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Mutation in dystrophin-encoding gene affects energy metabolism in mouse myoblasts

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ABSTRACT

Duchenne Muscular Dystrophy is characterized by severe defects in differentiated muscle fibers, including abnormal calcium homeostasis and impaired cellular energy metabolism. Here we demonstrate that myoblasts derived from dystrophic (*mdx*) mouse exhibit reduced oxygen consumption, increased mitochondrial membrane potential, enhanced reactive oxygen species formation, stimulated glycolysis but unaffected total cellular ATP content. Moreover, reduced amounts of specific subunits of the mitochondrial respiratory complexes and ATP-synthase as well as disorganized mitochondrial network were observed. Both the dystrophic and control myoblasts used were derived from a common inbred mouse strain and the only difference between them is a point mutation in the dystrophin-encoding gene, thus these data indicate that this mutation results in multiple phenotypic alterations demonstrating as early as in undifferentiated myoblasts. This finding sheds new light on the molecular mechanisms of Duchenne Muscular Dystrophy pathogenesis.

Introduction

Duchenne Muscular Dystrophy (DMD) is an inherited disease caused by deficiency of full-length dystrophin due to mutations in the dystrophin-encoding gene. In the mature muscle dystrophin localizes to the cytoplasmic face of the plasma membrane. Its N-terminus interacts with the actin cytoskeleton while C-terminal domains anchor the dystrophin associated protein complex (DAP). Thus, dystrophin connects the cytoskeleton with the membrane complex and indirectly, extracellular matrix components interacting with the DAP. Although DMD is generally recognized as primarily a muscle disease, the dystrophin deficiency results in multiple biochemical changes not only in muscle but also in various non-muscle cells. Among abnormalities observed in DMD, defects of the mitochondrial oxidative metabolism and altered cellular calcium homeostasis seem to be those most prominent.

The *mdx* mouse is a commonly used animal model of DMD. The *mdx* dystrophin gene has a point mutation in exon 23 that results in a stop codon and produces premature termination of translation of the full-length dystrophin transcript leading to dystrophin deficiency [1,2]. An impairment of energy metabolism in *mdx* mouse muscle was described many years ago [3]. It was also shown that activity of respiratory chain enzymes and the maximal rate of oxygen consumption were substantially lower in skeletal muscle cells derived from the *mdx* mouse. Interestingly, no alterations were observed in the cardiac muscle of the same mice [4]. Subsequently, high resolution respirometry of skeletal muscle fibers was proposed as an effective tool for evaluation of the defects in energy metabolism in muscle biopsies from children suffering from different neuromuscular disorders, including DMD [5].

Studies on DMD are usually conducted using differentiated muscle, i.e. myotubes or myofibers. This reflects the consensus that, since dystrophin expression in undifferentiated cells is very low or absent [6–10], no phenotypic consequences of mutation in the dystrophin gene are to be expected in such cells. Contrary to this belief, Ferrari et al. [11] described an increased sensitivity to purinergic stimulation in lymphoblastoid cells derived from DMD patients—cells which contain a small amount of dystrophin mRNA but no protein. More recently, we showed that myoblasts derived from the *mdx* mouse (SC-5 cells) were more susceptible to nucleotide stimulation than their normal equivalents (IMO

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DMD, Duchenne Muscular Dystrophy; DMSO, dimethylsulfoxide; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide; PBS, phosphate buffered saline; ROS, reactive oxygen species.

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cells). Importantly, dystrophin was not detected by Western blotting in either myoblast line while full-length dystrophin mRNA was found in the IMO but not in the SC-5 cells [10]. An arising question was whether the differences between normal and dystrophic myoblasts are limited to their susceptibility to nucleotide stimulation or whether they concern other basic functions of these cells as well. It is noteworthy that affected purinergic response, known to influence intracellular calcium homeostasis, may have an important impact on mitochondria critical to the latter. Thus, the aim of the present study was to compare energy metabolism and other mitochondrial parameters of normal and dystrophic mouse myoblasts. We found that the dystrophin gene mutation severely disturbs energy metabolism in the affected cells. This finding offers a novel insight into the biochemical mechanisms of DMD.

Materials and methods

Cell culture. Immortalized *mdx* mouse (SC-5) and dystrophin-positive control (IMO) myoblasts were used. These two cell lines were of the same genetic background, as the two mouse strains from which the cells were derived originated from the same parental inbred mouse strain *H2Kb-tsA58* ("immorto mouse"), expressing the SV40 large T antigen gene with the tsA58 mutation. The *H2Kb-tsA58 mdx* strain was established by crossing male mouse homozygous for *H2Kb-tsA58* with female *mdx* mice. SC-5 and IMO cells are highly homogenous populations differing only in the presence or absence of a point mutation in the dystrophin gene [10,12,13]. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma Chemical Company, St. Louis, MO, USA) supplemented with 20% fetal calf serum, 4 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 20 U/ml murine γ-interferon (Invitrogen, San Diego, CA, USA) at 33 °C.

Cell respiration. The rate of cell respiration was measured with the use of oxygraph-2K (OROBOROS) [14]. Myoblast monolayer was trypsinized, harvested and centrifuged for 3 min (500*g*). Oxygen consumption was measured in intact cells suspended in PBS in the presence of sequentially added 1 mM pyruvate, 5 mM glucose, 0.1 mg/ml oligomycin and 0.25 μM CCCP.

Mitochondrial membrane potential ($\Delta\Psi$). $\Delta\Psi$ in intact cell was measured fluorimetrically with JC-1 probe, using laser scanning cytometry [16]. The cells grown in 24-well plates were incubated in 2 ml of the culture medium containing 2 μl JC-1 solved in DMSO. After 15 min at 37 °C they were washed with PBS containing 1 mM pyruvate and 5 mM glucose. Fluorescence was measured using an iCys Laser Scanning Microscope (Compucyte), at 488 nm excitation and 530/590 nm emission wavelengths, in 10^4 cells. Data were expressed as the ratio of fluorescence at the two emission wavelengths.

ROS production. Reactive oxygen species were measured fluorimetrically with DCF probe using Multimode microplate reader (Tecan Infinite F200). Cell monolayers rinsed with PBS were incubated with 10 μM DCF at 37 °C for 30 min. Wavelengths for excitation and emission were 485 and 520 nM, respectively. Data computations were performed using Magellan software.

Intracellular ATP content. ATP was measured spectrofluorimetrically in neutralized acidic cellular extracts, using standard enzymatic assay with hexokinase and glucose-6-phosphate dehydrogenase [15].

Lactate synthesis. Lactate was measured spectrofluorimetrically using standard enzymatic assay with lactate dehydrogenase [17] in neutralized acidic extracts of cells previously incubated for 30 min at 33 °C in PBS containing 5 mM glucose.

Mitochondrial organization. Cells grown on coverslips were loaded with 300 nM MitoTracker CMX Ros (Invitrogen, San Diego, CA, USA) for 20 min at room temperature. After rinsing with medium cells were fixed with 4% paraformaldehyde, rinsed with PBS containing 10% serum, sealed in Glycergel Mounting Medium (DakoCytomation) and viewed under a Leica TCS SP2 Spectral Confocal and Multiphoton Microscope.

Western blotting for mitochondrial complexes. Subunits of mitochondrial complexes III and V were detected with MitoProfile Human Total OXPHOS Complex Detection Kit (MitoScience) following SDS–PAGE and blotting of total cellular lysates.

Protein assay. Protein was measured in cells solubilized in 0.5 M NaOH according to Bradford [18] using commercially available Protein Assay Kit (Bio-Rad).

Statistical analysis. Quantitative data are expressed as means ± standard deviations. Statistical significance was calculated using Student's *t*-test.

Results and discussion

Mutations in the dystrophin gene result in a broad spectrum of abnormalities, leading to cell malfunctioning and premature death. Among the various cell types, differentiated muscle cells are the most severely affected. The major deficiencies involve calcium homeostasis and energy metabolism [19–22]. It is assumed that these defects are caused by a lack of dystrophin proteins. Data shown here clearly indicate that immature dystrophic muscle cells, myoblasts also exhibit severe changes in crucial mitochondrial parameters in comparison to their healthy equivalents. These abnormalities cannot be due to a lack of dystrophin, as it is not present in normal myoblasts either.

The obvious difference in mitochondrial morphology between normal (IMO) and *mdx* myoblasts (SC-5) (Fig. 1) immediately suggest affected mitochondrial functioning in the latter. To verify this, several basic parameters relevant to mitochondria were compared between the two cell lines.

Respiration analysis (Fig. 2) shows that basal oxygen consumption with endogenous substrates is substantially slower in SC-5 cells than in IMO. This difference remains evident after sequential addition of pyruvate and glucose to the incubation. The reduction of the respiration rate triggered by glucose exclusively in SC-5 cells indicates that the Crabtree effect occurs in *mdx* myoblasts but not in normal ones [23]. The cause of this differential response is unknown, but it might be due to aberrant intracellular calcium signaling and homeostasis in *mdx* myoblasts (Zablocki, unpublished observation) [10,23]. Addition of oligomycin reduces respiration to exactly the same level in both cell lines, indicating that the difference observed in the absence of oligomycin does not reflect partial uncoupling of oxidative phosphorylation in IMO myoblasts. Similarly, the fast but identical oxygen consumption found in both cell lines after addition of a mitochondrial uncoupler (CCCP) demonstrates that the maximal activity of the respiratory chain is the same in both cell lines. These data suggest that the slower oxygen consumption by SC-5 cells reflects a higher respiratory control due to limited ATP synthesis in these cells compared with that in IMO myoblasts. The substantially higher mitochondrial membrane

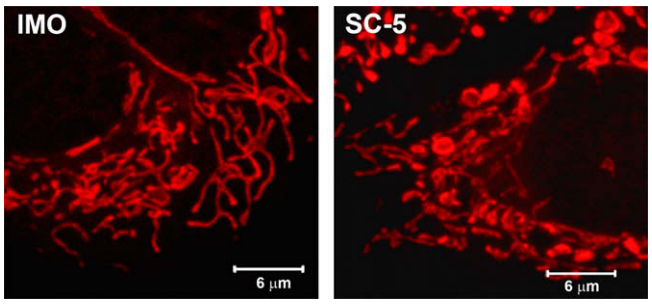

Fig. 1. Intracellular mitochondrial organization in SC-5 and IMO cells. Confocal images of mitochondria labeled with the MitoTracker CMX Ros.