Published in final edited form as:

Cell Calcium. 2015 August 1; 58(2): 186–195. doi:10.1016/j.ceca.2015.04.006.

Mitochondrial dysfunctions during progression of dystrophic cardiomyopathy

Victoria Kyrychenko^{§,*}, Eva Poláková^{#,*}, Radoslav Janíček, and Natalia Shirokova

Department of Pharmacology and Physiology, New Jersey Medical School - Rutgers University, Newark 07103, NJ, USA

Abstract

Duchenne muscular dystrophy (DMD) is a progressive muscle disease with severe cardiac complications. It is believed that cellular oxidative stress and augmented Ca^{2+} signaling drives the development of cardiac pathology. Some mitochondrial and metabolic dysfunctions have also been reported. Here we investigate cellular mechanisms responsible for impaired mitochondrial metabolism in dystrophic cardiomyopathy at early stages of the disease. We employed electrophysiological and imaging techniques to study mitochondrial structure and function in cardiomyocytes from *mdx* mice, an animal model of DMD. Here we show that mitochondrial matrix was progressively oxidized in myocytes isolated from *mdx* mice. Moreover, an abrupt increase in workload resulted in significantly more pronounced oxidation of mitochondria in dystrophic cells. Electron micrographs revealed a gradually increased number of damaged mitochondria in *mdx* myocytes. Degradation in mitochondrial structure was correlated with progressive increase in mitochondrial Ca^{2+} sequestration and mitochondrial depolarization, despite a substantial and persistent elevation in resting cytosolic sodium levels. Treatment of *mdx* cells with cyclosporine A, an inhibitor of mitochondrial permeability transition pore (mPTP), shifted both resting and workload-dependent mitochondrial redox state to the levels recorded in control myocytes. It also significantly reduced workload dependent depolarization of mitochondrial membrane in dystrophic cardiomyocytes. Overall, our studies highlight age

Address correspondence to: N. Shirokova, Department of Pharmacology and Physiology, NJMS-Rutgers University, 185 S. Orange Ave., Newark 07103, NJ, USA. nshiroko@njms.rutgers.edu.

[#]Eva Poláková current address: Department of Pharmacology & Systems Therapeutics, Mount Sinai School of Medicine, New York 10029, NY, USA

[§]Victoria Kyrychenko current address: Division of Cardiology, Department of Internal Medicine, UT Southwestern Medical Center, 6000 Harry Hines Blvd, NB11.106, Dallas, TX 75235, USA

*Victoria Kyrychenko and Eva Poláková contributed equally to this work.

Conflict of interest: none declared

*Co-author Approval Statement

The manuscript, or part of it, has neither been published nor is currently under consideration for publication by any other journal. The co-authors have read the manuscript and approved this submission. Authors have no conflict of interests.

*Conflict of Interest Statement

The manuscript, or part of it, has neither been published nor is currently under consideration for publication by any other journal. The co-authors have read the manuscript and approved this submission. Authors have no conflict of interests.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

dependent deterioration of mitochondrial function in dystrophic cardiomyocytes, which seems to be associated with excessive opening of mPTP due to oxidative stress and cellular Ca^{2+} overload.

Graphical Abstract

Keywords

Dystrophic cardiomyopathy; sodium overload; mitochondria; metabolism; oxidative stress

1. Introduction

Mitochondria are the major source of energy in cardiac muscle tissue. The organelles generate ATP from metabolites through oxidative phosphorylation. Reducing agents, such as NADH and FADH_2 , donate electrons to the mitochondrial respiratory chain resulting in the proton electrochemical gradient across the mitochondrial membrane. The gradient is further utilized by the mitochondrial ATP synthase to produce ATP. Mitochondrial ATP generation has to respond to the increased energy demand during mechanical activity of the heart. One way to do so is to increase the rate of oxidative phosphorylation. A moderate rise in cytosolic Ca^{2+} concentration (e.g. during workload) results in increased mitochondrial Ca^{2+} concentration ($[\text{Ca}^{2+}]_m$) which is known to increase the activity of tricarboxylic acid cycle (TCA) dehydrogenases, thus consequently increasing ATP synthesis. However, under some pathological conditions cytosolic and subsequent mitochondrial Ca^{2+} overload can promote irreversible opening of the mitochondrial permeability transition pore (mPTP), loss of functional mitochondria, reduction in cellular ATP production and even cell death. Mitochondrial Ca^{2+} levels mostly depend on coordinated function of several mitochondrial proteins (e.g. mitochondrial Ca^{2+} uniporter, mitochondrial Na^+ - Ca^{2+} exchanger (NCX_m) and mitochondrial Na^+ - H^+ exchanger) and are affected by cytosolic Ca^{2+} and Na^+ levels, pH values and redox potential (see [1–6] for review).

Duchenne muscular dystrophy (DMD) is a severe degenerative muscle disease. Most of the patients develop cardiomyopathy in their teens, and many of them succumb to cardiac complications [7–9]. The disease is caused by a genetic defect – the lack of a functional cytoskeleton protein dystrophin [10,11] and its phenotype exhibits numerous cellular

pathological features [12,13]. Recently we reported severe oxidative stress (resulting from overexpression of sarcolemmal NAD(P)H oxidase type II (NOX2)) and augmented intracellular Ca^{2+} signaling in hearts of *mdx* mice well before clinical manifestations of the disease [1,14]. Our results indicated that enhanced intracellular Ca^{2+} responses to mechanical challenges are associated with an increased sensitivity of sarcoplasmic (SR) Ca^{2+} release channels (a.k.a. ryanodine receptors, RyRs) due to their posttranslational modifications. We concluded that RyR oxidation followed by phosphorylation, first by CaMKII and later by PKA, synergistically contribute to RyR hypersensitivity and cardiac deterioration.

There are several reports of various mitochondrial abnormalities in hearts under conditions of intracellular Ca^{2+} overload and oxidative stress [10,15,16]. There is also increasing evidence of mitochondrial dysfunction in skeletal and cardiac muscle of DMD patients and *mdx* mice. In particular, there are indications of impaired oxidative phosphorylation, decreased ATP-generating capacity, premature stress-induced mPTP opening and mitochondrial depolarization [9,10,17–23]. Some of these features are present in dystrophic hearts at early stages of the disease. However, findings from different groups of investigators are somewhat controversial and causal links between pathophysiological manifestations of dystrophy in various intracellular compartments remain unclear.

The aim of this study was to establish cellular pathophysiological mechanisms leading to deterioration of mitochondria function and energy deprivation of dystrophic heart. For this, cytosolic and mitochondrial functions were assessed in cardiac myocytes isolated from *mdx* mice of different age groups. These studies were complemented by analyses of mitochondrial structure with transmission electron microscopy. Our results indicate an age dependent gradual degeneration of mitochondrial structure and function in dystrophic cardiomyocytes isolated from hearts that don't yet exhibit any sign of disease. They also suggest that mitochondrial dysfunctions are associated with increased activity of mPTP due to oxidative stress and cellular Ca^{2+} overload. Our results suggest that therapies targeting the mPTP may be helpful to DMD patients and improve not only skeletal muscle [21] but also cardiac performance. Some preliminary data of our study were reported in [24].

2. Materials and Methods

2.1. Cell isolation

All experiments conformed to the NIH Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH publication, 8th edition, 2011) and were approved by the Institutional Animal Care and Use Committee of the New Jersey Medical School, Rutgers University, USA. Mice used were C57BL10 mice (wild-type, WT, purchased from Harlan laboratory) and dystrophin-deficient *mdx* (C57BL/10ScSn-*mdx*, purchased from the Jackson Laboratory). Mice at the age of 1 (young) and 3–4 (adult) were used in this study. Ventricular myocytes were isolated enzymatically. Mice were heparinized (5000 U/kg), anesthetized with sodium pentobarbital (100 mg/kg), and checked to ensure the absence of movement, flexor, and pedal reflexes. Hearts were removed, mounted on a Langendorff apparatus and perfused with zero Ca^{2+} Tyrode solution containing collagenase and protease or Liberase. Ventricles were cut into small pieces from which cells were

mechanically dissociated and transferred to fresh solution followed by stepwise increasing Ca^{2+} concentration to 200 μM . Solution was changed to 1.8 mM CaCl_2 containing Tyrode solution shortly before each experiment. All experiments were carried out at room temperature and cells were used within 5 h after isolation. Solutions: *Zero Ca^{2+} Tyrode* (in mM): 140 NaCl, 5.4 KCl, 1.1 MgCl_2 , 5 HEPES, 1 NaH_2PO_4 , 10 glucose, pH 7.3, 300 mOsm. *Normal Tyrode (NT)* (in mM): 1.8 CaCl, 140 NaCl, 5.4 KCl, 1.1 MgCl_2 , 5 HEPES, 1 NaH_2PO_4 , 10 glucose, pH 7.4, 300 mOsm.

2.2. NADH measurement

NADH autofluorescence from the regions corresponding to mitochondria was measured with 2-photon confocal microscopy (Bio-Rad-2002-MP) in frame scanning mode at 0.1 Hz illuminations, using 720 nm excitation wavelength of the Mira-Verdi laser duo (Coherent, USA). After experimental manipulation, the resting NADH level in cells perfused with NT solution was calibrated. Maximal oxidation of NADH (a.k.a. 0% reduced NADH signal) was achieved by superfusing cells with NT solution supplemented with 1 μM FCCP and 1 $\mu\text{g/ml}$ oligomycin. Maximal reduction (a.k.a. 100% reduced NADH signal) was achieved by superfusing myocytes with NT supplemented with 5 μM rotenone and 10 mM β -hydroxybutyric acid. Resting NADH level was calculated and expressed as percentage of reduced NADH value for each cell, as in [3].

2.3. Electron microscopy

Hearts were removed, fixed with paraformaldehyde, sliced and imaged with a Phillips CM12 transmission electron microscope at Rutgers Core Imaging Lab.

2.4. Intracellular Na^+ calibration

Intracellular Na^+ signals ($[\text{Na}^+]_i$) were measured using either SBFI (10 μM SBFI-AM, 90 mins) or Asante NaTRIUM Green-2 (2 μM , 30 min) indicators. RatioMaster M-40 photometer (PTI, USA) was used to monitor the Na^+ -related SBFI fluorescence. Cells were sequentially illuminated at 340 and 380 nm at 1 Hz. Emitted light was collected above 500 nm and presented as excitation ratio. Changes in Asante NaTRIUM were recorded with confocal microscopy (Bio-Rad Radiance 2002MP) with the 488 nm excitation line of an Argon laser. After measurement of SBFI or Asante NaTRIUM Green-2 fluorescent intensity signals at resting conditions (cells perfused with NT), a calibration procedure was performed. For this, the perfusion solution was changed to Na^+ free solution followed by stepwise increases of extracellular Na^+ concentration (10, 20, 30, 50, 100, 145 mM). Resting $[\text{Na}]_i$ for each cell was taken from the corresponding calibration curve. Na^+ calibration solutions: *145 mM K^+ solution* (in mM): 135 K-gluconate, 10 KCl, 10 HEPES, 2 EGTA, 0.01 Ouabain, 0.01 Monensin, 0.01 Nigericin, 0.01 Gramicidin, 10 Glucose, pH 7.4, 300 mOsm. *145 mM Na^+ solution* (in mM): 135 Na-gluconate, 10 NaCl, 10 HEPES, 2 EGTA, 0.01 Ouabain, 0.01 Monensin, 0.01 Nigericin, 0.01 Gramicidin, 10 Glucose, pH 7.4, 300 mOsm. Mixtures of *145 mM K^+ and 145 mM Na^+ solution* solutions in different proportions were used to reach required Na^+ concentrations.

2.5. Measurements of mitochondrial Ca^{2+} content

Cells were loaded with cytosolic Ca^{2+} indicator fluo-4 (5 μM , 30 min). Changes in fluo-4 Ca^{2+} related fluorescence were recorded with an Olympus (FluoView-1000) confocal microscope using the 473 nm excitation of a diode laser. 2 μM FCCP and 1 $\mu\text{g/ml}$ oligomycin was added to promote mitochondrial Ca^{2+} efflux to the cytosol. To minimize contribution of Ca^{2+} -induced Ca^{2+} release from the SR, cells were pretreated with 10 μM ryanodine and 1 μM thapsigargin and field stimulated. The amplitude of FCCP-induced Ca^{2+} signal was used to evaluate mitochondrial Ca^{2+} content.

2.6. Mitochondrial membrane potential and mPTP opening

TMRE indicator (100 nM) was used to monitor mitochondrial membrane potential. TMRE fluorescence was measured with an Olympus (FluoView-1000) confocal microscope using the 559 nm excitation of a diode laser. 2 μM FCCP and 1 $\mu\text{g/ml}$ oligomycin were applied to completely depolarize mitochondria. The application resulted in redistribution of the indicator between mitochondria, cytosol and nuclei. To distinguish between cytosolic and mitochondrial signals, nuclear fluorescence (that is assumed to be the same as cytosolic) was subtracted from the fluorescence of cytosolic regions of cells that also included mitochondria. The opening of mPTP was monitored using fluorescent molecule calcein (473 nm excitation), which enter mitochondria after mPTP induction.

2.7. Statistics

Results are shown as mean $\pm$ standard error (SEM). All data sets contain results from a minimum of three mice. Statistical significance was evaluated by non-paired t-test or two-way ANOVA test. A p-value of < 0.05 was considered significant.

3. Results

To establish causal relationship between cytosolic and mitochondrial pathophysiological pathways in cardiac dystrophy, we analyzed several cytosolic and mitochondrial signals: cytosolic Ca^{2+} (determined in our previous work [10]) and Na^{+} concentrations, cellular redox state (determined by us in [14]), mitochondrial redox state, mitochondrial Ca^{2+} content and mitochondrial membrane potential. To follow the progression of this relationship at preclinical stages of the dystrophic cardiomyopathy, signals were monitored in cells isolated from young (1 month) and adult (3–4 months) WT and *mdx* mice, which do not show any sign of cardiac pathology. Functional studies were supplemented by the analysis of mitochondrial morphology assessed with transmission electron microscopy.

3.1. Mitochondrial matrix is oxidized in dystrophic cardiac myocytes

In order to obtain some insight into mitochondrial function in cells isolated from dystrophic and WT cells, we assessed the mitochondrial redox state of $\text{NADH}/\text{NAD}^{+}$ by measuring and calibrating NADH autofluorescence using 2 photon confocal system. Fig. 1A illustrates the experimental procedure. We recorded the NADH signal at rest (baseline) and subsequently under fully oxidized or reduced conditions, as detailed in methods. Images at the bottom show NADH signals in WT cell in completely oxidized or reduced settings. The baseline level of NADH autofluorescence was expressed as a percentage of the reduced value. The

redox state of mitochondrial NADH/NAD⁺ couple was substantially more oxidized in the *mdx* myocyte, compared to WT cell (32% vs. 51% of reduction, respectively). Fig. 1B summarizes our findings: the redox state of NADH/NAD⁺ is significantly more oxidized in dystrophic myocytes (26.7±2.7% (n=10) and 25.2±2.7% (n=21) in cells from young and adults hearts, respectively, vs. 42.7±2.7% (n=5) and 42.6±2.3% (n=28) in myocytes from young and adult WT mice, respectively).

In the next group of experiments, we tested the effects of increasing metabolic workload on the redox state of NADH/NAD⁺ couple. For this, cells were paced at 4Hz in the presence of 0.1 mM isoproterenol (Fig. 1C). An abrupt increase in workload resulted in oxidation of NADH both in *mdx* and WT cardiomyocytes. However, the extent of oxidation was significantly greater in dystrophic cells, especially in those isolated from adult animals. NADH signal decreased from 30.5±2.7 % at the baseline to 16.3±3.1% (n=10) at the end of the stimulation protocol in *mdx* cells from adult animals, whereas it reduced from 46.6±2.2% to 36.7±0.9% (n=7) in adult WT cells. When workload was discontinued, the mitochondrial matrix NADH quickly returned to baseline levels in WT but not always in *mdx* cells. Moreover, as the age of *mdx* animals increased, oxidation of mitochondrial matrix during transitions of workload became more pronounced (Fig. 1D).

3.2. Mitochondria morphology is abnormal in dystrophic cardiac myocytes

Next we aimed to determine if and to what extent deregulation of mitochondrial function in dystrophic cardiac tissue correlates with mitochondrial structural abnormalities. For this, sections of WT and *mdx* cardiac muscle were imaged with transmission electron microscopy (Fig. 2A). Electron micrographs revealed numerous mitochondria with increased area in *mdx* cardiac muscle, and many mitochondria with a loss of normal cristae structure (indicated by arrowheads). Figs. 2B and C quantify these observations. The number of mitochondria with a loss of cristae, as well as the average area of mitochondria was significantly higher in *mdx* cardiac myocytes. Moreover, the number of structurally abnormal mitochondria increased in cardiac tissue isolated from adult dystrophic animals, which parallels the worsening of mitochondrial function described above.

3.3. Intracellular [Na⁺] is elevated in dystrophic hearts

Previously we have shown that dystrophin-deficient cardiomyocytes, which are characterized by a fragile and leaky plasma membrane, have increased ion influx through several trans-membrane pathways in response to mechanical stretch [25]. A major component of this influx was proposed to be composed of Na⁺ ions. We assumed that enhanced Na⁺ influx eventually results in cellular Na⁺ overload, which in turn can influence mitochondrial function [1,3,4]. In particular, increased [Na⁺]_i may accelerate mitochondrial Ca²⁺ efflux and decrease mitochondrial Ca²⁺ accumulation, resulting from the larger Na⁺ gradient across the mitochondria and thus stimulation of mitochondrial NCX. The subsequent decrease in [Ca²⁺]_m may hamper the activation of mitochondrial TCA dehydrogenases, which in turn can affect the mitochondrial metabolic state and cause a pronounced NADH oxidation. However, the elevated cytosolic Na⁺ concentration may also cause additional Ca²⁺ influx *via* sarcolemmal NCX, which could partly or completely counteract the improved mitochondrial Ca²⁺ removal.

Cells were loaded with either of two fluorescent Na^+ indicators, SBFI or Asante NaTRIUM, and studied either with fluorescent photometry or confocal microscopy. Calibration was performed by superfusing cells with solutions containing various Na^+ concentrations, as described in Methods. Fig. 3A illustrates typical experiments with cardiomyocytes isolated from adult WT (top panels) or *mdx* (low panels) mice. First, we measured F_{340}/F_{380} ratio in intact cells under resting conditions. Then calibration procedure was applied. The SBFI ratio signal increased with increasing $[\text{Na}^+]$ in the superfusing solutions (left traces in Fig. 3A). Left traces are calibration curves obtained in these cells. Please note that while at lower Na^+ concentration calibration curves were linear, they become saturated at higher Na^+ levels. The calibration procedure revealed resting $[\text{Na}^+]_i$ values of 10.2 and 18.5 mM in these particular WT and dystrophic cardiomyocytes, indicating markedly elevated cytosolic Na^+ levels in dystrophic hearts.

The results with both Na^+ -sensitive indicators were similar, so data were pooled together and averaged (Fig. 3B). The averaged values of resting $[\text{Na}^+]_i$ were significantly elevated in myocytes isolated from *mdx* mice in two age groups studied (18.3 ± 1.4 (n=8), 24.4 ± 3.1 (n=11) vs. 12.4 ± 1.6 (n=6), 14.0 ± 1.7 (n=9) in dystrophic and WT cells from young and adult mice, respectively). Overall, dystrophic cardiomyocytes are overloaded with Na^+ . Whether or not this is the underlying reason for oxidation of mitochondrial matrix NADH was examined in the next group of experiments.

3.4. Mitochondrial Ca^{2+} content is increased in dystrophic hearts

As discussed above, the direct consequence of intracellular Na^+ overload could be an acceleration of mitochondrial Ca^{2+} extrusion. If this would be the case, it could consequently lead to an oxidation of the mitochondrial matrix NADH. Nevertheless, there are several reports of elevated Ca^{2+} content in mitochondrial of dystrophic skeletal and cardiac muscle [7,9]. In these experiments, we wanted to confirm that this is the case under our experimental conditions. The direct measurements of mitochondrial Ca^{2+} concentration in intact cells with conventional (non-genetically targeted) fluorescent indicators (e.g. rhod-2) is challenging, as large portions of the dye remain trapped in the cytosol obscuring the mitochondrial measurements. Therefore here we applied an indirect method. Like many other methods it has multiple pros and cons. The major advantage is technical simplicity. The major disadvantage is the specificity of intracellular targeting of fluorescent indicator. We evaluated the amount of Ca^{2+} released from the mitochondria after depolarization of the mitochondrial membrane. The contribution of CICR was minimized by pretreatment of cells with ryanodine and thapsigargin and pre-pacing. Cells were loaded with the cytosolic calcium indicator fluo-4. Our control experiments revealed slight decrease in fluo-4 signal after application of inhibitor of mitochondrial NCX CGP37157, indicating cytosolic targeting of fluo-4, as CGP37157 is known to increase mitochondrial and decrease cytosolic Ca^{2+} levels. Cells were superfused with extracellular solution supplemented with mitochondrial uncoupler FCCP in combination with oligomycin. Application of FCCP led to a significant increase in intracellular Ca^{2+} concentration (Fig. 4A). The amplitudes of FCCP-induced Ca^{2+} transients were associated with mitochondrial Ca^{2+} load. The amplitudes were not much different in cells from young animals (1.21 ± 0.02 (n=25) and 1.20 ± 0.03 (n=18) F/F_0 in WT and dystrophic cells, respectively) but were significantly

greater in cardiomyocytes isolated from hearts of adult *mdx* mice (1.30 ± 0.03 ($n=19$) and 1.51 ± 0.09 ($n=23$) F/F_0 , Figure 4B). Importantly, these findings were in good agreement with the previously observed cellular Ca^{2+} overload found in adult but not young *mdx* myocytes [10]. It should be mentioned that we, and others, have previously reported slightly elevated cytosolic Ca^{2+} levels in adult *mdx* myocytes compared to WT cells (~ 20 nM, or less than 0.1 F/F_0) [10,16]. However, this difference is not substantial enough to significantly influence the results described above.

Overall, our data supports previous finding by others that the mitochondrial Ca^{2+} levels are elevated in hearts of adult *mdx* mice. Therefore, elevated Na^+ levels cannot explain the oxidation of mitochondrial NADH/NAD $^+$ redox couple in a straightforward way. With the next experiments we tested for the alternative mechanisms.

3.5. Mitochondrial membrane is depolarized in dystrophic hearts

Several major and primary features of the cellular phenotype of cardiac dystrophy at early, preclinical stages of the disease result from severe oxidative stress, mostly due to overexpression of NOX2 and consequent posttranslational modification (oxidation and CaMKII phosphorylation) of Ca^{2+} release channels. As disease progresses, intracellular Ca^{2+} homeostasis gets more deregulated due to synergetic activation of additional mechanisms that contribute to increased sensitivity of RyRs [12]. Altogether the enhanced Ca^{2+} signals eventually lead to cytosolic and mitochondrial Ca^{2+} overload. These pathological conditions are some of key factors responsible for excessive opening of the mitochondrial permeability transition pore (mPTP). Prolonged opening of the pore leads to mitochondrial depolarization and dissipations of the proton gradient responsible for oxidative phosphorylation and ATP synthesis. Another consequence of mPTP opening is mitochondrial swelling and rupture owing to the sudden influx of water and solutes. Micrographs in Fig. 2 indicate swollen mitochondria in *mdx* but not in WT cardiac tissue. Here we tested whether the mitochondrial membrane potential (ψ_m) is changed in dystrophic cardiomyocytes.

Cells were loaded with the mitochondrial membrane potential sensitive indicator TMRE. The intensity of TMRE signal was recorded at resting conditions and then during application of FCCP with oligomycin. The signal after application of FCCP was assumed to originate from completely depolarized mitochondria. It was used to calibrate the TMRE signal under resting conditions. Dissipation of ψ_m resulted in redistribution (and equilibration) of the indicator between mitochondrial matrix and cytosol. Therefore the TMRE signal from the nuclei was subtracted from the mixed (cytosolic and mitochondrial) signal to correct for cytosolic TMRE contamination. Our data indicate that mitochondrial membrane is more depolarized in cells from *mdx* compared to WT mice but the difference was significantly larger only in cells from adult animals (in a.u. 614 ± 75 ($n=9$) vs. 576 ± 54 ($n=8$) and 517 ± 44 ($n=6$) vs 407 ± 22 ($n=6$) in myocytes from young and adult WT and *mdx* mice, respectively). This corresponds to a 6% and 20% greater TMRE signal in cells from young and adult WT mice compared to their dystrophic counterparts.

The greater extent of depolarization of mitochondrial membrane in *mdx* cardiomyocytes as well as larger number of swollen mitochondria (see Fig. 2) suggests excessive opening of mPTP in dystrophic hearts, which becomes exaggerated as disease progresses.

3.6. Inhibition of mPTP in dystrophic myocytes minimized oxidation of mitochondrial matrix during workload

Our results described above suggest that premature and excessive opening of mPTP, probably due to cellular oxidative stress and Ca^{2+} overload, results in dissipation of the proton gradient, inhibition of oxidative phosphorylation and oxidation of mitochondrial NADH/NAD⁺ couple. Here we directly test whether inhibition of mPTP minimizes oxidation of the mitochondrial matrix NADH pool and prevents ATP deprivation in dystrophic hearts.

Fig. 6 summarizes the experiments in which we evaluated the ability of the NADH/NAD⁺ redox couple to cope with an increased metabolic workload imposed on cardiomyocytes isolated from adult mice (as in Fig. 1C) in the presence of the mPTP inhibitor Cyclosporin A (1 μM). Treatment of dystrophic cells with Cyclosporin A normalized the mitochondrial redox state in adult dystrophic cardiomyocytes after increased workload.

3.7. Inhibition of mPTP in dystrophic myocytes reduced loss of mitochondrial membrane potential during workload

Opening of mPTP is usually associated with the loss of mitochondrial membrane potential. In order to obtain more support for our hypothesis implying involvement of mPTP in impaired mitochondrial metabolism, we tested 1) whether the mitochondrial membrane potential dissipates in *mdx* cardiomyocytes in response to increased workload and 2) whether treatment of dystrophic cells with Cyclosporin A counteracts the loss of ψ_m .

Fig. 7A shows representative images of TMRE fluorescence collected from two different *mdx* cells subjected to the workload protocol used before. As in experiments shown in Fig. 1C, cells were paced at 4Hz in the presence of 0.1 μM isoproterenol. Some mitochondria in resting *mdx* cells seemed to be already depolarized (no TMRE signal recorded). It should be also noted that the extent of ψ_m loss during the stimulation protocol significantly varied. Whereas some *mdx* cells lost only few to several dozen functional mitochondria ($n=9$, typical images are shown in Fig. 7A, top panels), others lost a significantly larger number of them or even all of the organelles ($n=3$, images are in Fig. 7A bottom). We suggested that the loss of TMRE fluorescence in individual mitochondria is associated with mPTP opening.

In the next group of experiments cells were loaded with two fluorescent indicators: TMRE and calcein. These two fluorescent markers have well separated excitation/emission properties and can be monitored simultaneously. The neutral fluorescent molecule calcein is often used to visualize mPTP openings. It readily enters mitochondria only after mPTP induction. Images on Fig. 7B illustrate accumulation of calcein (bottom panels) in mitochondria that irreversibly lost their membrane potential after the stimulation protocol (top panels). Graphs on the left show time course of TMRE and calcein signals in four selected mitochondria marked on the images by boxes. It is evident that depolarization of

mitochondrial membrane was immediately followed by accumulation of calcein, confirming mPTP opening.

Figs. 7C and 7D summarize data illustrated in Fig. 7A. An increase in workload resulted in a gradual depolarization of mitochondrial membrane both in WT and *mdx* cells (Fig. 7). However, depolarization was stronger in dystrophic cardiomyocytes. Treatment of *mdx* cells with Cyclosporin A prevented loss of ψ_m in *mdx* cells to some extent. On average, the TMRE signal decreased by $3\% \pm 0.08\%$ ($n=9$), $9\% \pm 4.7\%$ ($n=9$) and $4\% \pm 1.4\%$ ($n=8$) by the end of the stimulation protocol in WT, *mdx* and *mdx* treated with Cyclosporine A cells, respectively. Unfortunately, the significance of these results could not be established due to wide variability of the responses in the adult *mdx* cells.

Fig. 7D represents the fraction of depolarized mitochondria after increased workload in three groups of experiments. The loss of functional mitochondria was significantly greater in *mdx* cells, compared to WT and treated *mdx* myocytes. The treatment of *mdx* cardiomyocytes with Cyclosporine A substantially reduced the loss.

4. Discussion

In the present study we have defined changes in mitochondrial function during the development of cardiac dystrophy. Our experiments revealed that the mitochondrial matrix NADH/NAD⁺ redox couple is oxidized in cardiac muscle of *mdx* mice, well before the clinical onset of cardiac myopathy. In addition, the ability of dystrophic hearts to cope with increased metabolic workload is impaired. In parallel, we also found that the cytosol of dystrophic cardiomyocytes is overloaded with Na⁺. Therefore we first thought that oxidation of the mitochondrial matrix is a result of increased cytosolic Na⁺ level. As it was shown before [1], increased [Na⁺]_i could decrease Ca²⁺ sequestration in the mitochondria by stimulating Ca²⁺ efflux via mitochondrial Na⁺-Ca²⁺ exchanger, consequently reduce the activity of several enzymes of the mitochondrial respiratory chain and therefore shifting the redox status of NADH/NAD⁺ couple towards oxidation.

However, this conjecture was not supported by our experimental results. No significant difference in both cytosolic [10,16] and mitochondrial Ca²⁺ load was observed in myocytes isolated from young animals, despite increased cytosolic Na⁺ level. Moreover, as the disease progresses, the cytosol got steadily overloaded with Ca²⁺. It was accompanied by the increase in mitochondrial Ca²⁺ sequestration, gradual depolarization of mitochondrial membrane as well as by changes in mitochondrial morphology: many organelles appeared swollen and lost cristae.

The apparent contradiction between Na⁺ and Ca²⁺ handling in dystrophic cardiomyocytes, at least in part, could be explained assuming that mitochondrial Na⁺ influx and efflux pathways (NCX_m and Na⁺-H⁺ antiporter) are altered in dystrophy. Previous findings by others and us revealed that dystrophic hearts are under severe oxidative stress, even at preclinical stages of the disease. Increased expression and/or activity of sarcolemmal NOX2 were affirmed to be responsible for this phenomenon [10,17,20]. Oxidative stress in dystrophic cells can inhibit NCX_m [2,4–6,26,27] and reduce extrusion of Ca²⁺ from mitochondria. Another possibility is that elevated [Na⁺]_i could provoke acidification of the

cytosol and consequently increase Na^+ extrusion from the mitochondria, thus promoting their Ca^{2+} accumulation [8,28]. Nevertheless, this does not explain the oxidation of mitochondrial NADH/NAD⁺ system found in our experiments.

In dystrophic hearts, the oxidation of intracellular environment triggers a chain of events that leads to sensitization of RyRs, exaggerated intracellular Ca^{2+} signaling and eventually results in an increase in cytosolic Ca^{2+} levels. In addition, oxidation of the intracellular environment enhances Ca^{2+} influx through the sarcoplasmic membrane *via* several pathways (e.g. stretch-activated channels and membrane ruptures [11,25]), which may also contribute to cytosolic and consequently mitochondrial Ca^{2+} overload, mitochondrial depolarization and oxidation of NADH/NAD⁺ redox couple. Further, CaMKII was found to become activated by oxidative stress during dystrophy [13,14,29] which would further amplify several Ca^{2+} signaling pathways via modulation of channels and transporters (e.g. RyR2s [14,30]).

However, we and others observed no increase in $[\text{Ca}^{2+}]_i$ at the onset of dystrophy [10,15], suggesting that Ca^{2+} extrusion mechanisms are capable to counteract the increased Ca^{2+} influx into the cytosol. Similarly no increase in the mitochondrial Ca^{2+} level was recorded. Nevertheless, some functional and structural changes in the organelles were already detectable (Figs. 1 and 2). Hence, it appears that mitochondrial dysfunctions at this stage of disease are independent of mitochondrial Ca^{2+} overload. E.g. in response to cytosolic oxidative stress the distribution of NADH between cytosol and mitochondria can be shifted to compensate for oxidation of the cytosol thus shifting the mitochondrial NADH/NAD⁺ redox potential. In addition, excessive H_2O_2 (resulting from conversion of superoxide, produced by NOX2) in the cytosol can easily cross mitochondrial membrane to react with glutathione peroxidase and thus also contribute to oxidation of NADH pool.

At later stages of the disease, the oxidation of the cytosol becomes stronger, sensitization of RyRs due to additional post-translational modifications becomes greater, and Ca^{2+} leak from the SR increases [9,10,14,18,19,21–23]. The latter results in a significant increase in cytosolic [10,21] and mitochondrial [9,24] Ca^{2+} levels. It seems that at this time point, severe cellular oxidative stress and Ca^{2+} overload promote opening of mPTP leading to an impairment of oxidative phosphorylation and ATP synthesis.

Under normal physiological conditions, mitochondria use electron transport through the respiratory chain to generate an electrochemical gradient across the inner mitochondrial membrane. This gradient, which consists of a membrane potential and a proton gradient, is used by the ATP-synthase to phosphorylate ADP to ATP. Under pathological conditions, however, widespread opening of mPTP results in depolarization of the mitochondrial membrane, dissipation of the proton gradient consequently reversing the operation of ATP-synthase to the point where it consumes ATP instead of producing it. This, in turn, facilitates cell death by apoptotic or necrotic mechanisms.

In fact, excessive opening of mPTP, as well as its premature opening in response to stress, has been attributed to a wide range of cardiac diseases [3,15,31]. Various structural, biochemical and functional mitochondrial abnormalities have been reported for dystrophic

skeletal and cardiac muscle [10,32,33]. Among them is a predisposition of mitochondria from *mdx* hearts to an increased propensity of mPTP opening during ischemia-reperfusion [14,22]. Our results show that during the progression of dystrophic cardiac myopathy there is an accumulation of dysfunctional mitochondria. Gradual increase in oxidative stress and rise in cytosolic Ca^{2+} levels favor the irreversible opening of mPTP, which culminates in cellular death and loss of cardiac tissue, contributing to muscle fibrosis. Showing significant mitochondrial involvement in the pathology of dystrophy our data also provide additional support to the possibility for the mPTP to be a possible pharmacological target for treatment of the disease [25,34].

Acknowledgments

Funding

This work was supported by NIH (HL093342 and AR053933 to N.Sh.). Eva Polakova was recipient of a Postdoctoral Fellowship from AHA. We are grateful to Drs. L. Gaspers, E. Niggli and A.P. Thomas for useful comments on this project.

References

1. Maack C, Cortassa S, Aon MA, Ganesan AN, Liu T, O'Rourke B. Elevated cytosolic Na^+ decreases mitochondrial Ca^{2+} uptake during excitation-contraction coupling and impairs energetic adaptation in cardiac myocytes. *Circ Res*. 2006; 99:172–182. [PubMed: 16778127]
2. Williams GSB, Boyman L, Lederer WJ. Mitochondrial calcium and the regulation of metabolism in the heart. *J Mol Cell Cardiol*. 2014
3. Kohlhaas M, Liu T, Knopp A, Zeller T, Ong MF, Böhm M, et al. Elevated cytosolic Na^+ increases mitochondrial formation of reactive oxygen species in failing cardiac myocytes. *Circulation*. 2010; 121:1606–1613. [PubMed: 20351235]
4. Bay J, Kohlhaas M, Maack C. Intracellular Na^+ and cardiac metabolism. *J Mol Cell Cardiol*. 2013
5. Dedkova EN, Blatter LA. Calcium signaling in cardiac mitochondria. *J Mol Cell Cardiol*. 2013; 58:125–133. [PubMed: 23306007]
6. Eisner V, Csordás G, Hajnóczky G. Interactions between sarco-endoplasmic reticulum and mitochondria in cardiac and skeletal muscle - pivotal roles in Ca^{2+} and reactive oxygen species signaling. *J Cell Sci*. 2013; 126:2965–2978. [PubMed: 23843617]
7. Shkryl VM, Martins AS, Ullrich ND, Nowycky MC, Niggli E, Shirokova N. Reciprocal amplification of ROS and Ca^{2+} signals in stressed *mdx* dystrophic skeletal muscle fibers. *Pflugers Arch*. 2009; 458:915–928. [PubMed: 19387681]
8. Finsterer J, Stöllberger C. The heart in human dystrophinopathies. *Cardiology*. 2003; 99:1–19. [PubMed: 12589117]
9. Viola HM, Davies SMK, Filipovska A, Hool LC. L-type Ca^{2+} channel contributes to alterations in mitochondrial calcium handling in the *mdx* ventricular myocyte. *Am J Physiol Heart Circ Physiol*. 2013; 304:H767–H775. [PubMed: 23335798]
10. Jung C, Martins AS, Niggli E, Shirokova N. Dystrophic cardiomyopathy: amplification of cellular damage by Ca^{2+} signalling and reactive oxygen species-generating pathways. *Cardiovasc Res*. 2007; 77:766–773. [PubMed: 18056762]
11. Ervasti JM, Campbell KP. A role for the dystrophin-glycoprotein complex as a transmembrane linker between laminin and actin. *J Cell Biol*. 1993; 122:809–823. [PubMed: 8349731]
12. Niggli E, Ullrich ND, Gutierrez D, Kyrychenko S, Poláková E, Shirokova N. Posttranslational modifications of cardiac ryanodine receptors: Ca^{2+} signaling and EC-coupling. *Biochimica Et Biophysica Acta (BBA) - Molecular Cell Research*. 2012
13. Shirokova N, Niggli E. Cardiac phenotype of Duchenne Muscular Dystrophy: Insights from cellular studies. *J Mol Cell Cardiol*. 2013; 58:217–224. [PubMed: 23261966]

14. Kyrychenko S, Polakova E, Kang C, Pocsai K, Ullrich ND, Niggli E, et al. Hierarchical accumulation of RyR post-translational modifications drives disease progression in dystrophic cardiomyopathy. *Cardiovasc Res*. 2013; 97:666–675. [PubMed: 23263329]
15. Lisa FD, Canton M, Menabò R, Kaludercic N, Bernardi P. Mitochondria and cardioprotection. *Heart Fail Rev*. 2007; 12:249–260. [PubMed: 17516167]
16. Williams IA, Allen DG. Intracellular calcium handling in ventricular myocytes from mdx mice. *Am J Physiol Heart Circ Physiol*. 2006; 292:H846–H855. [PubMed: 17012353]
17. Williams IA, Allen DG. The role of reactive oxygen species in the hearts of dystrophin-deficient mdx mice. *Am J Physiol Heart Circ Physiol*. 2007; 293:H1969–H1977. [PubMed: 17573457]
18. Kuznetsov AV, Winkler K, Wiedemann FR, von Bossanyi P, Dietzmann K, Kunz WS. Impaired mitochondrial oxidative phosphorylation in skeletal muscle of the dystrophin-deficient mdx mouse. *Mol Cell Biochem*. 1998; 183:87–96. [PubMed: 9655182]
19. Robert V. Alteration in Calcium Handling at the Subcellular Level in mdx Myotubes. *Journal of Biological Chemistry*. 2000; 276:4647–4651. [PubMed: 11029464]
20. Prosser BL, Ward CW, Lederer WJ. X-ROS signaling: rapid mechano-chemo transduction in heart. *Science*. 2011; 333:1440–1445. [PubMed: 21903813]
21. Reutenauer J, Dorchies OM, Patthey-Vuadens O, Vuagniaux G, Ruegg UT. Investigation of Debio 025, a cyclophilin inhibitor, in the dystrophic mdx mouse, a model for Duchenne muscular dystrophy. *British Journal of Pharmacology*. 2009; 155:574–584. [PubMed: 18641676]
22. Ascah A, Khairallah M, Daussin F, Bourcier-Lucas C, Godin R, Allen BG, et al. Stress-induced opening of the permeability transition pore in the dystrophin-deficient heart is attenuated by acute treatment with sildenafil. *Am J Physiol Heart Circ Physiol*. 2011; 300:H144–H153. [PubMed: 20971771]
23. Percival JM, Siegel MP, Knowels G, Marcinek DJ. Defects in mitochondrial localization and ATP synthesis in the mdx mouse model of Duchenne muscular dystrophy are not alleviated by PDE5 inhibition. *Hum Mol Genet*. 2012; 22:153–167. [PubMed: 23049075]
24. Poláková E, Shirokova N. Abnormal sodium handling and mitochondrial metabolism in cardiac dystrophy. *Biophys J*. 2011; 100:81a.
25. Fanchaouy M, Polakova E, Jung C, Ogrodnik J, Shirokova N, Niggli E. Pathways of abnormal stress-induced Ca^{2+} influx into dystrophic mdx cardiomyocytes. *Cell Calcium*. 2009; 46:114–121. [PubMed: 19604578]
26. Jornot L, Maechler P, Wollheim CB, Junod AF. Reactive oxygen metabolites increase mitochondrial calcium in endothelial cells: implication of the $\text{Ca}^{2+}/\text{Na}^{+}$ exchanger. *J Cell Sci*. 1999; 112(Pt 7):1013–1022. [PubMed: 10198283]
27. Gandhi S, Wood-Kaczmar A, Yao Z, Plun-Favreau H, Deas E, Klupsch K, et al. PINK1-Associated Parkinson's Disease Is Caused by Neuronal Vulnerability to Calcium-Induced Cell Death. *Molecular Cell*. 2009; 33:627–638. [PubMed: 19285945]
28. Garcarena CD, Youm JB, Swietach P, Vaughan-Jones RD. H^{+} -activated Na^{+} influx in the ventricular myocyte couples Ca^{2+} -signalling to intracellular pH. *J Mol Cell Cardiol*. 2013; 61:51–59. [PubMed: 23602948]
29. Erickson JR, Joiner MLA, Guan X, Kutschke W, Yang J, Oddis CV, et al. A dynamic pathway for calcium-independent activation of CaMKII by methionine oxidation. *Cell*. 2008; 133:462–474. [PubMed: 18455987]
30. Shirokova N, Kang C, Fernandez-Tenorio M, Wang W, Wang Q, Wehrens XHT, et al. Oxidative stress and Ca^{2+} release events in mouse cardiomyocytes. *Biophys J*. 2014; 107:2815–2827. [PubMed: 25517148]
31. Weiss JN. Role of the Mitochondrial Permeability Transition in Myocardial Disease. *Circ Res*. 2003; 93:292–301. [PubMed: 12933700]
32. Ruegg UT, Nicolas-Métral V, Challet C, Bernard-Hélary K, Dorchies OM, Wagner S, et al. Pharmacological control of cellular calcium handling in dystrophic skeletal muscle. *Neuromuscul Disord*. 2002; 12(Suppl 1):S155–61. [PubMed: 12206810]
33. Burelle Y, Khairallah M, Ascah A, Allen BG, Deschepper CF, Petrof BJ, et al. Alterations in mitochondrial function as a harbinger of cardiomyopathy: Lessons from the dystrophic heart. *J Mol Cell Cardiol*. 2010; 48:310–321. [PubMed: 19769982]

34. Millay DP, Sargent MA, Osinska H, Baines CP, Barton ER, Vuagniaux G, et al. Genetic and pharmacologic inhibition of mitochondrial-dependent necrosis attenuates muscular dystrophy. *Nat Med*. 2008; 14:442–447. [PubMed: 18345011]

Research Highlights

Duchenne muscular dystrophy (DMD) is a progressive muscle disease with severe cardiac pathology.

We show that mitochondrial function and structure progressively deteriorate in this disease.

It is likely associated with excessive openings of mPTP due to oxidative stress and cellular Ca^{2+} overload.

Fig. 1.

Mitochondrial matrix is more oxidized in dystrophic cardiomyocytes. (A) Representative traces of averaged NADH autofluorescence acquired during the calibration procedure in cells from WT and *mdx* ventricles. At the bottom are typical images of a myocyte after complete reduction and oxidation of mitochondria matrix. (B) Averaged resting values of NADH pooled out from cardiomyocytes isolated from WT and *mdx* mice of different age groups, as indicated. Data are from young (n=5) and adult (n=12) WT and young (n=11), adult (n=15) *mdx* myocytes. (*p < 0.05). (C) Changes in NADH signals during increased workload in WT and *mdx* cells. Calibration procedure was applied to each cell at the end of experiment. Protocol is indicated at the top. (D) Averaged values of NADH levels at three time points: under resting conditions, after 1 min of isoproterenol application and after 5 min of 4Hz stimulation in the presence of the same concentration of isoproterenol. Data are from young (n=5) and adult (n=7) WT and young (n=10) and adult (n=10) *mdx* myocytes. (*p < 0.05).

Fig. 2. Abnormal mitochondrial morphology in dystrophic cardiomyocytes. (A) Typical transmission electron micrographs of heart slices from adult WT and *mdx* mice. Arrows indicate mitochondria with a loss of cristae. Scale bar corresponds to 500 nm. (B) Percent of defective mitochondria. 18–20 random fields with 50 to 110 mitochondria were analyzed in each experimental group. (C) Summary of mitochondria area in hearts from young and adult WT and *mdx* mice. 122 to 244 mitochondria from at least 7 different fields were analyzed in each group. Samples from two hearts were analyzed in each experimental cluster. (* $p < 0.05$, # $p < 0.001$).

Fig. 3. Intracellular [Na⁺] is elevated in *mdx* cardiomyocytes. (A) Calibration of intracellular [Na⁺]. Representative traces of SBFi fluorescence acquired during calibration procedures in cells from WT and *mdx* adult hearts. (B) Averaged values of intracellular [Na⁺] in cardiomyocytes isolated from WT and *mdx* mice of different age groups, as indicated. (*p < 0.05, #*p* < 0.001). Data are from young (*n*=6) and adult (*n*=11) WT and young (*n*=8) and adult (*n*=7) *mdx* myocytes. (*p < 0.05).

Fig. 4. Mitochondrial Ca^{2+} is elevated in adult *mdx* cardiomyocytes. (A) Typical traces of Fluo-4 fluorescence in adult WT and *mdx* cardiomyocytes during treatment with FCCP and oligomycin. (B) Average changes in cytosolic Ca^{2+} levels after FCCP application. (* $p < 0.05$). Data are from young ($n=25$) and adult ($n=19$) WT and young ($n=18$) and adult ($n=23$) *mdx* myocytes. (* $p < 0.05$).

Fig. 5.

Mitochondrial membrane is partially depolarized in adult *mdx* cardiomyocytes. (A) Representative images and time-course of TMRE fluorescence in adult WT cardiomyocytes during calibration procedure. Arrowheads indicate nuclei, from which extra-mitochondrial TMRE fluorescence was collected. Signals were recorded from cytosolic part, containing mitochondria and from nucleus. The difference in fluorescence between cytosolic and nucleus regions corresponds to mitochondria-derived signal. Scale bar corresponds to 15 μm . (B) The averaged changes in TMRE signals in young and adult WT and *mdx* cardiomyocytes after treatment with FCCP and oligomycin. (* $p < 0.05$). Data are from young ($n=9$) and adult ($n=6$) WT and young ($n=8$) and adult ($n=6$) *mdx* myocytes. (* $p < 0.05$).

Fig. 6.

Oxidation of mitochondrial matrix in *mdx* cardiomyocytes is prevented by inhibition of mPTP. Averaged values of NADH signals at three time points: under resting conditions, after 1 min of isoproterenol application and after 5 min of 4Hz stimulation in the presence of isoproterenol. Data was acquired from adult WT (n=12) and *mdx* cardiomyocytes without (n=10) and with (n=7) treatment with cyclosporine A.

Fig. 7.

Dissipation of mitochondrial membrane potential in *mdx* cardiomyocytes is diminished by inhibition of mPTP. (A) Representative images of *mdx* cardiomyocytes with different extent of depolarized mitochondrial. Scale bar corresponds to 20 μm . (B) Images of *mdx* cell dual-loaded with TMRE (inside mitochondria) and calcein-AM (localized to the cytosol). Graphs on the left show that cytosolic calcein enters mitochondrion after mPTP induction. (C) Averaged values of TMRE signals at five time points (indicated by * in panel B): under resting conditions, after 1 min of isoproterenol application, after 5 min of 4Hz stimulation, after following 30 s of isoproterenol application and after following washout in WT (n=9) and *mdx* cells with (n=8) and without (n=9) treatment with Cyclosporin A. (D) Fraction of mitochondria completely depolarized in three groups of cells during stimulation protocol. There is a significant difference in % of loss between WT and *mdx* at the end of stimulation protocol.