/yjmcc

Original article

Remodeling of the sarcomeric cytoskeleton in cardiac ventricular myocytes during heart failure and after cardiac resynchronization therapy

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ARTICLE INFO

Article history:
Received 11 February 2014
Received in revised form 13 March 2014
Accepted 14 March 2014
Available online 20 March 2014

Keywords:
Heart failure
Cardiac resynchronization therapy
Remodeling
Cardiac recovery
Alpha actinin

ABSTRACT

Sarcomeres are the basic contractile units of cardiac myocytes. Recent studies demonstrated remodeling of sarcomeric proteins in several diseases, including genetic defects and heart failure. Here we investigated remodeling of sarcomeric α-actinin in two models of heart failure, synchronous (SHF) and dyssynchronous heart failure (DHF), as well as a model of cardiac resynchronization therapy (CRT). We applied three-dimensional confocal microscopy and quantitative methods of image analysis to study isolated cells from our animal models. 3D Fourier analysis revealed a decrease of the spatial regularity of the α-actinin distribution in both SHF and DHF versus control cells. The spatial regularity of α-actinin in DHF cells was reduced when compared with SHF cells. The spatial regularity of α-actinin was partially restored after CRT. We found longitudinal depositions of α-actinin in SHF, DHF and CRT cells. These depositions spanned adjacent Z-disks and exhibited a lower density of α-actinin than in the Z-disk. Differences in the occurrence of depositions between the SHF, CRT and DHF models versus control were significant. Also, CRT cells exhibited a higher occurrence of depositions versus SHF, but not DHF cells. Other sarcomeric proteins did not accumulate in the depositions to the same extent as α-actinin. We did not find differences in the expression of α-actinin protein and its encoding gene in our animal models. In summary, our studies indicate that HF is associated with two different types of remodeling of α-actinin and only one of those was reversed after CRT. We suggest that these results can guide us to an understanding of remodeling of structures and function associated with sarcomeres.

1. Introduction

The cytoskeleton is a complex intracellular network of proteins essential for determining the shape and mechanical properties of cells [1]. It also helps to coordinate the function of subcellular proteins in all cell types. These functions range from anchoring cellular organelles such as the Golgi apparatus, nuclei and mitochondria to transmission of extracellular signals and coordinating contraction [1,2]. Cytoskeletal proteins form the sarcomeres, which are the basic contractile units of striated muscle cells. Within each sarcomere is a complex arrangement of proteins [2–4]. Each sarcomere is bounded by the Z-disk [5]. The Z-disk is crucial for maintaining sarcomeric structure and function. The main component of the Z-disk is the protein α-actinin, even though it accounts for less than 20% of Z-disk weight [4]. Alpha-actinin is an anti-parallel homodimer that anchors the actin filaments, which are essential for contraction in myocytes. It was initially believed that α-actinin solely provided an actin binding site [6]. However, subsequent studies demonstrated that α-actinin links to several transmembrane receptors, regulatory proteins, adherens junctions, focal adhesion sites and stress fibers [6]. Further studies revealed that α-actinin plays a pivotal role in the assembly of sarcomeres and the regular arrangement of myofilaments [7,8]. The studies suggest that sarcomere assembly is initiated by small Z-bodies comprising complexes of α-actinin and associated proteins. Subsequently, the Z-bodies expand, fuse and align in Z-bands. Similar as other proteins in the Z-disk α-actinin exhibits a surprisingly dynamic exchange with the cytosolic pool [8]. Exchange rates were larger in Z-bodies than Z-disks indicating that molecular interactions increase the stability of Z-disks. Four different isoforms of α-

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http://dx.doi.org/10.1016/j.yjmcc.2014.03.012
0022-2828/

actinin exist. The genes ACTN-1 and ACTN-4 encode α-actinin isoforms that are expressed in non-muscle cells, where these isoforms of α-actinin contribute to the actin cytoskeleton. ACTN-2 and ACTN-3 encode isoforms specific to the Z-disks of sarcomeres found in striated muscle fibers [4,9–11], with α-actinin-2 being the only cardiac specific isoform [4].

Remodeling of sarcomeric proteins in cardiac disease has been implicated in reduced ventricular function [2]. In particular, it has been suggested that the transition from hypertrophy to heart failure (HF) occurs in two consecutive stages. The first stage is reversible and involves an accumulation of cytoskeletal proteins to counteract the increased strain imposed on the myocardium. The latter stage becomes irreversible and is characterized by a loss of contractile filaments and crucial sarcomeric proteins, including α-actinin, titin, and myomesin. Several cardiac diseases have been associated with remodeling of α-actinin and the Z-disk [9–12]. Melo et al. demonstrated that *Trypanosoma cruzi* in mouse myocytes caused α-actinin distributions to lose their periodic structure and to localize to focal adhesion sites [11]. Hein et al. found depositions of α-actinin-1 in failing myocardium [10], while Oxford et al. showed an accumulation of electron dense material around Z-lines in canines with arrhythmogenic cardiomyopathy. Cardiac disorders have also been linked to mutations in the gene responsible for α-actinin-2, specifically dilated cardiomyopathy [13] and hypertrophic cardiomyopathy [9].

Here we investigated remodeling of α-actinin in two models of HF, synchronous (SHF) and dyssynchronous heart failure (DHF), as well as in a model of cardiac resynchronization therapy (CRT). Approximately 40% of patients suffering from HF develop delays of ventricular electrical activation that result in a dyssynchronous mechanical contraction of the ventricles. The electrical dyssynchrony results in a widened QRS interval, which has been shown to be an independent predictor of mortality and sudden cardiac death [14]. CRT is an established clinical therapy to treat DHF. CRT resynchronizes ventricular mechanical and electrical activity via biventricular pacing and has been proven effective at improving quality of life and reducing mortality in about 55% of patients [15]. A number of studies showed that CRT is associated with restoration of cardiac structure and function, for instance, the transverse tubular system and excitation–contraction coupling [16] as well as the electrophysiological and contractile properties of myocytes [17,18]. However, little is known about the reorganization of sarcomeric structures and associated protein distributions in DHF and CRT.

Our hypothesis is that DHF associated remodeling of sarcomeric organization is reversed after CRT. We used α-actinin as a marker of sarcomeric organization. We applied high-resolution three-dimensional confocal microscopy to image α-actinin distributions in ventricular tissues and cells. Analyses of image stacks allowed us to provide quantitative data on the structural arrangement of α-actinin and its remodeling. Our studies revealed alterations of the spatial regularity of α-actinin. We applied Fourier analysis to characterize remodeling of the spatial arrangement of α-actinin. Also, our studies revealed longitudinal depositions of α-actinin in HF. Using methods of pattern detection we quantified their occurrences. For further insights into the composition of the longitudinal depositions we investigated colocalization of α-actinin with sarcomeric proteins and measured the density of α-actinin based on super-resolution microscopy. To shed light on α-actinin expression in our animal models we performed gene expression analyses and Western blotting.

2. Methods

2.1. Animal model, tissue and isolated cell preparation

All procedures involving the handling of animals were approved by the Animal Care and Use Committees of the Johns Hopkins University and the University of Utah. Protocols complied with the published *Guide for the Use and Care of Laboratory* Animals published by the National Institutes of Health.

The applied animal models have been described previously [16,19–21]. In previous studies, successful implementation of the DHF and CRT models was confirmed by increased and normalized QRS duration, respectively (Table S1) [17]. In brief, adult male mongrel dogs were used as control and models of SHF, DHF and CRT. SHF and DHF animals underwent right atrial pacing for 6 weeks. DHF was caused by left bundle-branch radiofrequency ablation. CRT animals underwent left bundle-branch radiofrequency ablation and 3 weeks of right atrial pacing followed by 3 weeks of biventricular pacing. The pacing rate for SHF, DHF and CRT animals was 180 to 200 bpm. Hemodynamic data were measured at the time of explantation (Table S2).

Lateral and anterior tissue samples from left ventricular subepicardial myocardium were taken shortly after explantation of the heart. Tissue was fixed with paraformaldehyde for confocal imaging or flash frozen in liquid nitrogen for western blot analysis. Cardiac myocytes were isolated enzymatically via retrograde Langendorff perfusion [17]. Myocytes were sampled from the anterior and lateral mid-epi myocardium of the left ventricle.

2.2. Immunohistochemistry

The sarcolemma of isolated myocytes was labeled using wheat germ agglutinin (WGA) conjugated to Alexa Fluor-555 (Invitrogen, Carlsbad, CA, USA). After WGA labeling, cells were fixed and prepared for immunolabeling as previously described [16]. For studies of the subcellular distribution of α-actinin we incubated myocytes with anti-α-actinin (ab9465, clone# EA-53, Abcam, San Francisco, CA) diluted 1:200 in phosphate buffered saline (PBS) solution containing 2% bovine serum albumin, 2% normal goat serum and 0.05% Triton-X 100 overnight at 4 °C. We then applied a secondary goat anti-mouse IgG (H + L) antibody attached to Alexa Fluor-488 (Invitrogen) for confocal microscopy or Alexa Fluor-647 (Invitrogen) for super-resolution imaging. Incubation with either secondary antibody was performed for 1 h at room temperature. For colocalization analyses of α-actinin with proteins of the sarcomeric cytoskeleton we labeled for titin (anti-titin-T12 provided by Dr. Elizabeth Ehler, King's College, London), filamentous actin (A22284, Invitrogen) and heavy chain myosin (ab15, Abcam). Each colocalization study involved staining for α-actinin and one other sarcomeric protein at a time. For dual labeling of α-actinin and titin or heavy chain myosin, we used a polyclonal rabbit anti-sarcomeric α-actinin antibody (ab137346, Abcam) instead of the EA-53 mouse monoclonal antibody. These antibodies were diluted 1:100 and incubated overnight at 4 °C using the same PBS incubation solution as above. The secondary antibodies consisted of either a secondary goat anti-mouse IgG (H + L) antibody attached to Alexa Fluor-488 or 633 (Invitrogen) or goat anti-rabbit IgG (H + L) antibody attached to Alexa Fluor-488 or 633 (Invitrogen). Incubation with either secondary antibody was performed for 1 h at room temperature.

Tissue preparations were fixed in PBS containing 4% paraformaldehyde for 4 h at room temperature. The preparations were immersed in distilled water containing 30% sucrose for 2–3 d. The tissue was then flash-frozen using tissue-freezing medium (Triangle Biomedical Sciences, Durham, North Carolina, USA) at -20 °C and sectioned into 80 μm slices using a Leica CM1850 (Leica Biosystems, Wetzlar, Germany) cryostat. Afterwards tissue sections were stored in PBS, labeled with WGA and for α-actinin as described for isolated myocytes except with the incubation time for the secondary antibody prolonged to overnight at room temperature.

2.3. Confocal imaging and image preprocessing

Imaging was performed using a Zeiss LSM 5 Live Duo (Carl Zeiss, Jena, Germany) confocal microscope equipped with a 63×/1.4 Numerical Aperture oil immersion objective. Tissue sections were imaged with a size