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Original article

Dissociation of mitochondrial from sarcoplasmic reticular stress in *Drosophila* cardiomyopathy induced by molecularly distinct mitochondrial fusion defects

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ABSTRACT

Mitochondrial dynamism (fusion and fission) is responsible for remodeling interconnected mitochondrial networks in some cell types. Adult cardiac myocytes lack mitochondrial networks, and their mitochondria are inherently "fragmented". Mitochondrial fusion/fission is so infrequent in cardiomyocytes as to not be observable under normal conditions, suggesting that mitochondrial dynamism may be dispensable in this cell type. However, we previously observed that cardiomyocyte-specific genetic suppression of mitochondrial fusion factors optic atrophy 1 (Opa1) and mitofusin/MARF evokes cardiomyopathy in *Drosophila* hearts. We posited that fusion-mediated remodeling of mitochondria may be critical for cardiac homeostasis, although never directly observed. Alternately, we considered that inner membrane Opa1 and outer membrane mitofusin/MARF might have other as-yet poorly described roles that affect mitochondrial and cardiac function. Here we compared heart tube function in three models of mitochondrial fragmentation in *Drosophila* cardiomyocytes: Drp1 expression, Opa1 RNAi, and mitofusin MARF RNA1. Mitochondrial fragmentation evoked by enhanced Drp1-mediated fission did not adversely impact heart tube function. In contrast, RNAi-mediated suppression of either Opa1 or mitofusin/MARF induced cardiac dysfunction associated with mitochondrial depolarization and ROS production. Inhibiting ROS by overexpressing superoxide dismutase (SOD) or suppressing ROMO1 prevented mitochondrial and heart tube dysfunction provoked by Opa1 RNAi, but not by mitofusin/MARF RNAi. In contrast, enhancing the ability of endoplasmic/sarcoplasmic reticulum to handle stress by expressing Xbp1 rescued the cardiomyopathy of mitofusin/MARF insufficiency without improving that caused by Opa1 deficiency. We conclude that decreased mitochondrial size is not inherently detrimental to cardiomyocytes. Rather, preservation of mitochondrial function by Opa1 located on the inner mitochondrial membrane, and prevention of ER stress by mitofusin/MARF located on the outer mitochondrial membrane, are central functions of these "mitochondrial fusion proteins".

1. Introduction

It is axiomatic that mitochondria are dynamic, meaning they exist as interconnected networks that constantly undergo structural remodeling. The problem is that mitochondria of cardiomyocytes in adult hearts appear structurally homogenous and static, i.e. hypo-dynamic [1]. Yet, accumulation of smaller organelles after genetic suppression of mitochondrial outer and inner membrane fusion proteins in the cardiomyocytes of *Drosophila* is compelling indirect evidence for homeostatic mitochondrial fusion, at least in fly hearts [2]. Likewise, naturally occurring and experimental cardiomyopathies linked to genetic defects in mitochondrial fusion proteins [3,4] indicate that mitochondrial fusion in cardiomyocytes, however infrequent, is essential to heart health. What is not understood are the roles played by different mitochondrial fusion factors in sustaining normal cardiac performance.

Mitochondrial fusion is a three-step process: Initially, two mitochondria are reversibly tethered, and then the outer membranes irreversibly fuse. Both of these events are mediated by outer membrane mitofusins (Mfn1 and Mfn2 in vertebrates and MARF in *Drosophila*). Subsequent to outer membrane fusion, the two inner mitochondrial membranes fuse, which is mediated by Opa1. The presence of two mitofusins that are functionally redundant for promoting organelle tethering and outer membrane fusion, but not for other mitofusin functions, complicates interpretation of cardiac-specific single and double Mfn2 gene deletion studies in mice [5]. For this reason, we interrogated mitochondrial dynamism in *Drosophila* heart tubes, employing conditional cardiomyocyte-specific expression of RNAi to suppress either inner membrane Opa1 or outer membrane mitofusin (i.e. MARF); we observe cardiac contractile dysfunction after interrupting mitochondrial fusion at either the inner or outer mitochondrial membrane [2,6]. Our recent findings suggest that cardiomyopathy provoked by mitofusin deficiency cannot primarily

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be attributed to impaired mitophagy because the cardiac phenotypes caused by *Drosophila* mitofusin- and Parkin-deficiency differ [7]. Indeed, suppressing mitochondrial fusion actually delayed heart tube dysfunction in mitophagy-defective fly hearts [7].

Mitofusins not only form molecular tethers between mitochondria, but mammalian Mfn2 can bridge cardiomyocyte mitochondria and adjacent sarcoplasmic reticulum (SR), thereby facilitating calcium signaling between these two calcium-rich organelles [8–10]. In *Drosophila* heart tubes MARF can perform this same function [11]. Because SR-mitochondrial calcium signaling is an essential component of the ER stress response [12], we posited that the cardiomyopathy caused by interrupting fusion at the step of outer mitochondrial membrane tethering (to SR or other mitochondria) and fusion by mitofusin/MARF could result from ER stress instead of, or in addition to, mitochondrial stress [13]. We further hypothesized that the cardiomyopathy induced by interrupting fusion at the step of inner mitochondrial membrane fusion/assembly (by Opa1) could evoke mitochondrial stress without ER stress, because Opa1 is not known to direct mitochondrial–ER/SR interactions [14]. If these notions are correct, then effective therapeutics will need to be appropriately targeted to the specific molecular lesion at the inner or outer membrane, rather than indiscriminately applied to any condition in which fusion-defective (so called "fragmented") mitochondria are observed.

2. Materials and methods

2.1. Drosophila strains

w1118 (#6326), *UAS-mitoGFP (#8442)*, UAS-SOD1 (#33605 and #24750), and UAS-SOD2 (#24494) were obtained from the Bloomington Stock Center. UAS-MARF RNAi, UAS-Opa1 RNAi, and UAS-Drp1 TG were provided by M. Guo (University of California, Los Angeles, CA) [15]. UAS-Romo1 RNAi (#v101353 and #v9224) were obtained from the Vienna Drosophila RNAi Center [16]. The strain expressing XBP1 (XBP1 ^{d08698}) was obtained from Exelixis collection at Harvard. UAS transgenes were expressed in *Drosophila* cardiomyocytes using the tincΔ4-Gal4 driver provided by Rolf Bodmer (Sanford-Burnham Medical Research Institute, La Jolla, CA) [17].

2.2. Drosophila heart function

Optical coherence tomography (OCT) heart tube images of two week adult flies were acquired using a Michelson Diagnostics (Maidstone, UK) EX 1301 OCT microscope as described previously [2,18]. Image J was used to analyze B-mode images to measure the internal chamber diameter at end-systole (ESD) and end diastole (EDD). % Fractional Shortening (FS) was calculated as (EDD − ESD / EDD).

2.3. Confocal microscopy

Fly heart tubes were dissected and mounted in haemolymph as described (11) for live confocal imaging. A Nikon Eclipse Ti confocal system or Carl-Zeiss LSM510-Meta Laser Scanning confocal Microscope with Plan Apo VC 60×/1.40 Oil objective and 4× digital zoom were used to image mitochondria in *Drosophila* cardiomyocytes expressing *Tinc* Δ4-Gal4 driven UAS-mitoGFP.

Mitochondrial dimensions were measured using Image J (19.5 pixels/um). Size distribution curves were generated by grouping the data into .25 μm^2 bins (range: 0 to 1.5 μm^2). Median mitochondrial area was determined by averaging ten different samples with 150–300 mitochondria per sample.

Tetramethylrhodamine ethyl ester (TMRE) fluorescence was used to assess mitochondrial membrane potential. Heart tubes were dissected and incubated in TMRE (250 nM) (Life Technologies) for 20 min, and then washed for 10 min in Hank's balanced salt solution (HBBS) prior to confocal imaging. The numbers of orange and green mitochondria were determined by manual counting from ten separate heart tube images with a sample of at least 300 mitochondria from each image. To assess cardiomyocyte mitochondrial ROS production, dissected heart tubes were incubated in MitoSOX (2.5 mM) (Life Technologies) in PBS for 20 min at 25 °C, washed for 10 min with PBS, and visualized by confocal fluorescent microscopy. The mitoSOX (red) to mitotracker (green) ratio was determined by comparing the red/green fluorescence intensity in the confocal images using Image J.

2.4. Genetic crosses for the generation of flies with various genotypes

1. *tincΔ4-Gal4 > MARF RNAi, SOD1*
   The tincΔ4-Gal4 females were crossed to the 2nd and 3rd balancer chromosome SM5 and TM3 males, and balanced F1 progeny self-crossed to generate tincΔ4-Gal4/tincΔ4-Gal4 with 2nd chromosome balancer SM5. Similarly UAS-SOD1 (#33605) was crossed to the 2nd and 3rd balancer chromosome SM5 and TM3 and balanced F1 progeny self-crossed to generate UAS-SOD1/UAS-SOD1 with 3rd chromosome balancer TM3. +/SM5; tincΔ4-Gal4/tincΔ4-Gal4 females were then crossed to UAS-SOD1/UAS-SOD1; +/TM3 males. Males with the genotype UAS-SOD1/SM5; tincΔ4-Gal4/TM3 were crossed to MARF RNAi/MARF RNAi females to generate female UAS-SOD1/+; tincΔ4-Gal4/MARF RNAi flies for studies.
2. *tincΔ4-Gal4 > MARF RNAi, SOD2*
   UAS-SOD2 (#24494) was crossed to the 2nd and 3rd balancer chromosome SM5 and TM3, and balanced F1 progeny self-crossed to generate UAS-SOD2/UAS-SOD2 with 3rd chromosome balancer TM3. +/SM5; tincΔ4-Gal4/tincΔ4-Gal4 females were crossed to UAS-SOD2/UAS-SOD2; +/TM3 males and resulting males with the genotype UAS-SOD2/SM5; tincΔ4-Gal4/TM3 crossed to MARF RNAi/MARF RNAi females to generate UAS-SOD2/+; tincΔ4-Gal4/MARF RNAi for studies.
3. *tincΔ4-Gal4 > Opa1 RNAi, SOD1*
   UAS-SOD1 (#33605) was crossed to the 2nd and 3rd balancer chromosome SM5 and TM3 and balanced F1 progeny self-crossed to generate UAS-SOD1/UAS-SOD1 with 3rd chromosome balancer TM3. +/SM5; tincΔ4-Gal4/tincΔ4-Gal4 females were crossed to UAS-SOD1/UAS-SOD1; +/TM3 males producing males with the genotype UAS-SOD1/SM5; tincΔ4-Gal4/TM3 that were crossed to Opa1 RNAi/Opa1 RNAi females to generate UAS-SOD1/Opa1 RNAi; tincΔ4-Gal4/+ females for studies.
4. *tincΔ4-Gal4 > Opa1 RNAi, SOD2*
   +/SM5; tincΔ4-Gal4/tincΔ4-Gal4 females were crossed to UAS-SOD2/UAS-SOD2; +/TM3 males. The progeny were screened for UAS-SOD2/SM5; tincΔ4-Gal4/TM3 males, which were crossed to Opa1 RNAi/Opa1 RNAi females t generate UAS-SOD2/Opa1 RNAi; tincΔ4-Gal4/+ females for studies.
5. *tincΔ4-Gal4 > UAS-mito-GFP, MARF RNAi, SOD1*
   MARF RNAi/MARF RNAi females were crossed to the 2nd and 3rd balancer chromosome SM5 and TM3 males and balanced F1 progeny were self-crossed to generate MARF RNAi/MARF RNAi with 2nd chromosome balancer SM5. Similarly, UAS-SOD1 (#33605) was crossed to the 2nd and 3rd balancer chromosome SM5 and TM3. The F1 balanced progeny were self-crossed to generate UAS-SOD1/UAS-SOD1 with 3rd chromosome balancer TM3. +/SM5; MARF RNAi/MARF RNAi males were crossed to UAS-SOD1/UAS-SOD1; +/TM3 females to generate UAS-SOD1/SM5; MARF RNAi/TM3 males, which were crossed to UAS-mito-GFP/CyO; tincΔ4-Gal4/TM6 females. The resulting progeny were screened for UAS-SOD1/UAS-mito-GFP; MARF RNAi/tincΔ4-Gal4 females, which were studied.
6. *tincΔ4-Gal4 > UAS-mito-GFP, Opa1, SOD1*
   Opa1 RNAi/Opa1 RNAi females were crossed to the 2nd and 3rd balancer chromosome SM5 and TM3 males, and balanced F1 progeny self-crossed to generate Opa1 RNAi/Opa1 RNAi with 3rd chromosome balancer TM3. Similarly, UAS-SOD1 (#24730) was crossed to