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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 $35 \pm 1$ °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,

10% (v/v) glycerol, protease inhibitor cocktail, 1% digitonin, adjusted to pH 7), as previously described [40] and centrifuged at 600*g* at 4 °C for 10 min to remove tissue debris. Supernatants were dialyzed overnight in a 10,000 MWCO dialysis cassette (Thermo Fisher Scientific Inc., Waltham, MA, USA) with 0.3% digitonin in BN-lysis buffer. Protein quantification was performed using the Bradford method (Bio-Rad protein assay) with BSA as a standard.

*Blue Native PAGE*

Samples of 200 μg of proteins were homogenized using a NativePAGE sample preparation kit (Invitrogen, Carlsbad, CA, USA), according to the instructions of the manufacturer, but without digitonin. Proteins were separated in a first dimension in a 4–15% acrylamide-bisacrylamide gel, incubated for 15 min at room temperature in SDS sample buffer made of 12.5 mM Tris, 4% (w/v) SDS, 20% (w/v) glycerol, and 0.02% (w/v) bromophenol blue, reincubated for 20 min in the same SDS sample buffer, boiled at 100 °C, and then separated in second dimension in 12% (v/v) polyacrylamide SDS-PAGE gel and stained by Coomassie blue.

*Data acquisition and analysis of Western blots and Blue Native PAGE*

For 2D carbonylated protein Western blot, spots were quantified by densitometry in nitrocellulose membranes using ImageJ software. Spots were normalized with corresponding Coomassie blue values. These are reported in Supplementary Table 2.

*In-gel digestion, mass spectrometry protein identification, database searches*

All the following step were performed by the "Plateforme de protéomique de l'Université Paris-Descartes 3P5" (France) and described in detail in the supplementary material and methods. The identity of detected proteins is reported in Supplementary Table 1.

*Coimmunoprecipitation*

Coimmunoprecipitation was performed using Novex Dynabeads protein G immunoprecipitation kit (Life Technologies Japan Ltd., Tokyo, Japan) according to the manufacturer's instructions. Corresponding antibody was used to detect the prey protein in Western blotting, depending on the bait antibody [41].

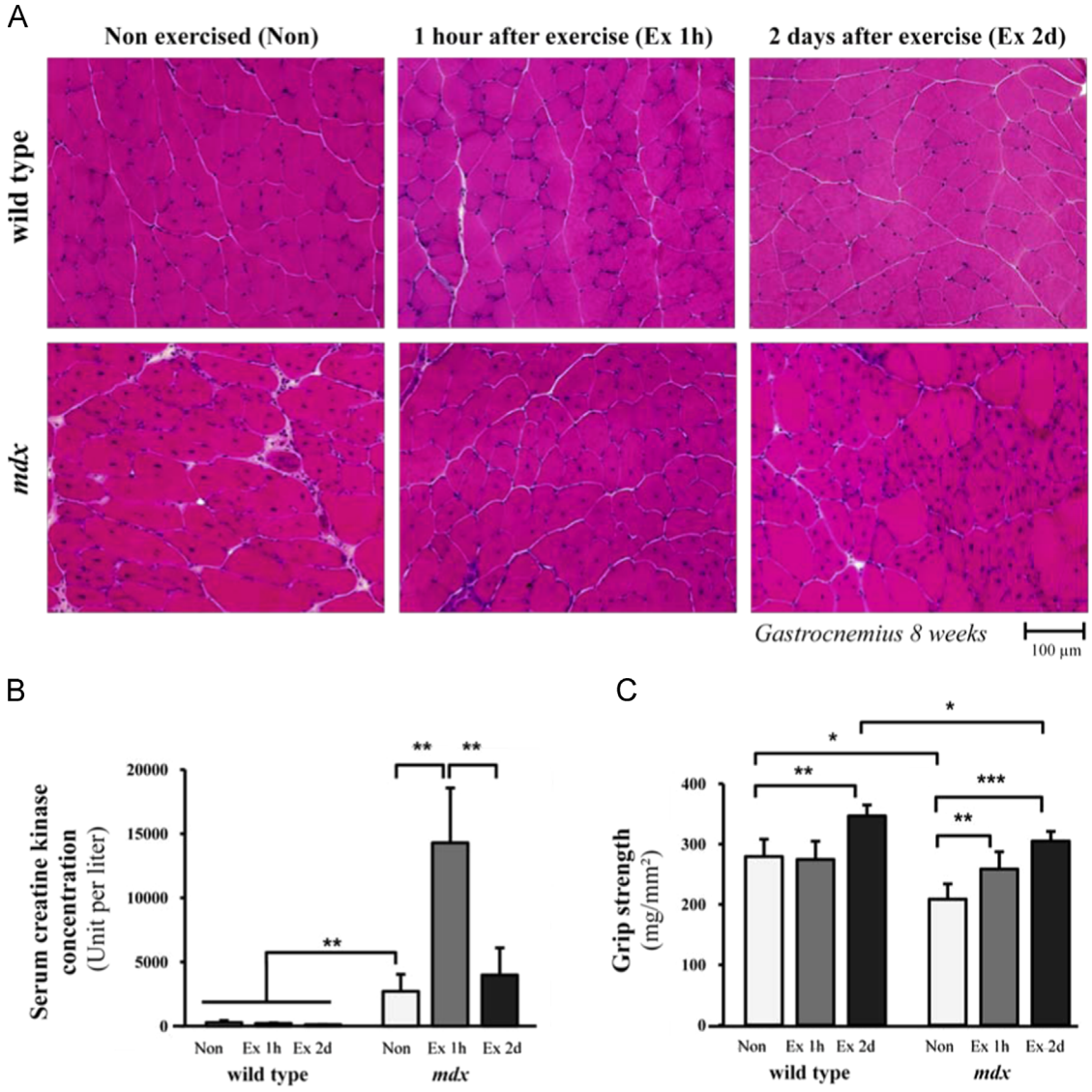


**Fig. 1.** Profile of 8-week-old wild-type and *mdx* mice after 4 weeks of swimming exercise. H&E staining of gastrocnemius muscle (A), serum creatine kinase concentration (B), and grip strength (C) from 8-week-old wild-type and *mdx* mice, nonexercised (Non) or after 4 weeks of swimming exercise, 1 h (Ex 1h) or 2 days (Ex 2d) after the last session. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ means a significant difference between two groups. $n = 4$ to 6 per group. Scale bar represents 100 μm.

*Sample preparation for PCR*

Total RNA was extracted from muscles using TRIzol (Invitrogen). One microgram of total RNA template was used for PCR with a QuantiTect reverse transcription kit (Qiagen, Crawley, UK), according to the manufacturer's instructions. The cDNA product (1 μl) was then used as template for PCR in a 25 μl reaction solution with 0.125 unit of TaqDNA polymerase (Qiagen). The reaction mixture comprised 10X PCR

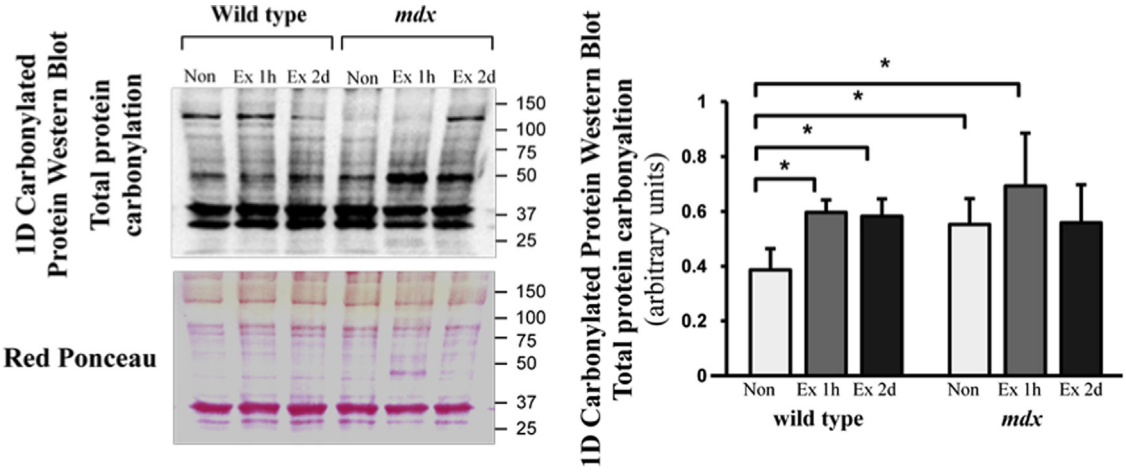


**Fig. 2.** Total protein carbonylation level in wild-type and *mdx* gastrocnemius muscle. Carbonylated proteins were detected by 1D carbonylated protein Western blot in gastrocnemius muscle of 8-week-old wild-type and *mdx* mice, nonexercised (Non) or after 4 weeks of swimming exercise, 1 h (Ex 1h) or 2 days (Ex 2d) after the last session. Ponceau red staining is shown as loading control. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ means a significant difference between two groups. $n = 4$ to 6 per group.

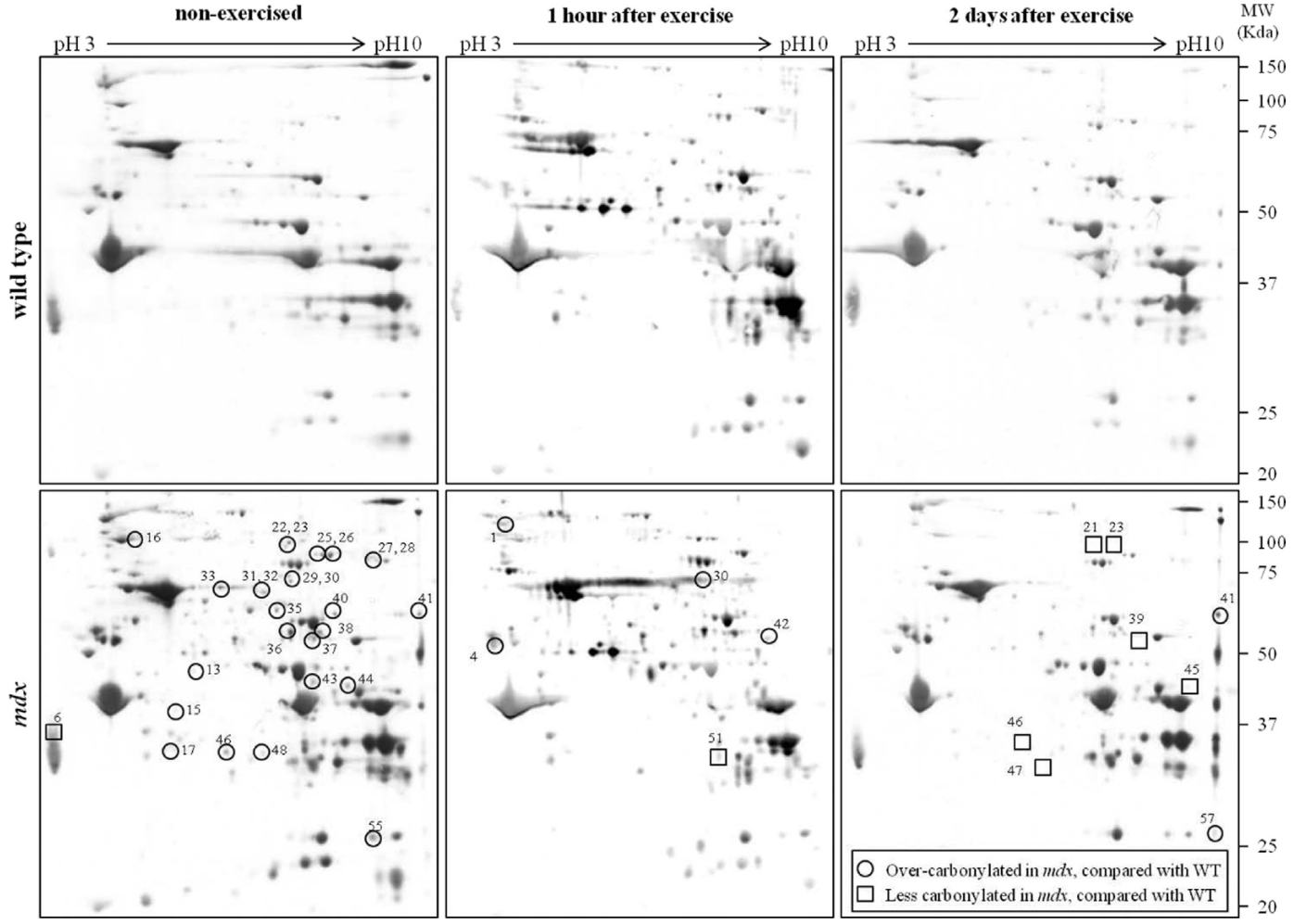


**Fig. 3.** Representative 2D carbonylated protein Western blot of gastrocnemius muscle from 8-week-old nonexercised and exercised wild-type and *mdx* mice. Proteins were separated by IEF and carbonylated proteins were derivatized with DNPH in the strip (13 cm, 3–10 NL). Second dimension was performed in 12% acrylamide SDS PAGE. Protein carbonylation was compared two by two between wild-type and *mdx* mice for each condition: nonexercised, 1 h after the last swimming session or 2 days after. ○ represents overcarbonylated spots in *mdx* in comparison with wild-type. □ represents less carbonylated spots in *mdx* in comparison with wild-type. Numbers indicate the spots identified by MS (Supplementary Table 1A) and listed in Table 1. For each experiment, gastrocnemius muscles from five different mice were pooled and electrophoresis was performed in triplicate.

buffer (Roche, Basel, Switzerland), 10 mmol/L of each dNTP (Qiagen), and 10 μmol/L of each primer. The primers for PCR were synthesized by Operon Biotechnologies (Tokyo, Japan) and are listed in Supplementary Table S2. The cycling conditions were 95 °C for 4 min, 35 cycles at 94 °C for 1 min, 60 °C for 1 min, 72 °C for 1 min, and finally 72 °C for 7 min. The intensity of PCR bands was analyzed by ImageJ software. Relative gene expression levels were normalized to those of 18S rRNA.

### *Statistics*

Values are reported as mean ± SD (standard deviation). The number of mice analyzed per group is shown in the figure legends. Statistically significant differences between two groups were determined by Student's *t* test, with a *P* value of $P < 0.05$ considered significant. Statistically significant differences between more than two groups were determined by ANOVA test followed by Tukey's range test, a *P* value of $P < 0.05$ was considered significant for ANOVA test, and a result superior to the minimum significant difference was considered significant for the Turkey test.

## Results

### *Effects of low intensity swimming on 8-week-old mice*

We assessed the effects of swimming exercise on wild-type and *mdx* muscle. No changes were observed in sections from gastrocnemius muscle after 1 h past the last session of 4 weeks of swimming (Fig. 1A), even though creatine kinase levels were significantly increased ($P < 0.01$) at this time. Creatine kinase levels returned to nonexercised values after 2 days (Fig. 1B). Of note, exercise led to an increase in grip strength of wild-type and *mdx* mice (Fig. 1C).

### *Total protein carbonylation level after low intensity swimming*

The level of oxidative stress in muscle was quantified by measurement of protein carbonylation [12]. Carbonyls groups were derivatized into their DNP adducts using DNPH and these were detected by carbonylated protein Western blot. In wild-type muscle, total protein carbonylation levels increased 1 h after exercise ($P < 0.05$) and remained elevated 2 days after, as observed in previous studies [42]. Interestingly, in *mdx* muscle, total protein carbonylation level was not significantly different after exercise (Fig. 2).

### *Influence of low intensity training on the proteome of gastrocnemius muscle*

Proteomic analysis of wild-type and *mdx* gastrocnemius muscles was performed by 2D carbonylated protein Western blot to detect alterations in protein carbonylation (Fig. 3), 2D-PAGE to examine protein expression (Fig. 4), and BN-PAGE to detect protein–protein

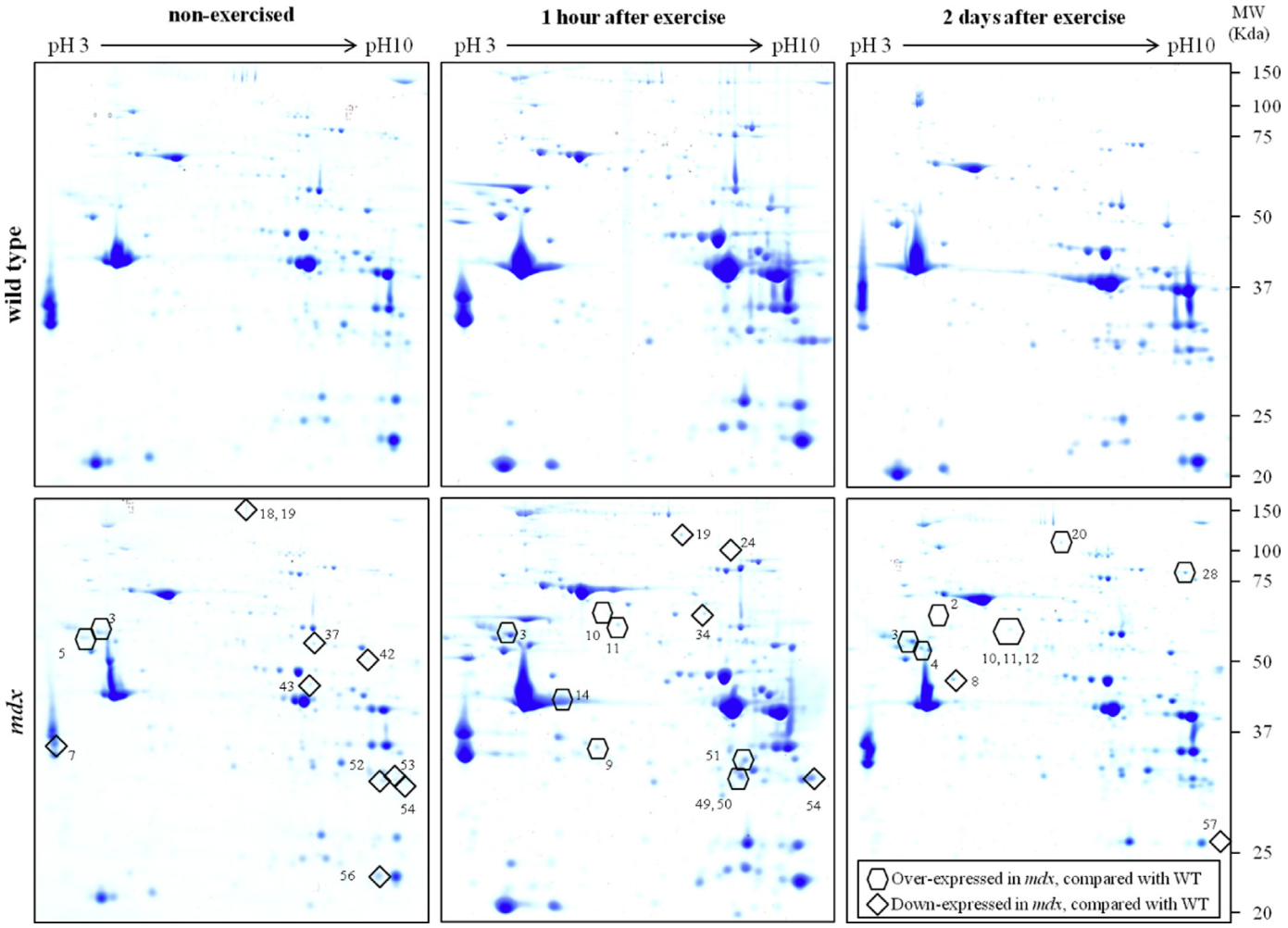


**Fig. 4.** Representative Coomassie blue staining of gastrocnemius muscle from 8-week-old nonexercised and exercised wild-type and *mdx* mice. Corresponding Coomassie blue gels related to Fig. 3. Protein expression was compared two by two between wild-type and *mdx* mice. ⬡ represents overexpressed spots in *mdx* in comparison with wild type. ◇ represents downregulated spots in *mdx* in comparison with wild-type muscle. Numbers indicate spots identified by MS (Supplementary Table 1A) and listed in Table 2.

interaction (Fig. 5). In a first step, we compared nonexercised wild-type muscle with nonexercised *mdx* muscle and in a second one, nonexercised muscle with exercised ones in both types of mice. Spots displaying a change of at least 50% were selected for mass spectrometrical identification. A total of 76 spots were identified (Supplementary Table 1) and grouped by categories according to the SwissProt/UniProtKB data base (Tables 1–4).

### *Proteome of 8-week-old nonexercised wild-type and mdx gastrocnemius muscle*

Overcarbonylated proteins in *mdx* muscle were mainly involved in the citric acid cycle, for example, succinate dehydrogenase, aconitate hydratase, in muscle contraction such as the fast isoforms of troponin T and MyBP-C, in glycogen metabolism, such as glycogen phosphorylase (GP) and glycolysis and in cytoskeleton (Table 1A and Fig. 3).

Downregulated proteins were involved in the respiratory chain, muscle contraction, glycogen metabolism, and the stress response. Interestingly, fast isoforms of troponin T and MyBP-C and glycogen phosphorylase were both carbonylated and downregulated. Overexpressed proteins were involved in glycolysis and in the microtubular cytoskeleton (Tables 2 and 3A and Figs. 4 and 5).

Protein–protein interaction analysis by BN-PAGE revealed the absence of ATP synthase subunits α and β in *mdx* muscle (Table 3D and Fig. 5).

### *Proteome of nonexercised and exercised gastrocnemius in wild-type or mdx muscle*

We compared nonexercised muscle with exercised muscle 2 days after the last swimming session (Table 4 and Figs. 3–5). In wild-type muscle, LIT increased protein carbonylation, but had little influence on protein expression. Proteins involved in the citric acid cycle, the fast isoforms of troponin T and MyBP-C, and UTP-glucose-1-phosphate uridylyl transferase were more carbonylated in exercised wild-type muscle (Fig. 3) as compared to control, whereas beta-enolase was overexpressed (Figs. 4 and 5).

In *mdx* muscle (Table 4 and Figs. 3–5), LIT decreased the carbonylation and enhanced the expression of specific proteins. While total protein carbonylation remained unchanged by exercise (Fig. 2), voltage-dependent anion-selective channel protein 1, fast isoforms of troponin T and MyBP-C, and phosphoglucomutase-1 were less carbonylated in exercised *mdx* muscle (Fig. 3). In contrast to wild-type muscle, LIT increased protein expression of some respiratory chain proteins, the fast isoforms of troponin T and

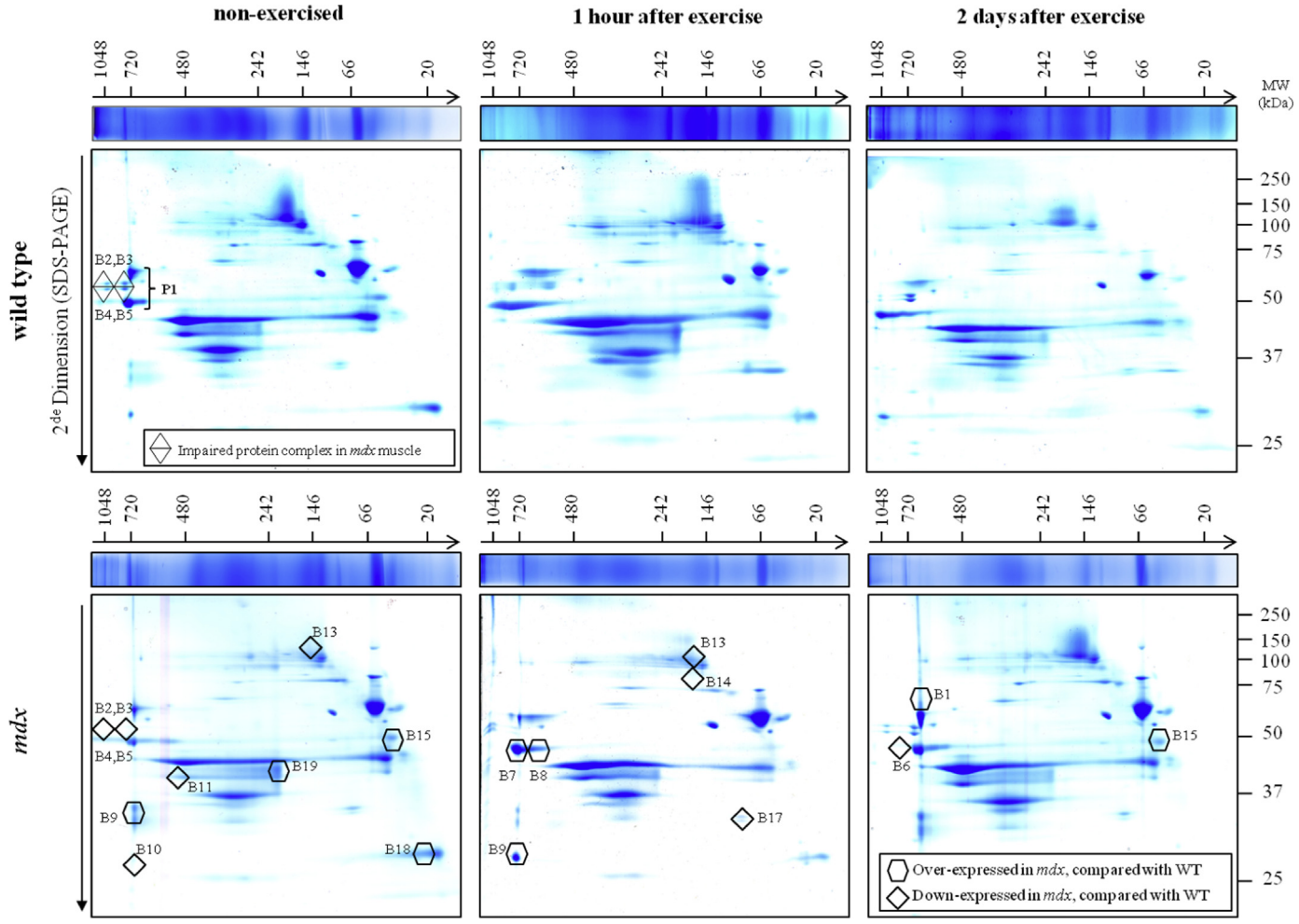


**Fig. 5.** Representative Blue Native PAGE of gastrocnemius muscle from 8-week-old nonexercised and exercised wild-type and *mdx* mice. Proteins were separated under nondenaturing conditions in 4–15% acrylamide-bisacrylamide gel. Second dimension was performed in 12% acrylamide SDS PAGE. ◇ represents the protein complexes present in wild-type, but absent in *mdx* gels. P numbers represent protein complexes listed in Table 3D. As for Fig. 3, protein expression was compared two by two between wild-type and *mdx* mice. ⬡ represents overexpressed spots in *mdx* in comparison with wild type. ⋄ represents downregulated spots in *mdx* in comparison with wild type. B numbers indicate spots identified by MS (Supplementary Table 1B) and listed in Table 3. For each experiment, gastrocnemius muscles from five different mice were pooled and electrophoresis was performed in triplicate.

**Table 1**
Identity of proteins whose carbonylation differs in mdx gastrocnemius, in comparison with wild type.

Protein carbonylation in mdx muscle (in comparison with wild type)

| | A. Nonexercised | | | | B. Exercised, 1 h after the last session | | | | C. Exercised, 2 days after the last session | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Spot No. [a] | Accession No. [b] | Protein name | Fold change [c] | Spot No. [a] | Accession No. [b] | Protein name | Fold change [c] | Spot No. [a] | Accession No. [b] | Protein name | Fold change [c] |
| ↑ | Mitochondria | | | | Mitochondria | | | | Muscle contraction | | | |
| | 29 | Q8CAQ8 | Mitochondrial inner membrane protein | 20 | 30 | Q8CAQ8 | Mitochondrial inner membrane protein | 5.26 | 57 | P05977 | Myosin light chain 1/3, skeletal muscle | Only mdx |
| | 30 | Q8CAQ8 | Mitochondrial inner membrane protein | 14.29 | 42 | Q91YT0 | NADH dehydrogenase flavoprotein 1 | 3.85 | 41 | Q9JIF9 | Myotilin | Only mdx |
| | 31 | Q8K2B3 | Succinate dehydrogenase flavoprotein sub | 14.29 | Cytoskeleton | | | | Others | | | |
| | 32 | Q8K2B3 | Succinate dehydrogenase flavoprotein sub | 14.29 | 4 | P31001 | Desmin | 3.13 | 57 | O70250 | Phosphoglycerate mutase 2 | Only mdx |
| | 38 | Q9D0K2 | Succinyl-CoA:3-ketoacid coA transferase 1 | 14.29 | Other | | | | 57 | Q9CQA3 | Succinate dehydrogenase iron-sulfur subunit | Only mdx |
| | 43 | P35486 | Pyruvate dehydrogenase E1 subunit alpha | 7.69 | 1 | P59242 | Cingulin | 3.23 | ↓ | Mitochondria | | |
| | 33 | P13707 | Glycerol-3-phosphate dehydrogenase | 5 | ↓ | Glycolysis | | | 45 | Q9DB77 | Cytochrome b-c1 complex subunit 2 | -6.06 |
| | 25 | Q99KI0 | Aconitate hydratase, mitochondrial | 4.76 | 51 | P05064 | Fructose-bisphosphate aldolase A | -3.72 | 39 | Q03265 | ATP synthase subunit alpha, mitochondrial | -3.07 |
| | 26 | Q99KI0 | Aconitate hydratase, mitochondrial | 4.35 | 51 | P16858 | Glyceraldehyde-3-phosphate dehydrogenase | -3.72 | Glycogen metabolism | | | |
| | 36 | O08749 | Dihydrolipoyl dehydrogenase, mitochondrial | 4.35 | | | | | 21 | Q9WUB3 | Glycogen phosphorylase, muscle form | -5.01 |
| | Muscle contraction | | | | | | | | 23 | Q9WUB3 | Glycogen phosphorylase, muscle form | -5.16 |
| | 48 | Q9QZ47 | Troponin T, fast skeletal muscle | 7.69 | | | | | 39 | Q91ZJ5 | UTP-glucose-1-phosphate uridylyltransferase | -3.07 |
| | 16 | Q5XKE0 | Myosin-binding protein C, fast-type | 5.56 | | | | | Muscle contraction | | | |
| | 41 | Q9JIF9 | Myotilin | 5 | | | | | 46 | Q9QZ47 | Troponin T, fast skeletal muscle | -3.31 |
| | 46 | Q9QZ47 | Troponin T, fast skeletal muscle | 5 | | | | | Others | | | |
| | Glycogen metabolism | | | | | | | | 47 | P14152 | Malate dehydrogenase, cytoplasmic | -7.27 |
| | 35 | Q9D0F9 | Phosphoglucomutase-1 | 8.33 | | | | | 46 | P07310 | Creatine kinase M-type | -3.31 |
| | 23 | Q9WUB3 | Glycogen phosphorylase, muscle form | 7.69 | | | | | 39 | P97384 | Annexin A11 | -3.07 |
| | 22 | Q9WUB3 | Glycogen phosphorylase, muscle form | 6.25 | | | | | | | | |
| | 37 | Q91ZJ5 | UTP-glucose-1-phosphate uridylyltransferase | 6.25 | | | | | | | | |
| | Glycolysis | | | | | | | | | | | |
| | 40 | P52480 | Pyruvate kinase isozymes M1/M2 | 33.33 | | | | | | | | |
| | 55 | O70250 | Phosphoglycerate mutase 2 | 7.69 | | | | | | | | |
| | 48 | P21550 | Beta-enolase | 7.69 | | | | | | | | |
| | 13 | P21550 | Beta-enolase | 4 | | | | | | | | |
| | Cytoskeleton | | | | | | | | | | | |
| | 29 | P48678 | Prelamin-A/C | 20 | | | | | | | | |
| | 31 | O88342 | WD repeat-containing protein 1 | 14.29 | | | | | | | | |
| | 15 | P68033 | Actin, alpha cardiac muscle 1 | 7.14 | | | | | | | | |
| | 28 | Q9JKS4 | LIM domain-binding protein 3 | 5 | | | | | | | | |
| | 27 | Q9JKS4 | LIM domain-binding protein 3 | 4.76 | | | | | | | | |
| | 17 | P47753 | F-actin-capping protein subunit alpha-1 | 4 | | | | | | | | |

| | | | | |
|---|---|---|---|---|
| Others | | | | |
| 44 | P07310 | Creatine kinase M-type | 10 | |
| 43 | P62196 | 26S protease regulatory subunit 8 | 7.69 | |
| 48 | P10518 | Delta-aminolevulinic acid dehydratase | 7.69 | |
| ↓ Muscle contraction | | | | |
| 6 | P58774 | Tropomyosin beta chain | - 5.60 | |

↑ Refer to proteins whose carbonylation is higher in *mdx* than in wild-type muscle.
↓ Refer to proteins whose carbonylation is lower in *mdx* than in wild-type muscle.
[a] Spots refer to annoted spots in Fig. 3.
[b] Accession number in UniProtKB/Swiss-prot.
[c] Fold change between mdx and wild-type values. A positive fold change means an increased value in mdx, a negative means a decreased value.

MyBP-C, UTP-glucose-1-phosphate uridylyltransferase, and carbonic anhydrase 3 (Figs. 4 and 5).

The difference in carbonylation levels between exercised wild-type and *mdx* muscles was reduced 1 h after the last session of LIT (Tables 1–3B) and 2 days later, ATP synthase subunit α, fast isoform of troponin T, and GP were less carbonylated in *mdx* muscle (Table 1C and Fig. 3). In contrast, expression of tubulin, vimentin, and associated proteins, as well as stress response proteins, was higher in exercised *mdx* muscle as of 1 h after the last session, and remained higher 2 days after (Tables 2 and 3B and Tables 2 and 3C; Figs. 4 and 5). Protein–protein interaction analysis showed that the two ATP synthase complexes absent in nonexercised *mdx* muscle were restored 1 h after LIT in *mdx* muscles (P1 in Table 3D and Fig. 5).

### *Validation of proteomic results by 1D carbonylated protein Western blot and Western blot*

The above results point to differences in protein carbonylation and expression between wild-type and *mdx* muscles that are altered by LIT. To validate these results, we investigated these levels by coimmunoprecipitation (co-IP) followed by 1D carbonylated protein Western blot analysis. Two critical proteins were selected, namely GP (Fig. 4, spot 24; Fig. 5, spot B13), and the fast isoforms of MyBP-C (Fig. 4, spot 18).

We confirmed that GP (Fig. 6A) and MyBP-C (Fig. 6B) were significantly more carbonylated in nonexercised *mdx* muscle than in wild-type muscle, consistent with results shown in Table 1A. GP carbonylation was not significantly different between exercised wild-type and *mdx* muscles, 1 h after LIT (Table 1B) but significantly lower in exercised *mdx* muscle after 2 days (Table 1C). MyBP-C carbonylation was not significantly different between exercised wild-type and *mdx* muscles (Table 1B and C).

Expression was assessed by Western blot. We confirmed that GP (Fig. 7A) and MyBP-C (Fig. 7B) were downregulated in nonexercised *mdx* muscles, compared with nonexercised wild-type muscles, as found previously (Tables 2 and 3A). GP expression was lower in exercised *mdx* muscle than in wild-type muscle, 1 h after the last session (Table 2B) and after 2 days, it was similar in both types of mice (Table 3B). MyBP-C expression was not significantly different between exercised wild-type and *mdx* muscles (Table 2B and C). Altogether, results of co-IP and Western blot analysis were in accordance with the results of the proteomic study.

### *Increased expression of fast skeletal muscle isoforms in exercised mdx gastrocnemius muscle*

Gastrocnemius muscle is composed of about 95% of fast type fibers [43]. Proteomic analysis revealed a lower expression of fast isoforms of troponin T and MYBP-C in *mdx* gastrocnemius muscle as compared to wild-type muscle (Table 2). Using Western blot analysis, we confirmed this result and showed that slow isoforms were more highly expressed in nonexercised *mdx*, compared with wild-type muscle (Fig. 7B). After swimming, a higher expression of slow isoforms was detected in both exercised wild-type and *mdx* muscles. Interestingly, in *mdx* muscle, we found a higher expression of fast isoforms, similar to the level of exercised wild-type muscle. This result showed that LIT stimulated the expression of slow type isoforms, and also restored the expression of fast isoforms in *mdx* muscle. These observations at the protein level were supported by a similar pattern at the mRNA level (Fig. 7C).

## Discussion

Our study links, for the first time, alterations in protein carbonylation and expression levels, induced by low intensity

**Table 2**
Identity of proteins whose expression differs in mdx gastrocnemius, in comparison with wild type.

Protein expression in mdx muscle (2D-PAGE)
(in comparison with wild type)

| A. Nonexercised | | | | B. Exercised, 1 h after the last session | | | | C. Exercised, 2 days after the last session | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Spot No. [a] | Accession No. [b] | Protein name | Fold change [c] | Spot No. [a] | Accession No. [b] | Protein name | Fold change [c] | Spot No. [a] | Accession No. [b] | Protein name | Fold change [c] |
| ↑ Cytoskeleton | | | | ↑ Muscle contraction | | | | ↑ Cytoskeleton | | | |
| 3 | P05213 | Tubulin alpha-1B chain | 3.45 | 9 | P58774 | Tropomyosin beta chain | 2.63 | 12 | P11983 | T-complex protein 1 subunit alpha | 3.45 |
| 3 | P20152 | Vimentin | 3.45 | 50 | Q9QZ47 | Troponin T, fast skeletal muscle | 1.67 | 10 | P11983 | T-complex protein 1 subunit alpha | 3.03 |
| 5 | P68372 | Tubulin beta-4B chain | 1.79 | 49 | Q9QZ47 | Troponin T, fast skeletal muscle | 1.54 | 10 | P80316 | T-complex protein 1 subunit epsilon | 3.03 |
| ↓ Mitochondria | | | | Glycolysis | | | | 4 | P31001 | Desmin | 2.56 |
| 43 | P35486 | Pyruvate dehydrogenase E1 subunit alpha | -2.35 | 51 | P05064 | Fructose-bisphosphate aldolase A | 2.86 | 28 | Q9JKS4 | LIM domain-binding protein 3 | 2.44 |
| 56 | Q9DCS9 | NADH dehydrogenase 1 β subcomplex sub 10 | -2.21 | 51 | P16858 | Glyceraldehyde-3-phosphate dehydrogenase | 2.86 | 3 | P05213 | Tubulin alpha-1B chain | 1.82 |
| 42 | Q91YT0 | NADH dehydrogenase flavoprotein 1 | -2.18 | 9 | P21550 | Beta-enolase | 2.63 | 3 | P20152 | Vimentin | 1.82 |
| 54 | Q60932 | Voltage-dependent anion-selective channel 1 | -2.03 | Cytoskeleton | | | | Stress response | | | |
| Muscle contraction | | | | 10 | P11983 | T-complex protein 1 subunit alpha | 2.17 | 11 | P27773 | Protein disulfide-isomerase A3 | 4.17 |
| 53 | Q9QZ47 | Troponin T, fast skeletal muscle | -2.78 | 10 | P80316 | T-complex protein 1 subunit epsilon | 2.17 | 2 | P63038 | 60 kDa heat shock protein, mitochondrial | 2.86 |
| 56 | Q9QZ47 | Troponin I, fast skeletal muscle | -2.21 | 3 | P05213 | Tubulin alpha-1B chain | 1.64 | Others (cell cyke) | | | |
| 18 | Q5XKE0 | Myosin-binding protein C, fast-type | -2.12 | 3 | P20152 | Vimentin | 1.64 | 20 | Q9WU78 | Programmed cell death 6-interacting protein | 4.17 |
| 54 | Q9QZ47 | Troponin T, fast skeletal muscle | -2.03 | Stress response | | | | ↓ Mitochondria | | | |
| 52 | Q9QZ47 | Troponin T, fast skeletal muscle | -1.74 | 11 | P27773 | Protein disulfide-isomerase A3 | 3.45 | 57 | Q9CQA3 | Succinate dehydrogenase iron-sulfur subunit | -2.93 |
| 7 | P58771 | Tropomyosin alpha-1 chain | -1.61 | 14 | Q60854 | Serpin B6 | 2.17 | 8 | Q9CZ13 | Cytochrome b-c1 complex subunit 1 | -1.54 |
| Glycogen metabolism | | | | ↓ Mitochondria | | | | Glycolysis | | | |
| 37 | Q91ZJ5 | UTP-glucose-1-phosphate uridylyltransferase | -1.71 | 54 | Q60932 | Voltage-dependent anion-selective channel 1 | -2.29 | 57 | O70250 | Phosphoglycerate mutase 2 | -2.93 |
| Stress response | | | | 7 | Q60597 | 2-oxoglutarate dehydrogenase | -1.67 | Others | | | |
| 43 | P62196 | 26S protease regulatory subunit 8 | -2.35 | Muscle contraction | | | | 57 | P05977 | Myosin light chain 1/3. skeletal muscle isoform | -2.93 |
| Others | | | | 54 | Q9QZ47 | Troponin T, fast skeletal muscle | -2.29 | 8 | P60710 | Actin. cytoplasmic 1 | -1.54 |
| 43 | Q9DCL9 | Multifunctional protein ADE2 | -2.35 | Glycogen metabolism | | | | | | | |
| 43 | P07310 | Creatine kinase M-type | -2.35 | 24 | Q9WUB3 | Glycogen phosphorylase, muscle form | -1.72 | | | | |
| | | | | 34 | Q9D0F9 | Phosphoglucomutase-1 | -1.65 | | | | |
| | | | | Stress response | | | | | | | |
| | | | | 24 | P58252 | Elongation factor 2 | -1.72 | | | | |

↑ Refer to proteins whose expression is higher in *mdx* than in wild-type muscle.
↓Refer to proteins whose expression is lower in *mdx* than in wild-type muscle.

[a] Spots refer to annoted spots in Fig. 4.
[b] Accession number in UniProtKB/Swiss-prot.
[c] Fold change between mdx and wild-type values. A positive fold change means an increased value in mdx, a negative means a decreased value.

training at the molecular level in muscle of *mdx* mice. We hypothesized first that overcarbonylated proteins in *mdx* muscle would also be downregulated and that protein–protein interactions would be lost. We also thought that LIT would rescue these changes, rendering LIT a physiotherapeutic approach to treatment.

In agreement with our first hypothesis, we found that proteins involved in muscle contraction and glycogen metabolism were both overcarbonylated and downregulated in nonexercised *mdx* muscle. However, this was not associated with a loss of protein–protein interactions.

**Table 3**
Identity of proteins whose expression and protein-protein interactions differs in *mdx* gastrocnemius, in comparison with wild type.

Protein expression in mdx muscle (BN-PAGE)
(in comparison with wild type)

A. Nonexercised

| | Spot No. [a] | Accession No. [b] | Protein name | Fold change [c] |
|---|---|---|---|---|
| ↑ | Glycolysis | | | |
| | B9 | P06151 | L-lactate dehydrogenase A chain | 2.56 |
| | B15 | P21550 | Beta-enolase | 1.75 |
| | B19 | P05064 | Fructose-bisphosphate aldolase A | 1.75 |
| | Others | | | |
| | B19 | P07310 | Creatine kinase M-type | 1.75 |
| | B16 | P07310 | Creatine kinase M-type | 1.64 |
| ↓ | Mitochondria | | | |
| | B5 | P56480 | ATP synthase subunit beta, mitochondrial | -3.72 |
| | B4 | Q03265 | ATP synthase subunit alpha, mitochondrial | -3.26 |
| | B3 | P56480 | ATP synthase subunit beta, mitochondrial | -2.57 |
| | B2 | Q03265 | ATP synthase subunit alpha, mitochondrial | -2.42 |
| | Glycogen metabolism | | | |
| | B13 | Q9WUB3 | Glycogen phosphorylase, muscle form | -1.66 |
| | Stress response | | | |
| | B10 | P16015 | Carbonic anhydrase 3 | -3.9 |
| | Others | | | |
| | B11 | P07310 | Creatine kinase M-type | -2.02 |
| | B13 | Q8R429 | Sarco/endoplasmic reticulum calcium ATPase 1 | -1.66 |

B. Exercised, 1 h after the last session

| | Spot No. [a] | Accession No. [b] | Protein name | Fold change [c] |
|---|---|---|---|---|
| ↑ | Mitochondria | | | |
| | B8 | P55084 | Trifunctional enzyme subunit beta, mitochondrial | 1.86 |
| | Glycolysis | | | |
| | B8 | P21550 | Beta-enolase | 1.86 |
| | B8 | P05064 | Fructose-bisphosphate aldolase A | 1.86 |
| | B7 | P21550 | Beta-enolase | 1.81 |
| ↓ | Mitochondria | | | |
| | B14 | Q99KI0 | Aconitate hydratase, mitochondrial | -3.36 |
| | B17 | Q60932 | Voltage-dependent anion-selective channel prot 1 | -1.59 |
| | Glycogen metabolism | | | |
| | B13 | Q9WUB3 | Glycogen phosphorylase, muscle form | -1.72 |
| | Stress response | | | |
| | B14 | P07901 | Heat shock protein HSP 90-alpha | -3.36 |
| | Other | | | |
| | B13 | Q8R429 | Sarco/endoplasmic reticulum calcium ATPase 1 | -1.72 |
| | B17 | P07310 | Creatine kinase M-type | -1.59 |
| | B17 | P14152 | Malate dehydrogenase, cytoplasmic | -1.59 |

C. Exercised, 2 days after the last session

| | Spot No. [a] | Accession No. [b] | Protein name | Fold change [c] |
|---|---|---|---|---|
| ↑ | Glycolysis | | | |
| | B15 | P21550 | Beta-enolase | 1.59 |
| | B1 | P52480 | Pyruvate kinase isozymes M1/M2 | 1.54 |
| ↓ | Glycolysis | | | |
| | B6 | P21550 | Beta-enolase | -1.51 |

D. Identity of the proteins which compose the complexes absents in nonexercised *mdx* muscle

| Spot No. [a] | Accession No. [b] | Protein name |
|---|---|---|
| P1 complexes | | |
| B2 | Q03265 | ATP synthase subunit alpha, mitochondrial |
| B3 | P56480 | ATP synthase subunit beta, mitochondrial |
| B4 | Q03265 | ATP synthase subunit alpha, mitochondrial |
| B5 | P56480 | ATP synthase subunit beta, mitochondrial |

↑Refer to proteins whose expression is higher in *mdx* than in wild-type muscle.
↓Refer to proteins whose expression is lower in *mdx* than in wild-type muscle.

[a] Spots refer to annoted spots in Fig. 5.
[b] Accession number in UniProtKB/Swiss-prot.
[c] Fold change between mdx and wild-type values. A positive fold change means an increased value in mdx, a negative means a decreased value.

Furthermore, we found that LIT rescued, at least in part, *mdx* muscle at the protein level, consistent with our second hypothesis. Specifically, proteins from mitochondria, muscle contraction, and glycogen metabolism, highly carbonylated and downregulated in nonexercised *mdx* muscle, were less carbonylated and highly expressed after LIT. LIT of *mdx* mice also restored carbonylation and expression levels of fast isoforms of troponin T and MyBP-C at the level of exercised wild-type muscle, while expression of slow isoforms increased, also consistent with our second hypothesis.

*Protein carbonylation in skeletal muscle of nonexercised mdx mice*

Elevated total protein carbonylation levels have been reported in muscle of *mdx* mice and DMD patients [17,20]. However, the targets

**Table 4**
Identity of proteins whose carbonylation or expression changes 2 days after exercise, in comparison with nonexercised, in wild-type or *mdx*.

| | Protein carbonylation after LIT (2 days after the last exercise) | | | | Protein expression after LIT (2 days after the last exercise) | | | |
|---|---|---|---|---|---|---|---|---|
| | Spot No. [a] | Accession No. [b] | Protein name | Fold change [c] | Spot No. [d] | Accession No. [b] | Protein name | Fold change [c] |
| Wild type | ↑ Mitochondria | | | | ↑ Glycolysis | | | |
| | 38 | Q9D0K2 | SuccinylCoA:3ketoacid coenzyme A transferase 1 | 10 | B8 | P21550 | Beta-enolase | 5.26 |
| | 26 | Q99KI0 | Aconitate hydratase, mitochondrial | 2.85 | | | | |
| | Muscle contraction | | | | | | | |
| | 46 | Q9QZ47 | Troponin T, fast skeletal muscle | 4.16 | | | | |
| | 18 | Q5XKE0 | Myosin-binding protein C, fast-type | 1.66 | | | | |
| | Glycogen metabolism | | | | | | | |
| | 37 | Q91ZJ5 | UTP-glucose-1-phosphate uridylyltransferase | 5 | ↓ | | | |
| | Others | | | | | | | |
| | 46 | P07310 | Creatine kinase M-type | 4.16 | | | | |
| | ↓ Cytoskeleton | | | | | | | |
| | 5 | P68372 | Tubulin beta-4B chain | -5.93 | | | | |
| | Spot No. [a] | Accession No. [b] | Protein name | Fold change [c] | Spot No. [d] | Accession No. [b] | Protein name | Fold change [c] |
| *mdx* | ↑ | | | | ↑ Mitochondria | | | |
| | | | | | B7 | P56480 | ATP synthase subunit beta, mitochondrial | 3.57 |
| | | | | | B6 | Q03265 | ATP synthase subunit alpha, mitochondrial | 3.33 |
| | ↓ Mitochondria | | | | 42 | Q91YT0 | NADH dehydrogenase [ubiquinone] flavoprotein 1 | 2.63 |
| | 54 | Q60932 | Voltage-dependent anion-selective channel prot 1 | -8.22 | 25 | Q99KI0 | Aconitate hydratase, mitochondrial | 2 |
| | Muscle contraction | | | | Muscle contraction | | | |
| | 54 | Q9QZ47 | Troponin T, fast skeletal muscle | -8.22 | 53 | Q9QZ47 | Troponin T, fast skeletal muscle | 3.03 |
| | 46 | Q9QZ47 | Troponin T, fast skeletal muscle | -3.83 | 52 | Q9QZ47 | Troponin T, fast skeletal muscle | 2.27 |
| | 18 | Q5XKE0 | Myosin-binding protein C, fast-type | -1.76 | 18 | Q5XKE0 | Myosin-binding protein C, fast-type | 1.96 |
| | Glycogen metabolism | | | | Glycogen metabolism | | | |
| | 35 | Q9D0F9 | Phosphoglucomutase-1 | -4.25 | 37 | Q91ZJ5 | UTP-glucose-1-phosphate uridylyltransferase | 1.64 |
| | 24 | Q9WUB3 | Glycogen phosphorylase, muscle form | -3.03 | Stress response | | | |
| | Glycolysis | | | | 10 | P21550 | Carbonic anhydrase 3 | 4.16 |
| | 40 | P52480 | Pyruvate kinase isozymes M1/M2 | -6.27 | ↓ Glycolysis | | | |
| | Cytoskeleton | | | | 16 | P21550 | Beta-enolase | -1.63 |
| | 4 | P31001 | Desmin | -2.4 | | | | |
| | Others | | | | | | | |
| | 46 | P07310 | Creatine kinase M-type | -3.83 | | | | |

↑Refer to proteins whose carbonylation is higher in *mdx* than in wild-type muscle.
↓Refer to proteins whose carbonylation is lower in *mdx* than in wild-type muscle.
X spots refer to annoted spots in Fig. 3, BX to annoted spots in Fig. 4.

[a] Spots refer to annoted spots in Fig. 2.
[d] Spots refer to annoted spots in Fig. 2.

of protein carbonylation were not identified until late. Consistent with our observations (spots 25 and 26 in Fig. 3), a recent study on tibialis anterior muscle of DMD patients [16] showed aconitate hydratase to be overcarbonylated. Also in agreement with our study, mitochondrial proteins appeared to be preferential targets of carbonylation in dystrophic muscle. However, we showed that these proteins were not equally affected by oxidative stress. We found two major groups of proteins: those from the citric acid cycle (Table 1A) and those from the respiratory chain (Tables 2 and 3A). Citric acid cycle proteins were overcarbonylated, consistent with the fact that the function of these proteins was impaired in *mdx* muscle [44]. In contrast, respiratory chain proteins were not overcarbonylated. This suggests that the impact of oxidative stress on mitochondria of *mdx* muscle depends on protein location, since citric acid cycle proteins are mainly located in the matrix, whereas those of the respiratory chain are located in the inner membrane [45].

We also found that other groups of proteins were over carbonylated in *mdx* muscle: those involved in the modulation of contraction, in glycogen metabolism, and in the formation of the cytosketelon. A functional impairment of the proteins of the first group, namely the fast isoforms of troponin T and MyBP-C, has not been reported in DMD. On the other hand and consistent with a recent study, in which a reduced activity of glycogen phosphorylase was observed in *mdx* muscle [46], we found this enzyme to be overcarbonylated. Among proteins involved in formation of the cytoskeleton, we found overcarbonylated levels of actin-associated proteins, such as LIM domain-binding protein 3 and F-actin-capping protein subunit alpha-1. This is consistent with the finding that the actin filament architecture is severely damaged in *mdx* muscle [47]. Taken together our results suggest that protein carbonylation could cause a functional impairment in *mdx* muscle.

### *Protein expression in skeletal muscle of nonexercised mdx mice*

Differences in protein expression between wild-type and *mdx* muscles have been widely documented. Our results (Tables 2 and 3A;

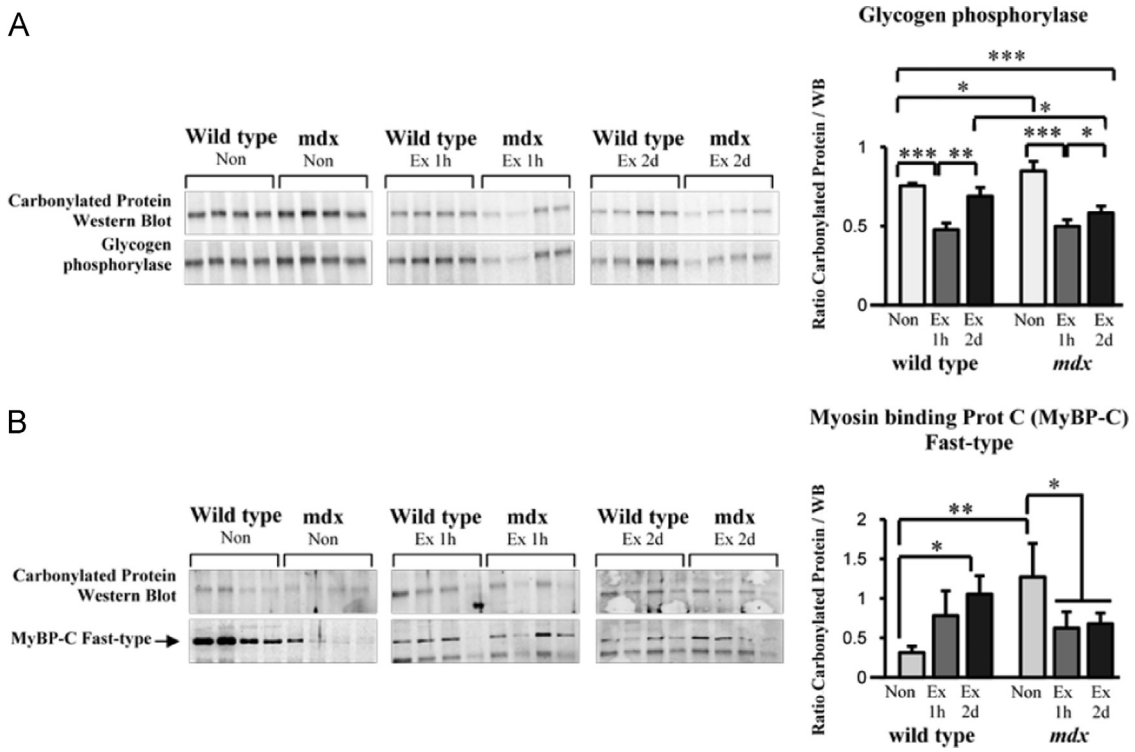


**Fig. 6.** Validation of proteomic results by 1D carbonylated protein Western blot. Carbonylation levels of glycogen phosphorylase (A) or myosin binding protein C (B) were confirmed by coimmunoprecipitation with corresponding antibodies, followed by 1D carbonylated protein Western blot. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ means a significant difference between two groups. $n = 4$–6 per group.

Figs. 4 and 5) are in agreement with previous ones. In particular, we found a downregulation of proteins of the respiratory chain [48] as well as glycogen metabolism [46]. Again consistent with previous reports, vimentin [49], tubulin [50], several enzymes involved in glycolysis [51], and lactate deshydrogenase [52] were overexpressed. Downregulation of fast isoforms of troponin T and MyBP-C have also been reported [53]. As hypothesized, the majority of overcarbonylated proteins were downregulated. However, some proteins of the citric acid cycle, of glycolysis, and of the actin cytoskeleton (27, 28, 29, and 40, 55 in Table 1A and Fig. 3) were overcarbonylated but not downregulated. We suppose the turnover of these proteins to be slower and therefore they might accumulate more oxidative modifications before being degraded.

*Protein–protein interactions in skeletal muscle of nonexercised mdx mice*

Our BN-PAGE analysis showed, for the first time, that ATP synthase subunits α and β were absent in nonexercised *mdx* muscle (Table 3D and Fig. 5). The molecular weights of these complexes correspond to fully assembled monomeres and dimers of ATP synthase, namely 597 and 1194 kDa [54]. This result is consistent with previous findings reporting that the expression of ATP synthase subunit α was not changed in *mdx* muscle, but ATP production was reduced because of a proton leak in the inner mitochondrial membrane [55]. Our study suggests that incomplete formation of the ATP synthase complex in *mdx* muscle could be a cause of this proton leak.

*Effect of low intensity training on skeletal muscle of mdx mice*

The major result of our study is that overcarbonylation, downregulation, and loss of protein–protein interactions in *mdx* muscle are fully corrected by LIT. Swimming is an endurance exercise, and known to affect proteins involved in the respiratory chain, glucose uptake, citric acid cycle, fatty acid metabolism, glycolysis, and oxygen transfer [56]. We found that LIT reduced carbonylation levels and increased the expression of proteins involved in mitochondria function, muscle contraction, glycogen metabolism, and glycolysis (Table 4), but not of proteins involved in glucose uptake, oxygen transfer, or fatty acid metabolism. Previous studies revealed that the destabilization of microtubule networks affects the glucose uptake in *mdx* muscle [57]. LIT was not able to counterbalance this effect, consistent with the fact that cytoskeleton protein remained overexpressed in exercised *mdx* muscle (Table 2).

*Low intensity training is more efficient on mdx muscle than on wild type*

Interestingly, the effects of LIT were more pronounced in *mdx* than in wild-type muscle. In the latter, exercise increased protein carbonylation but had little influence on their expression. In contrast, in *mdx* muscle, exercise reduced protein carbonylation and increased their expression. These results highlight differences in sensitivity to training between wild-type and *mdx* muscle.

*Swimming improves expression of slow and fast isoforms of troponin T and MyBP-C*

Pharmacologic agents have been developed during the past years in an attempt to mimic the effects of aerobic exercise on wild-type [58] or *mdx* muscle [59]. Some of these agents improved *mdx* muscle strength and increased the expression of utrophin A and slow myosin heavy chain isoforms through a shift from fast to slow fibers. In our study, we showed that LIT decreased carbonylation and increased the expression level of fast isoforms of troponin T and MyBP-C, and also stimulated the expression of their slow isoforms (Fig. 7). These results encourage investigating the effects of exercise mimicking drugs on a larger scale of muscle proteins, especially regarding their isoforms in fast muscle.

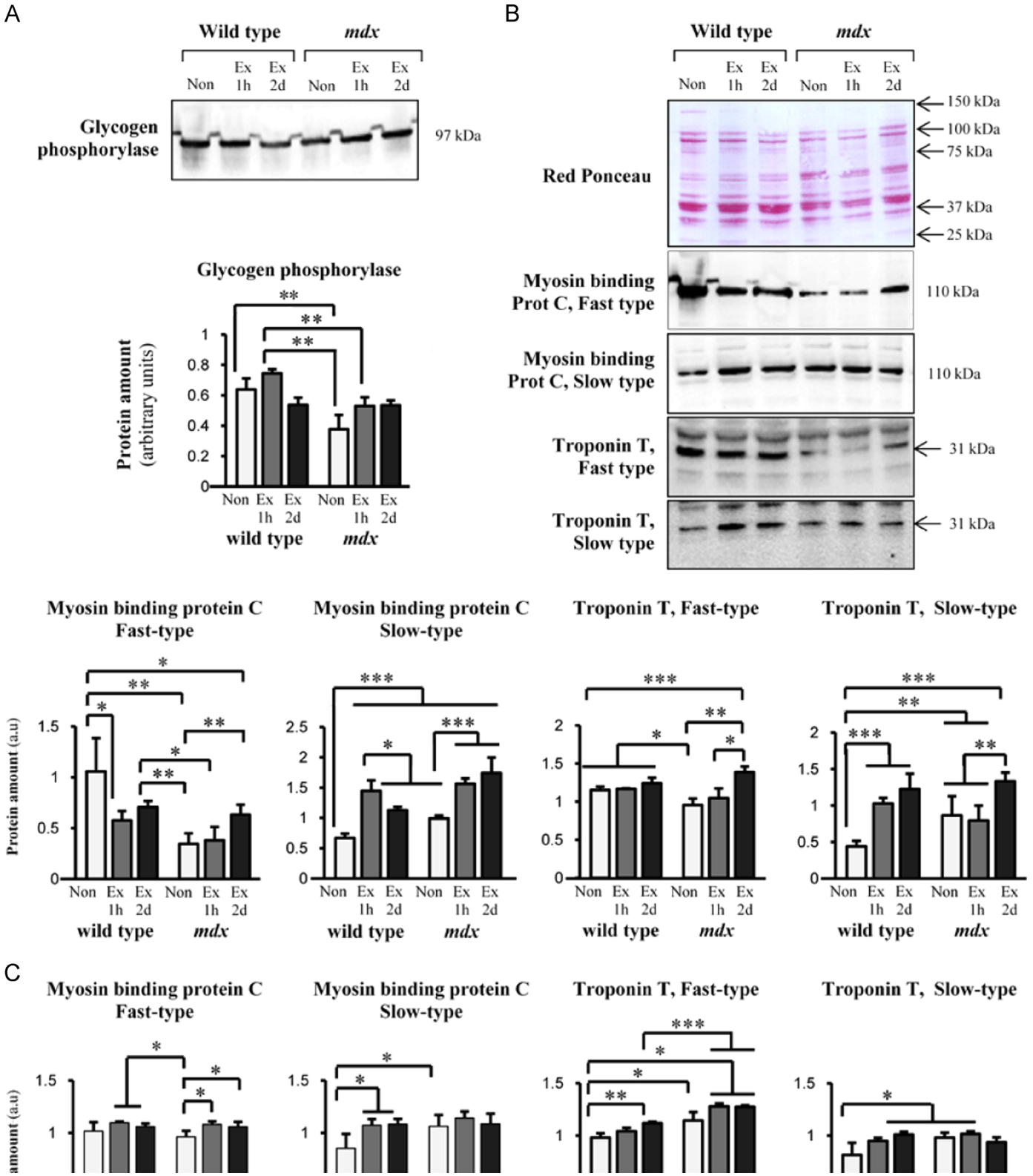


**Fig. 7.** Expression of slow and fast troponin T and myosin binding protein C isoforms. Protein expression level of glycogen phosphorylase (A), then troponin T and myosin binding protein C slow and fast isoforms (B), was assessed by Western blot using corresponding antibodies. Ponceau red staining is shown as loading control. mRNA level was assessed by PCR analysis (C). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ means a significant difference between two groups. $n = 4$ per group.

*Multiple proteins identified in a single spot*

High sensitivity MALDI TOF/TOF analysis [60] revealed that 22% of spots from 2D electrophoresis gels and 33% of spots from BN-PAGE gels contained multiple proteins (Supplementary Fig. 1). Because it was difficult to determine which proteins underwent changes, we limited our analysis to observations made in previous publications or consistent with other results. Using this approach, we detected, for example, an increased expression of vimentin and tubulin alpha1B chain in nonexercised mdx muscle (spot 3 in

Figs. 3 and 4), as reported previously [50]. This result is consistent with the increased expression of desmin and tubulin beta 4B chain (spot 4 and 5 in Figs. 3 and 4). Along similar lines, glycogen phosphorylase and SERCA1 were identified in the same spot (B13 in Fig. 5). Downregulation of glycogen phosphorylase has been previously reported [46] and is noted under Results. However, downregulation of SERCA1 has not been documented and is not noted.

*Influence of infiltrated immune cells on proteomic analysis of exercised mdx mice*

In dystrophic muscle, infiltration of immune cells occurs during early stages of the disease and plays a role in progression of DMD pathology [61]. In *mdx* mice, this infiltration reaches a peak between 4 and 8 weeks of age, which corresponds to the period of LIT. We need to evaluate the extent of infiltrated cells, since they may influence results of our proteomic analysis on nonexercised and exercised wild-type and *mdx* samples.

According to Evans et al., at 8 weeks of age, macrophages are the principal immune cells that infiltrate *mdx* muscles [61]. For this reason, we immunostained macrophages in sections of gastrocnemius muscles and determined the stained area using ImageJ software. Results showed that the area of infiltrated immune cells was less than 1% of the total muscle area, even in exercised *mdx* muscle (J. Hyzewicz et al., personal communication). As a consequence, the influence of the infiltrated immune cells on the proteomic study is negligible.

## Conclusion

In our study, we have used an extensive proteomic method to assess the effects of 4 weeks of LIT on carbonylation, expression, and protein–protein interactions of proteins in gastrocnemius muscle of 8-week-old *mdx* mice. We found that proteins of mitochondria, muscle contraction, and glycogenolysis were overcarbonylated and downregulated in nonexercised *mdx* muscle. Furthermore, we demonstrated that LIT corrected these impairments by decreasing carbonylation and increasing expression levels of fast isoforms of troponin T and myosin binding protein C, as well as increasing the expression of slow type isoforms. In addition, the results point to different sensitivities of wild-type and *mdx* muscle in response to LIT.

The present research confirms the beneficial effects of LIT at the protein level and provides pertinent information which could help to design exercise mimicking drugs for DMD therapy.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.freeradbiomed.2015.01.023.

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