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Proteome analysis in dystrophic mdx mouse muscle reveals a drastic alteration of key metabolic and contractile proteins after chronic exercise and the potential modulation by anti-oxidant compounds

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ABSTRACT

Weakness and fatigability are typical features of Duchenne muscular dystrophy patients and are aggravated in dystrophic mdx mice by chronic treadmill exercise. In the present study, we describe, the pattern of differentially abundant spots that is associated to the worsening of dystrophy phenotype induced by chronic exercise. Our proteomic analysis pointed out 34 protein spots with different abundance between sedentary and exercised mdx mice. These proteins belong mostly to glucose metabolism, energy production and sarcomere structure categories. Interestingly exercise induced an increase of typical fast twitch fiber proteins (Troponin T fast skeletal muscle, Troponin I fast skeletal muscle and Myozenin-1) combined with an increase of several glycolytic enzymes. Concerning energy transfer, Adenylate kinase, showed a marked decrease when compared with non-exercised mdx. The decline of this enzyme correlates with increased Creatin kinase enzyme, suggesting that a compensatory energy metabolism mechanism could be activated in mdx mouse skeletal muscle following exercise. In addition, we analysed muscles from exercised mdx mice treated with two natural anti-oxidant compounds, apocynin and taurine, that in our previous study, were proved to be beneficial on some pathology related parameters, and we showed that these compounds can counteract exercise-induced changes in the abundance of several proteins.

Significance: Mdx mouse model of Duchenne muscular dystrophy shows a phenotype of the disorder milder than in human sufferers. This phenotype can be worsened by a different protocols of chronic exercise. These protocols can mimic the muscle progressive damage observed in humans, can allow studying the effects of inadequate training on dystrophic muscles and have been largely used to assess the ability of a drug to reduce the damage induced by exercise. In this study, we describe for the first time, the pattern of protein variation associated with the worsening of dystrophy phenotype induced by chronic exercise. Our proteomic analysis pointed out 34 protein spots with different amount between sedentary and exercised mdx mice. These proteins belong mostly to glucose metabolism, energy production and sarcomere structure categories and their variation indicates that mdx exercised muscle are not able to carry out the metabolic changes associated to fast-to-slow transition typically observed in aerobically trained muscle.

1. Introduction

Duchenne Muscular Dystrophy (DMD) is a severe, X-chromosome linked disease inherited muscle disorder caused by the absence of functional dystrophin protein. It affects 1 in 5000 boys worldwide; no definitive treatment for this disease is available yet. DMD is characterized by muscle membrane fragility, progressive myofiber death and replacement of skeletal muscle by fibrous and connective tissue (due to failed regeneration). This results in extensive wasting, weakness and loss of muscle function leading to premature death.

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The dystrophin-deficient mdx mouse is the most common animal model for DMD [1], and it is characterized by a nonsense mutation in exon 23 of the dystrophin gene. The mdx mice phenotype of the disorder is milder than in human sufferers. This difference arises from differences in size, mechanical loading and lifespan [2]. These mice present an acute onset of pathology around 3–4 weeks of age, that is reduced to a chronic low level of damage by 8 weeks and persists throughout life [3]. Compared to DMD patients, mdx mouse muscles show less accumulation of connective and adipose tissue and are characterized by a better recover from the wasting. In fact, although the necrotic process persists throughout their life, the regenerative capacity does not decline until an advanced age (around one year) [4].

Since regular exercise has a plethora of positive effects on health and muscle performance, exercise.

has been proposed as treatment for DMD, but this recommendation has not been unanimously accepted because inappropriate exercise damages dystrophic muscles [5,6]. The establishment of the exact boundary line between a proper and unsuitable exercise is challenging. The effect of exercise in mdx mouse model has been largely used for basically three purposes: i) assessing the physical capacities of the mice, ii) investigating the effects of training on dystrophic muscles, iii) worsening the phenotype in order to assess the effects of a drug [5]. In the context of worsen the mild dystrophic phenotype of mdx mice, various models of exercise-induced muscle damage, including forced wheel or treadmill running, and swimming, were used. These protocols can mimic the muscle progressive damage observed in humans, can allow studying the effects of inadequate training on dystrophic muscles and have been largely used to assess the ability of a drug to reduce the damage induced by exercise [3,5]. Several proteomic studies, comparing wild type and mdx muscles were performed [7–12], however only few investigated the effect of exercise [13,14]. In particular, these studies were focused on the beneficial effect of a low intensity exercise, while the effect of high intensity protocols has never been investigated by a proteomic approach. In a previous study, we analysed how a chronic protocol of treadmill exercise affects the *in vivo* performance and the expression of specific molecules involved in muscle damage and regeneration in mdx mice [15]. We demonstrated that this protocol reduces functional performance *in vivo* in mdx mice. In parallel, we demonstrated that exercised mdx mice showed changes in expression genes indicating a failure in mechanical metabolic coupling. In particular, we observed an impaired ability of adaptation to exercise of protective metabolic oxidative pathways, while the gene expression of proteins involved in damaging signals, such as oxidative stress and inflammation, remains upregulated. Importantly, no change was detected in wild-type mice, the protocol being too mild to induce training effects in healthy animals [15]. The more severe phenotype induced by chronic exercise is useful to test the beneficial effects of drugs and natural compounds, among which anti-oxidant and anti-inflammatory drugs [16–18]. In the present study, we describe, for the first time, the pattern of differentially abundant spots that is associated to the worsening of dystrophy phenotype induced by chronic exercise. Our investigation includes the protein profile in tibialis anterior muscle of wild type, mdx mice and mdx mice undergoing a standard 4 weeks' protocol of exercise on treadmill. In particular, we focused our attention to changes induced by exercise in the mdx phenotype, showing significant alteration of several metabolic pathways including glucose one, as well as sarcomere structure organization. In addition, we also analysed muscle from exercised mdx mice treated with two natural anti-oxidant compounds, apocynin and taurine, proved to be beneficial on some pathology related parameters [16], in order to assess, at proteomic level, if the exercise and pathology related changes could be counteracted by these compounds.

2. Materials and methods

2.1. Animal model and exercise

The present study was performed using the hind limb muscle samples of experimental mice groups previously used [16,19]. In particular, muscle samples used in this study are referred to the following mice and experimental groups: male mdx mice (C57BL/10ScSn-Dmdmdx/J from Jackson Laboratories) divided in sedentary mdx (mdx) mice or exercised mdx (mdx exe) mice, mdx exercised mice treated with taurine (mdx exe tau) or apocynin (mdx exe apo) and C57/BL wild-type (wt) mice. The age of all mice at the beginning of the study was 4–5 weeks while muscle sampling was performed at 8–10 weeks of age. The training protocol consisted of a 30 min running on a horizontal treadmill (Columbus Instruments, USA) at 12 m/min, twice a week (keeping a constant interval of 2–3 days between each trial), for 4 weeks. The doses of taurine and apocynin were 1 g/kg (orally) and 38 mg/kg (1.5 mmol/L in drinking water) respectively. The treatment started one day before the beginning of the exercise protocol, lasted at least 4 weeks and continued until the day of sacrifice. A group of age-matched male wild-type mice (C57BL/10) has also been used as control. The animals were sacrificed after 48–72 h after the last exercise session to avoid acute effects of exercise to be detected [19]. Muscles were removed, immediately frozen in liquid nitrogen and stored at − 80 °C until use for biochemical evaluations. The animals used in this proteomic study were 5 for each group.

2.2. Muscle sample preparation and two-dimensional gel electrophoresis

Muscle protein extracts were prepared as previously described [20]. Briefly, frozen muscles were ground in dry ice in a cooled mortar, suspended in lysis buffer (50 mM Tris–HCl pH 7.0, 150 mM, NaCl, 2 mM EGTA, 100 mM NaF, 1% (*v*/v) NP-40, 0.5% (*w*/*v*), deoxycholate, 0.1% (w/v) SDS containing a cocktail of protease inhibitors (Sigma) and solubilized by sonication on ice.

After centrifugation (8000 RCF, for 5 m) proteins were precipitated following a chloroform/methanol protocol [21] and suspended in 8 M urea, 4% (*w*/*v*) CHAPS, 50 mM DTT. Protein samples (60 µg for silver stained gels and 700 µg for preparative gels) were separated on 18-cm immobilized pH gradient (IPG) strips (pH 3–10 NL) on PROTEAN® i12™ IEF System (BIO-RAD). In particular, the strips were actively rehydrated (at 50 V), for 14 h, in the presence of sample in rehydration solution (8 M urea, 2% (*w*/*v*) CHAPS, 0.5% (w/v) DTE) supplemented with 0.5% (*v*/v) carrier ampholyte (Bio-Rad) and a trace of bromophenol blue. The strips were then focused at 16 °C according to the following electrical conditions: 250 V for 20 m (rapid), from 250 V to 8000 V for 1 h, 8000 V until a total of 43,000 V/h was reached, with a limiting current of 50 µA/strip. Analytical gels were stained with ammoniacal silver nitrate as previously described [22]; MS-preparative gels were stained with colloidal Coomassie blu silver [23].

2.3. Image analysis and statistics

In this study we used 5 animals for group (biological replicates) and for each animal two 2DE gels were carried out (technical replicates), so that, in total 50 gels were analysed. Silver-stained gels were scanned using the Epson expression 1680 PRO scanner. The gel images were saved with a resolution of 300 dpi and in 16-bit TIFF format. Image analysis was carried out using the Progenesis SameSpots software v4.0 (Nonlinear Dynamics, UK), which allows spot detection, background subtraction and protein spot volume quantification. The gel image showing the highest number of spots and the best protein pattern was chosen as the reference image and its spots were then matched across all gels. This reference image was used to quantify and normalize the spot volumes. The spot volumes were normalized in each gel as relative volume (volume percentage), by dividing the raw quantity of each spot