Journal of Molecular and Cellular Cardiology 60 (2013) 151–160

Contents lists available at SciVerse ScienceDirect

Journal of Molecular and Cellular Cardiology

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

Original article

Altered regional cardiac wall mechanics are associated with differential cardiomyocyte calcium handling due to nebulette mutations in preclinical inherited dilated cardiomyopathy ☆

K. Maiellaro-Rafferty [a], J.P. Wansapura [b], U. Mendsaikhan [a], H. Osinska [a], J.F. James [a], M.D. Taylor [a], J. Robbins [a], E.G. Kranias [c], J.A. Towbin [a], E. Purevjav [a,*]

[a] The Heart Institute, Department of Pediatrics, Cincinnati Children's Hospital Medical Center, Cincinnati, OH, USA
[b] Department of Radiology, Imaging Research Center, Cincinnati Children's Hospital Medical Center, Cincinnati, OH, USA
[c] Department of Pharmacology and Cell Biophysics, University of Cincinnati, College of Medicine, Cincinnati, OH, USA

ARTICLE INFO

Article history:
Received 22 November 2012
Received in revised form 19 April 2013
Accepted 20 April 2013
Available online 28 April 2013

Keywords:
Nebulette
Dilated cardiomyopathy
Sarcomere
Cardiac magnetic resonance imaging
Connexin43
Desmin

ABSTRACT

Nebulette (NEBL) is a sarcomeric Z-disk protein involved in mechanosensing and force generation *via* its interaction with actin and tropomyosin–troponin complex. Genetic abnormalities in *NEBL* lead to dilated cardiomyopathy (DCM) in humans and animal models. The objectives of this study are to determine the earliest preclinical mechanical changes in the myocardium and define underlying molecular mechanisms by which *NEBL* mutations lead to cardiac dysfunction. We examined cardiac function in 3-month-old non-transgenic (non-Tg) and transgenic (Tg) mice (WT-Tg, G202R-Tg, A592E-Tg) by cardiac magnetic resonance (CMR) imaging. Contractility and calcium transients were measured in isolated cardiomyocytes. A592E-Tg mice exhibited enhanced *in vivo* twist and untwisting rate compared to control groups. *Ex vivo* analysis of A592E-Tg cardiomyocytes showed blunted calcium decay response to isoproterenol. CMR imaging of G202R-Tg mice demonstrated reduced torsion compared to non-Tg and WT-Tg, but conserved twist and untwisting rate after correcting for geometric changes. *Ex vivo* analysis of G202R-Tg cardiomyocytes showed elevated calcium decay at baseline and a conserved contractile response to isoproterenol stress. Protein analysis showed decreased α-actinin and connexin43, and increased cardiac troponin I phosphorylation at baseline in G202R-Tg, providing a molecular mechanism for enhanced *ex vivo* calcium decay. Ultrastructurally, G202R-Tg cardiomyocytes exhibited increased I-band and sarcomere length, desmosomal separation, and enlarged t-tubules. A592E-Tg cardiomyocytes also showed abnormal ultrastructural changes and desmin downregulation. This study showed distinct effects of *NEBL* mutations on sarcomere ultrastructure, cellular contractile function, and calcium homeostasis in preclinical DCM *in vivo*. We suggest that these abnormalities correlate with detectable myocardial wall motion patterns.

1. Introduction

Approximately 30–50% of patients with various forms of cardiomyopathy and a large "at risk" population consisting of healthy relatives of patients with cardiomyopathy exhibit inherited mutations in genes encoding cardiomyocyte sarcomeric, Z-disk, desmosomal, and cytoskeletal proteins. Most pediatric (66%) dilated cardiomyopathy (DCM) cases are idiopathic [1] and individuals carrying sarcomeric gene mutations may not present clinical signs of cardiomyopathy until adulthood, supporting a temporal mechanism by which chronically altered mechanosensing and mechanotransduction lead to cardiomyopathy. Currently, mutation identification *via* genetic screening is not a reliable predictor of the cardiomyopathy phenotype or severity. Different cardiomyopathy clinical phenotypes can be observed in individuals or family members carrying the same gene mutations, particularly when the genetic abnormality is in a gene encoding a multifunctional protein. Moreover, cardiac function and symptoms of congestive heart failure (CHF) at the time of DCM diagnosis can be prognostic for death and cardiac transplantation rates in children. An increase in the risk of death and cardiac transplantation by 2.2-fold was evident in DCM cases with moderate left ventricular (LV) systolic dysfunction as compared to patients with normal LV systolic function, suggesting that recognition and diagnosis of DCM at

☆ Author Contributions: K.M-R maintained all animals, acquired the *ex vivo* and protein expression data, analyzed CMR images, and prepared all figures and text for this manuscript. JW acquired and analyzed CMR images. UM analyzed electron microscopy images, and performed immunohistochemical and protein analyses. HO acquired and analyzed the electron microscopy images. JJ performed echocardiography. MDT and JR edited this manuscript. EGK provided equipment and input on the cardiomyocyte contractile mechanics and calcium transient data and edited the manuscript. JAT and EP supervised all experimentation and edited this manuscript.

* Corresponding author at: MLC 7020, 3333 Burnet Avenue, Cincinnati, OH 45229, USA. Tel.: +1 5138032576.
E-mail address: Enkhsaikhan.Purevjav@cchmc.org (E. Purevjav).

0022-2828/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.yjmcc.2013.04.021

the preclinical or subclinical stages are important in terms of potentially impacting outcome [1]. In addition, approximately one third of the asymptomatic "at risk" population have serum anti-heart autoantibodies at baseline, one third has echocardiographic abnormalities, such as LV enlargement and depressed fractional shortening (FS), and the remainder of such relatives develop DCM [2]. This suggests that apparently healthy relatives may have latent, early, or undiagnosed disease. Delineation of "preclinical" alterations in cardiac function or defining useful biomarkers would be extremely useful in correlating early effects of genetic abnormalities to the development of a cardiomyopathy phenotype.

We and others have used cardiovascular magnetic resonance (CMR) imaging to detect occult alterations in regional myocardial wall motion in early manifestations of DCM and CHF [3]. Recent clinical echocardiography data show that early diastolic dysfunction, despite normal LV size and ejection fraction (EF), can be captured during the preclinical stage of DCM [4]. Thus, advanced cardiac imaging and computation of regional wall mechanics may open a window into our understanding of subclinical cardiomyopathic events, prediction of disease development and outcome.

Alterations in cardiomyocyte calcium regulation and morphology are linked to cardiac mechanical behavior and, upon detection, may expose preclinical events associated with DCM. Failing human myocardium expresses decreased calcium handling proteins and the proportion of calcium flux through intracellular and extracellular calcium stores is modified [5]. Mechanical stretch increases intracellular calcium in cardiomyocytes [6] and cardiac overload-induced hypertrophy is attenuated by calcium-dependent proteins, such as calcineurin and CaMKII [7]. Furthermore, thin filament calcium sensitivity is directly coupled to cardiomyocyte physiologic contractility [8]. Genetic variations in sarcomeric proteins may thus reveal themselves as changes in calcium signaling prior to presenting a detectable cardiomyopathy phenotype.

Nebulette (*NEBL*), which encodes a sarcomeric Z-disk protein, contributes to muscle force generation *via* its interaction with actin and cardiac tropomyosin/troponin (cTm/cTn) complex, as well as participating in force transmission of the cardiac myofibril *via* the Z-disk assembly [9]. Humans carrying either A592E-NEBL or G202R-NEBL mutation manifest DCM. Transgenic mice with cardiomyocyte-restricted expression of either human mutant (A592E-Tg and G202R-Tg, respectively) develop DCM at 6 months of age [10]. The G202R-NEBL mutation is localized in the nebulin-repeat region that extends into the I-band, while the A592E-NEBL mutation resides nearest the sarcomeric Z-disk (Fig. 1). In part due to their distinct locations, these two NEBL mutations are thought to lead to DCM by different mechanisms *via* mutation-induced alterations in protein–protein interactions.

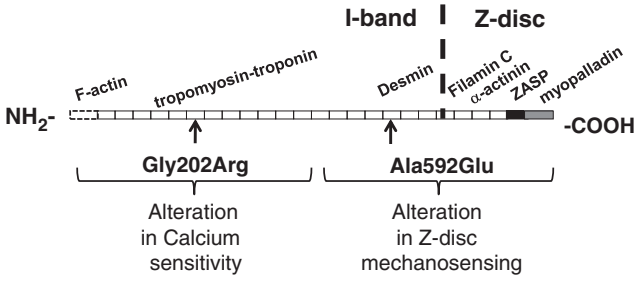

Fig. 1. Structure of NEBL protein and its interacting partners. The G202R mutation is located in the force-transducing nebulin-repeat modules at the N-terminal, while the A592E mutation is located in the C-terminal nebulin-repeat modules closest to the Z-disk. The functional domains are indicated as follows: dotted rectangle — F-actin binding domain; white rectangles — nebulin repeats; black — linker domain; and gray — SH3 domain. Interacting proteins are shown to the corresponding binding nebulin repeats and domains.

In this study, we hypothesized that G202R mutation affects the interaction of the NEBL protein with the cTm/cTn complex, and may lead to early mechanical dysfunction by destabilizing the calcium–cTn interaction, while the A592E mutation may alter Z-disk mechanosensing. To differentiate the earliest mutation-specific mechanistic basis of DCM, our objective was to correlate these molecular and cellular changes with occult subclinical alterations in cardiac function that could serve as diagnostic predictors for the DCM phenotype onset. The ultimate significance of this study is to advance the development of tools for improved prevention and clinical management of cardiac disease, particularly by identifying and treating the disease at its earliest stages. Our data show differential calcium-mediated mechanisms of cardiac function in A592E-Tg and G202R-Tg NEBL mutant hearts. Furthermore, our data suggest that CMR strain analysis of myocardial untwisting rate may serve as a sensitive functional indicator of cardiac dysfunction in the preclinical stage of murine DCM.

2. Methods

2.1. Animals

The transgenic mice with specific cardiomyocyte expression of mutant human, G202R-Tg and A592E-Tg, and NEBL develop DCM at 6 months after birth [10]. In this study, 3-month-old G202R-Tg and A592E-Tg mice and non-transgenic (non-Tg) littermates were investigated. To confirm expression of NEBL transgene at 3-month-old hearts as well as to exclude possible "unstable" cardiac transgene expression, Western blot analysis was performed prior to planned studies. As a control for transgene expression, age-matched mice with specific cardiomyocyte expression of wild-type human NEBL (WT-Tg) were used. All procedures were approved by the Cincinnati Children's Hospital Medical Center Institutional Animal Care and Use Committee and were in compliance with the standards for the *Care and Use of Laboratory Animals*, Institute of Laboratory Animal Resources, National Academy of Sciences, Bethesda, MD.

2.2. Functional assessment with echocardiography and CMR imaging

Cardiac function was assessed by echocardiography and CMR imaging. Mice were anesthetized with 1.5–2.5% isofluorane. Echocardiograms were obtained with a Vevo 770 High-Resolution *In Vivo* Micro-Imaging System and RMV 707B Scanhead (VisualSonics Inc.). Left ventricle (LV) dimensions, including end-diastolic and end-systolic dimensions (LVED and LVES, respectively), interventricular septal thickness in diastole and systole (IVSd and IVSs, respectively) and LV posterior wall thickness in diastole and systole (LVPWd and LVPWs, respectively) were measured directly. Fractional shortening (FS), EF and the LVPW to wall thickness ratios in systole and diastole were then calculated.

CMR was performed as previously described [11]. Tagged images were acquired in the basal and apical levels in the short axis plane. Tagged images were analyzed using HARP (Diagnosoft Plus, Diagnosoft Inc., CA, USA) to assess the ventricular rotation. Ventricular dimensions were calculated using Segment v1.x (http://segment.heiberg.se, E. Heiberg, In Proceedings of IEEE Computers in Cardiology 2005). LV torsion, ρ, was calculated as rotation in degrees (°) of the heart apex relative to the base and LV twist was calculated as torsion normalized by a geometric constant, as described previously [12]. Ventricular dimensions were calculated using Segment v1.x (http://segment.heiberg.se, E. Heiberg, In Proceedings of IEEE Computers in Cardiology 2005). LV twist was calculated as

$$\text{Geometric constant} = \left(r_{\text{apex}} + r_{\text{base}}\right)/2\,l \tag{1}$$

$$\text{Twist} = \rho \times \text{Geometric constant} \tag{2}$$