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Original Contribution

Low intensity training of *mdx* mice reduces carbonylation and increases expression levels of proteins involved in energy metabolism and muscle contraction

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ABSTRACT

High intensity training induces muscle damage in dystrophin-deficient *mdx* mice, an animal model for Duchenne muscular dystrophy. However, low intensity training (LIT) rescues the *mdx* phenotype and even reduces the level of protein carbonylation, a marker of oxidative damage. Until now, beneficial effects of LIT were mainly assessed at the physiological level. We investigated the effects of LIT at the molecular level on 8-week-old wild-type and *mdx* muscle using 2D Western blot and protein–protein interaction analysis. We found that the fast isoforms of troponin T and myosin binding protein C as well as glycogen phosphorylase were overcarbonylated and downregulated in *mdx* muscle. Some of the mitochondrial enzymes of the citric acid cycle were overcarbonylated, whereas some proteins of the respiratory chain were downregulated. Of functional importance, ATP synthase was only partially assembled, as revealed by Blue Native PAGE analysis. LIT decreased the carbonylation level and increased the expression of fast isoforms of troponin T and of myosin binding protein C, and glycogen phosphorylase. In addition, it increased the expression of aconitate hydratase and NADH dehydrogenase, and fully restored the ATP synthase complex. Our study demonstrates that the benefits of LIT are associated with lowered oxidative damage as revealed by carbonylation and higher expression of proteins involved in energy metabolism and muscle contraction. Potentially, these results will help to design therapies for DMD based on exercise mimicking drugs.

Introduction

Duchenne muscular dystrophy (DMD) is a lethal inherited neuromuscular disease caused by mutations in the *DMD* gene. A lack of dystrophin in skeletal muscle of DMD patients causes injuries through multiple pathogenic mechanisms, including mechanical weakening of the sarcolemma [1], inappropriate calcium flux [2], and increased oxidative stress [3].

Physical exercise causes mechanical stress, calcium flux, and oxidative stress in skeletal muscle [4] and thereby, high intensity training (forced, above fatigue threshold, and damaging) was used to injure muscles of dystrophin-deficient *mdx* mice, an animal model for DMD [5]. In contrast, low intensity training (LIT) (voluntary, short, and nondamaging) rescued *mdx* mice phenotypes. Improved force output, tetanic tension, and endurance capacities of *mdx* muscles were reported after low intensity swimming [6] and running [7,8]. Another study also showed a reduction of markers of oxidative stress in *mdx* gastrocnemius after low intensity running [9]. This effect of low intensity training was especially interesting, given the fact that oxidative stress was thought to play a role in exacerbation of DMD pathology [10].

Oxidative stress is defined as "an imbalance between oxidants and antioxidants in favor of the oxidants, leading to a disruption of redox signaling and control, and/or molecular damages" [11]. One of the most common types of oxidative modification is protein carbonylation, the introduction of carbonyl groups (C=O) in a protein [12]. We chose protein carbonylation as a marker of oxidative stress, because it is a reliable indicator of oxidative damages [13], suitable for proteomic analysis [14] and commonly used on *mdx* muscle [15,16]. Studies reported an abnormal oxidative stress in skeletal muscle of DMD patients and *mdx* mice [17,18]. Indeed, myofibers lacking dystrophin were highly susceptible to oxidant-induced injury [19] and thus, the

Abbreviations: 2D-PAGE, two dimensional polyacrylamide gel electrophoresis; BN-PAGE, Blue Native polyacrylamide gel electrophoresis; Co-IP, coimmunoprecipitation; DMD, Duchenne muscular dystrophy; DNPH, 2,4-dinitrophenylhydrazine; IEF, isoelectric focusing; LIT, low intensity training; GP, glycogen phosphorylase; MyBP-C, myosin binding protein C

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protein carbonylation level correlated with the degree of the disease [20]. In the absence of dystrophin, oxidative stress acts together with mechanical stress to worsen fiber damage [17].

In healthy muscle, physical exercise leads to a production of oxidants through the mitochondrial electron transport chain [21], sarcolemmal NADPH oxidase [22], and xanthine oxidase [23]. These oxidants participate in cell signaling through MAPK and JNK pathways [24], leading to muscle adaptation to training (for example, overexpression of mitochondrial enzymes) [25]. In *mdx* muscle, this production of oxidants is known to be abnormally amplified by a mitochondrial overload of Ca^{2+} [4] and an overactivation of the NADPH oxidase 2 [26]. As a consequence, MAPK and JNK signaling pathways have been shown to be altered [27].

We aimed to clarify how low intensity training improved *mdx* phenotypes despite an abnormal oxidative environment. Thus, we investigated, for the first time, the effects of low intensity training at the protein level. Protein downregulation has been previously reported in nonexercised *mdx* muscle [28]. Our first hypothesis was that overcarbonylated proteins in nonexercised *mdx* muscle would be also downregulated and would lose protein-protein interactions, since carbonyl adducts target proteins for proteasomal degradation [29] and potentially affect interactions between proteins [30]. Our second hypothesis was that low intensity training would rescue proteins impaired in nonexercised *mdx* muscle, because physical exercise upregulates antioxidant defenses [31] and stimulates muscle plasticity [32].

We performed an extensive proteomic study on gastrocnemius muscle of 8-week-old *mdx* mice using 2D electrophoresis, known for its excellent reproducibility [33] and its reliability in skeletal muscle protein analysis [21,34,35]. Carbonylated proteins were detected by 2D carbonylated protein Western blot, protein expression was measured by 2D-PAGE, and protein–protein interactions were assessed by Blue Native PAGE (BN-PAGE). Detected proteins were identified by mass spectrometry.

Here we show that in nonexercised *mdx* muscle, proteins from muscle contraction and glycogen metabolism were both overcarbonylated and downregulated. Also, two complexes composed of ATP synthase subunits were absent. In exercised *mdx* muscle, these proteins were less carbonylated and higher expressed, and the ATP synthase complex was restored. Specifically, expression of the slow isoforms of the muscle contraction proteins troponin T and myosin binding protein C (MyBP-C) was increased, while carbonylation and expression level of fast isoforms were restored to the level of exercised wild-type mice. Thus, we demonstrated that the benefits of LIT are associated with lower carbonylation and higher expression of proteins involved in energy metabolism and muscle contraction.

Materials and methods

Animals

Eight-week-old male *mdx* mice with C57BL/6 background and age-matched wild-type C57BL/6 male control mice were used in this study. All experimental protocols were approved by The Experimental Animal Care and Use Committee of the National Institute of Neuroscience, National Center of Neurology and Psychiatry (NCNP), Tokyo, Japan.

Low intensity training protocol

Mice underwent training when they reached 4 weeks old. According to previously described protocol [7], mice were introduced into a tank filled with water (maintained at 35 ± 1 °C) to a depth enough to allow them to swim. Animals completed a 4 week program, in which they exercised 4 days (Monday, Tuesday, Thursday, and Friday) in a week for 30 min per day. A rest was given the three other days. Animals were not forced to move and were free to stand by at will.

Physiological tests

For serum creatine kinase measurement, blood was taken from the tail artery and centrifuged at 3000*g* for 10 min. Creatine kinase was assayed with the Fuji Drychem system (Fuji Film Medical Co. Ltd, Tokyo, Japan) as previously described [36]. Grip strength of both forelimb and hind limb was assessed by a grip strength meter, to determine the effects of LIT on whole body musculature of the mice (MK-380M; Muromachi Kikai), as previously described [37].

Then, mice were sacrificed by cervical dislocation. Gastrocnemius muscles were dissected and flash-frozen for histology or stored at −80 °C for 2D electrophoresis, Western blot, and PCR analysis. We assessed the effects of LIT on gastrocnemius, a muscle predominantly activated during swimming exercise [38].

Hematoxylin and eosin (H&E) staining

Frozen gastrocnemius muscles were cut in 20 μm sections using a cryostat and stained using Harris H&E as previously described [37].

Protein sample preparation for 1D and 2D carbonylated protein Western blot or Western blot

Muscles were homogenized using a lysis buffer made of 8 M urea, 2 M thiourea, 4% (w/v) Chaps, 12 μl/ml Destreak (Invitrogen, Carlsbad, CA), and clarified by centrifugation. Protein concentration was determined by the Bradford method (Bio-Rad Life Science, Hercules, CA). Twenty micrograms of proteins were prepared according to the Millipore protein oxidation detection kit instructions for 1D carbonylated protein Western blot, or prepared for classical Western blot. For 2D carbonylated protein Western blot, 200 μg of proteins was diluted in a rehydration solution made of 8 M urea, 1 M thiourea, 2% (w/v) Chaps, 12 μl/ml Deastreak, 0.5% (v/v), IPG buffer (GE Healthcare, Tokyo, Japan), and 0.001% of Coomassie blue, for a final volume of 250 μl. Then, they were charged on 13 cm (carbonylated protein Western blot) IGP strips, pH 3–10 Non Linear, overnight at room temperature, and isofocused with IPGphor (GE Healthcare) at the following profile: 500 V at 500 V/h, 1000 V in gradient at 1000 V/h, 6000 V in gradient at 20,000 V/h, and 6000 V at 12,000 V/h. After that, strips were prepared as previously described [39]. Briefly, strips were incubated for 20 min in derivatization solution (10 mM DNPH, 2 M HCl) and washed for 10 and 30 min in neutralizing solution (2 M Tris, 30% (v/v) glycerol).

Electrophoresis and immunoblotting

Proteins were separated in SDS-PAGE gels (12% (v/v) polyacrylamide). For each condition, two gels were performed in parallel, one for colloidal blue staining of total proteins and the other one for electroblotting onto nitrocellulose membrane. After blocking, membranes were incubated with corresponding antibody (see supplementary material and methods) and developed using an Amersham ECL Plus Western blotting detection system. Films were digitized with Epson GT-X900 scan and densitometric analyses were performed using ImageJ software (developed by U.S. National Institutes of Health and available at http://imagej.nih.gov/ij/).

Protein sample preparation for Blue Native PAGE

For sample preparation, muscles were homogenized using a BN-lysis buffer (20 mM Tris-HCl, 137 mM NaCl, 0.2 mM EDTA,