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Regulation of mitochondrial fission by intracellular Ca^{2+} in rat ventricular myocytes

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ABSTRACT

Mitochondria are dynamic organelles that constantly undergo fission, fusion, and movement. Increasing evidence indicates that these dynamic changes are intricately related to mitochondrial function, suggesting that mitochondrial form and function are linked. Calcium (Ca^{2+}) is one signal that has been shown to both regulate mitochondrial fission in various cell types and stimulate mitochondrial enzymes involved in ATP generation. However, although Ca^{2+} plays an important role in adult cardiac muscle cells for excitation–metabolism coupling, little is known about whether Ca^{2+} can regulate their mitochondrial morphology. Therefore, we tested the role of Ca^{2+} in regulating cardiac mitochondrial fission. We found that neonatal and adult cardiomyocyte mitochondria undergo rapid and transient fragmentation upon a thapsigargin (TG)- or KCl-induced cytosolic Ca^{2+} increase. The mitochondrial fission protein, DLP1, participates in this mitochondrial fragmentation, suggesting that cardiac mitochondrial fission machinery may be regulated by intracellular Ca^{2+} signaling. Moreover, the TG-induced fragmentation was also associated with an increase in reactive oxygen species (ROS) formation, suggesting that activation of mitochondrial fission machinery is an early event for Ca^{2+}-mediated ROS generation in cardiac myocytes. These results suggest that Ca^{2+}, an important regulator of muscle contraction and energy generation, also dynamically regulates mitochondrial morphology and ROS generation in cardiac myocytes.

1. Introduction

Cardiac myocytes, with their high metabolic demands, are comprised of approximately 35% mitochondrial volume per cell [1]. These mitochondria, mostly situated among the contractile filaments and next to the sarcoplasmic reticulum (SR), perform the important role of providing ATP and regulating Ca^{2+} in microdomains [2]. Mitochondrial shape varies greatly within the adult cardiomyocyte. Based on electron microscopic observations, interfibrillar mitochondria are relatively large and oval shaped and evenly distributed in a crystal-like pattern along the contractile apparatus, whereas the subsarcolemmal and perinuclear mitochondria vary in size and shape, and appear less organized [1].

Despite the variations in mitochondrial size and shape in individual myocytes, very little is known about mitochondrial dynamics in the adult heart. Several recent studies show the presence of mitochondrial fission and fusion proteins in heart tissue, indicating that mitochondrial structure may be dynamic in the heart as well [3–7]. In addition, the high incidence of abnormal mitochondrial morphologies associated with many cardiac diseases suggests a role for these processes in normal cardiac function and abnormal disease etiology [8–14]. However, due to the highly organized and more static structure of the mitochondria in adult cardiac myocyte, it remains unclear whether these mitochondria are capable of undergoing fusion and fission and if mitochondrial dynamics plays a physiological role in the heart.

In many cell types, the dynamic interconversion between mitochondrial fusion and fission is important for normal cellular function. This becomes apparent as mutations or knockouts of the genes involved in mitochondrial dynamics have been shown to be lethal as well as cause human diseases [15–22]. Each process requires a different cohort of proteins. For example, mitochondrial fission requires the 17-kDa mitochondrial outer membrane protein, hFis1, and the recruitment of the 80- to 85-kDa large cytosolic GTPase DLP1 to the mitochondrial surface via transient interaction with hFis1 [23,24]. DLP1 is presumed to self-assemble into a homo-oligomeric ring around the mitochondrion [25] and possibly acts as a mechanoenzyme to pinch the mitochondrial membranes. GTP hydrolysis is required for the DLP1 constriction and disassembly, and a point mutation in this domain, such as DLP1-K38A, leads to a dominant-negative fission-defective mutant and elongation of mitochondria due to reduced fission [26–29]. DLP1-K38A also has other effects on cellular physiology, such as changing the distribution and morphology of the endoplasmic reticulum (ER) [27].

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In this study, we addressed the question of mitochondrial dynamics in the cardiac myocyte using thapsigargin (TG), a sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) inhibitor, which has been previously shown to increase intracellular Ca^{2+} leading to mitochondrial fission via a DLP1-dependent process [30,31]. We demonstrated that not only does Ca^{2+} regulate a DLP1-dependent mitochondrial fission but also ROS production. These findings are the first evidence to indicate the role of Ca^{2+} regulation of mitochondrial fragmentation and ROS generation in cardiac muscle cells.

2. Materials and methods

2.1. Neonatal rat ventricular cardiomyocyte culture

As modified from a previously published procedure [32], the hearts from 1- to 3-day-old anesthetized Sprague–Dawley rats were aseptically removed and transferred to HBSS containing 0.1% BSA and rinsed. Ventricles were minced and digested in BSA-free HBSS, 0.5 mg/mL collagenase (Worthington, type II) solution at 37 °C. The cells were filtered, pelleted, and resuspended in DMEM with 50U/mL penicillin G, 10% calf serum, and 10% horse serum. Cells were pre-plated once for 30 min and again for 60 min. Cells unattached to the plate were collected and plated on glass coverslips. After 24 hours, the cultures were treated with 1:100 arabinosylcytosine (ara-C) for 48 hours to reduce the growth of fibroblasts. Cells were treated with fresh medium and used for experiments from days 5 to 7 in culture. In some cases, cells were transfected with a vector expressing either untagged or GFP-tagged mutant DLP1-K38A using FuGENE (Roche) according to manufacturer's instructions as described previously [27]. For treatments and visualization, untransfected (control) and transfected (GFP positive) cells were examined from the same specimen.

2.2. Isolation of adult rat ventricular myocytes

The procedure was described in a published paper [33].

2.3. Ca^{2+} imaging

Modified from the procedure published previously [34], cells were loaded with 7 μM Fura-2/AM and 0.02% Pluronic F-127 (Invitrogen) in HEPES buffer (10 mM HEPES, 10 mM glucose, 140 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 2 mM $MgCl_2$, pH 7.4) at 37 °C for 30 min to allow the dye to accumulate in the cytosol. Cells were washed for 30 min at 37 °C to allow for de-esterification before experiments. After TG (Sigma-Aldrich, Co) addition, cells were sequentially excited at 340 nm and 380 nm, and cytosolic free Ca^{2+} was detected at 510 nm. Mitochondrial Ca^{2+} was measured by loading cells with 5 μM Rhod-2/AM (Invitrogen) at 4 °C for 15 min to allow the dye to accumulate in the mitochondria, for 15 min at room temperature, and were washed for 15 min. Cells were incubated with 10 μM Ru360 for 30 min at room temperature to block mitochondrial Ca^{2+} uptake. All graphs were plotted as $F/F_0 \pm$ SEM.

2.4. Fluorescence imaging

Indirect immunofluorescence was performed as described previously [29,35]. Cells were fixed with 4% paraformaldehyde, permeabilized, and incubated in blocking buffer containing 5% horse serum for 1 hour at 37 °C. Rabbit anti-DLP-N (described previously [36]) or mouse monoclonal anti-cytochrome *c* antibodies (Pharmingen) and Alexa 488 or 594-conjugated antibodies (Invitrogen) were used for primary and secondary antibodies, respectively. After appropriate washing, coverslips were mounted in ProLong antifade reagent (Invitrogen) on glass slides.

Live mitochondria were visualized by either loading with 200 nM Mitotracker Red CMXRos or 5 μM MitoSOX Red (Invitrogen) in cultured neonatal ventricular myocytes at 37 °C in HEPES buffer. With a 150-W xenon lamp from the illumination unit polychrome [36] V, single cell images were taken by fluorescent microscopy (TILL Photonics LLC, Pleasanton, CA). The fluorescent imaging system uses a Nikon TE2000s inverted microscope with a 40× oil objective. Neonatal ventricular myocyte mitochondria were analyzed as described previously [31,37].

2.5. Mitochondrial isolation

Cardiomyocytes were collected at 700×*g* for 5 min at 4 °C before and at various times after treatment with TG. The supernatant was discarded and the cell pellet was homogenized in 1 ml isolation buffer (10 mM HEPES pH 7.4, 1 mM EDTA, 320 mM sucrose, protease inhibitor). The homogenate was centrifuged at 700×*g* for 8 min at 4 °C and the supernatant was collected. The pellet was subjected to re-homogenization, centrifuged, and the supernatants were pooled. The supernatants were centrifuged at 17,000×*g* for 11 min at 4 °C and the supernatant (cytosol) and the pellet (mitochondria) were separated.

2.6. SDS–PAGE and Western blotting

SDS–PAGE [38] and Western blotting was performed as described previously [36]. We used anti-DLP1-N [39], anti-Fis1 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-GAPDH (Millipore, Billerica, MA), and anti-VDAC (Calbiochem, San Diego, CA) antibodies. Blots were developed with supersignal chemiluminescence detection substrate (Pierce, Rockford, IL).

2.7. Electron microscopy

Specimens were prepared for and analyzed by electron microscopy at the University of Rochester Core Facility, as described previously [40]. Mitochondria from adult ventricular myocytes were analyzed using AnalySIS software (University of Rochester, Electron Microscope Core Facility) for form factor, aspect ratio, and area.

2.8. Statistical analysis

The data were statistically analyzed by parametric (two tailed *t*-test, one way analysis of variance with Dunnett's post hoc testing) and non-parametric methods (Kruskal–Wallis with Dunn's post hoc testing) using either Prism (GraphPad Software, La Jolla, CA) or SPSS (SPSS Inc., Chicago, IL). A P value <0.05 was considered statistically significant.

2.9. Ethical aspects

Due to the lack of appropriate cell lines for cardiomyocytes, it was necessary to isolate cardiac cells in primary culture from newborn rats and adult rats. The animals were kept in the University of Rochester vivarium and treated humanely. Each animal was sacrificed consistent with UCAR protocol 2006063 and the Panel of Euthanasia of the American Veterinary Medical Association.

3. Results

Both adult and neonatal rat ventricular myocytes were used for experiments. Cultured neonatal rat ventricular myocytes were used for live cell imaging of mitochondrial morphology and function, taking advantage of their thin morphology with a lower density of mitochondria and relative ease of transfection. In contrast, in order to evaluate mitochondrial morphology in adult ventricular myocytes, electron microscopy was used because their thicker cell diameter with