

Reduction of mdx mouse muscle degeneration by low-intensity endurance exercise: a proteomic analysis in quadriceps muscle of exercised versus sedentary mdx mice

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Running title: Endurance exercise and mdx muscle proteomic analysis

Synopsis

In our recent study was shown a significant recovery of damaged skeletal muscle of mice with x-linked muscular dystrophy (*mdx*) following low-intensity endurance exercise, probably by reducing the degeneration of dystrophic muscle. Consequently, in the present work we aimed to identify proteins involved in the observed reduction of degenerating fibers. To this end, we used proteomic analysis to evaluate changes in the protein profile of quadriceps dystrophic muscles of exercised versus sedentary *mdx* mice. Four protein spots were found to be significantly changed and were identified as three isoforms of Carbonic anhydrase 3 (CA3) and superoxide dismutase [Cu-Zn] (SODC). Protein levels of CA3 isoforms were significantly up-regulated in quadriceps of sedentary *mdx* mice and were completely restored to wild type mice values, both sedentary and exercised, in quadriceps of exercised *mdx* mice. Protein levels of SODC were down-regulated in quadriceps of sedentary *mdx* mice and were significantly restored to wild type mice values, both sedentary and exercised, in quadriceps of exercised *mdx* mice. Western blot data were in agreement with those obtained using proteomic analysis and revealed the presence of one more CA3 isoform that was significantly changed. Based on data found in the present study, it seems that low-intensity endurance exercise may in part contribute to reduce cell degeneration process in *mdx* muscles, by counteracting oxidative stress.

Key words: carbonic anhydrase, exercise, *mdx*, muscle oxidative stress, muscle proteomic, muscular dystrophy

Abbreviations used:

Carbonic anhydrase 3 (CA3); superoxide dismutase [Cu-Zn] (SODC); Duchenne muscular dystrophy (DMD); mice with x-linked muscular dystrophy (*mdx*); exercised mice with x-linked muscular dystrophy (MDX-Ex); sedentary mice with x-linked muscular dystrophy (MDX-Sed); sedentary wild type mice (WT-Sed); exercised wild type (WT-Ex); two-dimensional gel electrophoresis (2DE)

INTRODUCTION

Duchenne muscular dystrophy (DMD) is a lethal X-linked muscle disease due to a defect in the sub-sarcolemmal protein dystrophin, which leads to membrane fragility, muscle necrosis, motor weakness, myofibre death and replacement of skeletal muscle by fibrous and fatty connective tissue, due to failed regeneration [1]. In patients with DMD, muscle biopsy characteristically demonstrates areas of necrotic or degenerating muscle fibres, often observed in clusters surrounded by macrophages and lymphocytes, and areas with small immature centrally nucleated fibres reflecting muscle regeneration from myoblasts [2]. Over the last few years, mass spectrometry-based proteomics has been successfully applied to investigate normal and pathologically altered skeletal muscle tissue [3], including the proteomic profiling of muscle tissues from *mdx* mice that has revealed differential degrees of perturbed protein expression patterns in dystrophin-deficient fibers [4]. Many of these proteomic analyses on muscles of mice with x-linked muscular dystrophy (*mdx*) and DMD have been performed with the aim of unravelling the molecular pathogenesis of muscular dystrophy [5] and these analyses discovered several proteins that were differentially expressed in dystrophic muscles [6]. This large number of proteins change has hampered the possibility of developing therapeutic approach to muscular dystrophy by targeting proteins modified.

The lack of dystrophin in *mdx* mice leads to cycles of muscle degeneration and regeneration processes and various strategies have been proposed in order to reduce the muscle-wasting component of muscular dystrophy [7,8], including implementation of an exercise program. In our recent study it was shown that following low-intensity endurance exercise there was a significant recovery of damaged skeletal muscle in *mdx* mice, probably by reducing the degeneration of dystrophic muscle [9]. Consequently, in the present work we aimed to identify proteins involved in the observed reduction of degenerating fibers by using proteomic analysis to evaluate changes in the protein profile of quadriceps dystrophic muscles of exercised *mdx* versus sedentary *mdx* mice.

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MATERIAL AND METHODS

Preparation of skeletal muscle proteins

This study was performed using the same quadriceps muscle samples of experimental mice groups used in our previous work [9] in order to better compare results of proteomic analysis with previous data showing a reduction of degeneration process in mice subjected to low-intensity endurance exercise. Therefore, muscle samples used in this study are referred to experimental condition of our previous work [9] in which the following mice and experimental groups were used: male *mdx* mice (C57BL/10ScSn-Dmd^{mdx}/J from Jackson Laboratories) 8 weeks old, divided in sedentary *mdx* (MDX-Sed) mice or exercised *mdx* (MDX-Ex) mice, and C57/BL wild type mice, divided in sedentary wild type (WT-Sed) and exercised wild type (WT-Ex), used as control mice. The training protocol, using motorized rotating treadmill (Rota-Rod; Ugo Basile, Biological Research Apparatus, Comerio Varese, Italy) for low-intensity endurance exercise, is described in Frinchi et al. [9]. Shortly, mice ran 5 days/week for 6 weeks: the first 2 weeks all exercised mice underwent a period of acclimatization with very low speed (16 rotations/min) for 15 min during the first week and for 30 min during the second week [9]. This acclimatization trial was followed by 30 days of low-intensity endurance training with increasing duration (session time) and intensity (rotation time) of weekly training (Table 1). For this study, we used quadriceps muscles derived from mice groups killed following 30 days of training. Thick cryosections (60 μ m), sampled at five different levels, were prepared from muscles of three mice for each group and used for protein extraction [10]. The sampled muscle sections were homogenized in cold buffer containing 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% triton, 0.5% SDS, H₂O and protease inhibitor cocktail (P8340, Sigma-Aldrich S.r.l., Milan, Italy). The homogenate was left on ice for 30 min and then centrifuged at 13,000 rpm for 30 min at 4 °C. The supernatants were stored at -80°C and aliquots were taken for protein determination. All Procedures involving animals and their care were conducted in conformity with the Italian institutional guidelines (D.L. no. 116, G.U., suppl. 40, February 18, 1992, and D. LGS. no. 26, GU n.61, March, 2014).

Two-dimensional gel electrophoresis and image acquisition and analysis

Two-dimensional gel electrophoresis (2DE) was carried out in the IPGphor system (GE Healthcare) as described by Fontana et al. [11]. Analytical gels were stained with ammonium silver nitrate [12] and preparative gels with Coomassie blue.

Stained gels were scanned by a ImageScanner II (GE Healthcare) previously calibrated with a greyscale marker (Kodak, Rochester, NY, USA), digitalized with Labscan 5.00 (v 1.0.8) software (GE Healthcare) and analyzed with the ImageMaster™ 2D Platinum v 6.0 software (GE Healthcare). Within each group (WT-Sed, MDX-Sed and MDX-Ex) three silver stained gels obtained from three independent experiments were analyzed to guarantee representative results. After automated spot detection, spots were checked manually to eliminate any possible artifacts, such as streaks or background noise. Biological replicates showed less than 10% of variability in the number of protein spots detected. The patterns of each sample were overlapped and matched, using landmark features, to detect potential differentially expressed proteins. The gel presenting the highest spot number was selected as Master gel and the coefficient of determination (r^2) for each gel pair was calculated in order to ensure the analyses reliability (pair average r^2 : 0.85). In order to reduce experimental error, quantitative analysis was based on evaluation of relative spot volume ($\%V = V_{\text{single spot}}/V_{\text{total spots}}$, where V is the integration of the optical density over the spot area). In the comparison of MDX-Sed mice vs WT-Sed mice and of MDX-Ex mice vs MDX-Sed mice, proteins were

considered differentially expressed when the ratio of the %Vol average (3 gels) was higher than 1.5 and the p-value deduced from the t-test was <0.05 .

In-gel digestion and protein identification by MALDI-TOF/TOF-MS

Coomassie blue-stained protein spots were excised from the preparative gels and cut into 1-mm pieces. In-gel digestion was performed as described by Shevchenko et al. [13] with minor modifications [11].

The digested peptides were desalted and cleaned with ZipTip C18 pipette tips (Millipore Corp.) before obtaining the mass spectrum of the peptide mixture. All analyses were performed using a Bruker Daltonics Autoflex (Bruker Daltonics) operated in the delayed extraction and reflectron mode with the following parameters: 20kV acceleration voltage, 95% grid voltage, 100ns delay time, and 500m/z low-mass gate. For acquisition of a mass spectrometric peptide map, a 1- μ L aliquot from the peptide extracts was premixed with 1 μ L of matrix (10mg/mL CHCA in 35% ACN and 0.1% TFA) and spotted onto a MALDI target plate. Measurements were externally calibrated with a standard peptide mixture (Peptide Calibration Standard - Bruker Daltonics). Both MS and MS/MS data were acquired with a N₂ laser at a 25-Hz sampling rate. The monoisotopic masses were processed for identification. For MS/MS spectra, the peaks were calibrated by default and smoothed. Data were analyzed using MASCOT software (Matrix Science, London, UK), by searching against the Swiss-Prot/UniprotKB database. Taxonomy was limited to *Mus musculus* and the following search parameters were used: enzyme, trypsin; allowed missed cleavages, carbamidomethyl cysteine as fixed modification by the treatment with iodoacetamide; variable modifications, oxidation of methionine; mass tolerance for precursors was set to 50 ppm and for MS/MS fragment ions to ± 0.5 Da. The identifications were accepted if the extent of sequence coverage was at least 10% and the number of matched peptides was at least 5. Only individual scores greater than 55, defined by MASCOT probability analysis ($p \leq 0.05$) were accepted.

Validation of proteomic data by Western Blotting (WB) analysis

For 1D-WB, 20 μ g of protein extracts were separated by 12%SDS-PAGE and transferred onto Hybond ECL Nitrocellulose Membrane (GE Healthcare Life Sciences). The membrane was incubated in blocking solution (5% non-fat dry milk, 20 mM Tris, 140 mM NaCl, 0.1% Tween-20), and probed overnight at room temperature with specific antibodies against superoxide dismutase [Cu-Zn] (SODC; Cell Signaling Technology) and β -actin [14]. After incubation with blocking solution, the membrane was probed overnight at room temperature with specific antibodies against carbonic anhydrase-3 (CA3; Cell Signaling Technology).

The results were confirmed by at least three independent experiments. The blots were subjected to densitometric analysis by using Image J (1D-WB) or ImageMaster 2D Platinum 6.0 (2D-WB) software. For the quantification, the intensity of the immunostained bands/spots corresponding to SODC and to CA3 were normalized with the bands/spots corresponding to the β -actin from the same gel. Statistical analysis of the data was performed by Student's t-test; p-values ≤ 0.05 were considered statistically significant.

RESULTS

Proteome profile characterization of muscle quadriceps.

Since previous data [9] showed that both in gastrocnemius and quadriceps muscle there was remarkably significant reduction of inflammatory-necrotic areas in mdx mice exercised for 30 days, in the present work we decided to restrict the proteomic survey to the experimental mice groups exercised for 30 days and to focus our attention to quadriceps muscle. Quadriceps proteins purified from WT-Sed, WT-Ex, MDX-Sed and MDX-Ex mice were analyzed by 2DE analysis. Although 2-D gels of muscle fiber homogenates do not represent the entire muscle protein complement, optimized electrophoretic procedures can separate a large and representative proportion of key metabolic, structural, regulatory and contractile elements [15]. Therefore, this procedure is the most powerful tools for conducting comparative proteins profiling investigations, although very low-density components and proteins with extreme *pI* values may be under-represented in 2-D gels [16].

As shown in the Supplementary Figure 1, the silver stained proteomic maps of each group of mice revealed an overlapping spot pattern within the range of *pI* 4-8 and *Mr* from 150 to 9 kDa. In order to validate the quality of our extracts we compared the spot pattern of our samples to the reference proteomic maps of muscle tissues. We found that the proteome profile of our extracts was comparable to well-characterized 2DE maps of mouse and rat gastrocnemius, soleus, vastus lateralis and diaphragm (see: <http://world-2dpage.expasy.org/swiss-2dpage/viewer>) [17]. To further characterize the protein profile of quadriceps we performed a MALDI-TOF/TOF-MS analysis and using the Mascot search program we could identified 36 protein spots randomly selected in the sedentary WT-Sed mice 2DE gel. Moreover, by Western Blotting we identified 8 isoforms of β -actin (Supplementary Figure 2). Altogether, we identified 44 protein spots corresponding to 17 different proteins, marked with their UniProt accession number in Figure 1 and listed by this number in the Supplementary Table 1, where the protein name, *pI*-values and relative molecular masses, identification method, number of matched peptide sequences, percentage sequence coverage, and Mascot score are also reported. All identified proteins in the quadriceps of WT and MDX mice confirm previous available data from proteomics map of several types of muscle tissue [4,17-19].

Comparative proteomic analysis of 2-DE maps of quadriceps from wild type and mdx mice.

To investigate the potential proteomic alterations induced by low intensity endurance exercise in *mdx* mouse muscles, proteome profile of quadriceps isolated from WT-Sed and MDX-Sed or MDX-Ex mice were compared by using Image Master 2D Platinum 6.0. As first step, we searched variation in protein expression profile of quadriceps in MDX-Sed mice as compared to WT-Sed mice. We found that eight protein spots showed significant differences ($p < 0.05$) in %Vol (Figure 2). Among these eight proteins, seven were identified by MS and the protein corresponding to spot 4, for which the MS failed the identification, was identified by WB. Based on its position in the map, we hypothesized that the spot 4 could represent an isoform of CA3. As shown in Supplementary Figure 3, WB analysis confirmed the identity of this spot. The identities and fold-changes of all eight modulated protein spots are listed in Table 1. These data showed that the proteins altered in quadriceps of MDX-Sed belonged to six class of cellular components involved in muscle contraction and in cellular oxidative stress response (CA3 and SODC).

By comparing quadriceps from MDX-Sed and MDX-Ex mice, only four spots showed modulation in protein levels in response to the low intensity endurance exercise. These spots

were the three CA3 isoforms (pI 6.72/Mr 28122Da; pI 6.74/Mr 27613 Da and pI 6.82/Mr 27782Da), and the SODC (Figure 2).

The data concerning CA3 showed that protein levels of CA3 isoforms, found to be significantly up-regulated in quadriceps of MDX-Sed mice, were significantly or completely restored to WT-Sed values in quadriceps of MDX-Ex mice (Figure 3).

The data concerning SODC showed that the down-regulated protein levels found in quadriceps of MDX-Sed mice were significantly but not completely restored to WT-Sed values in quadriceps of MDX-Ex mice (Figure 3).

In order to evaluate if the low-intensity endurance exercise had specific effect on the quadriceps of dystrophic *mdx* mice, we assessed the expression levels of CA3 and SODC in WT-Ex mice. As reported in Figure 3, our analyses showed that no significant modulation of both CA3 and SODC was found in quadriceps of WT-Ex mice as compared to WT-Sed mice.

Proteomic data validation by western blotting analysis.

In order to validate key proteomic findings, comparative 1 and 2-D western blotting analyses were carried out. As shown in Figure 4, western blot data showed that the levels of two CA3 isoforms, pI 6.72/Mr 28122Da and pI 6.74/Mr 27613 Da, are up-regulated in the quadriceps of MDX-Sed mice and the exercise restore them to WT-Sed basic levels, confirming data obtained with proteomic analysis. By contrast to proteomic analysis, CA3 isoform with pI 6.82/Mr 27782 Da was found unchanged in MDX-Ex mice as compared to MDX-Sed mice. Moreover, WB analysis of CA3 revealed the presence of a more acidic isoform of this enzyme, which was not detectable in the silver stained map. The values of pI and Mr (pI6.62/Mr 28122 Da) of this CA3 isoform (Figure 4) were established by matching the WB with the corresponding silver stained maps. The levels of this isoform are significantly up-regulated in quadriceps of MDX-Sed mice in comparison to WT-Sed mice and appear restored to the levels of WT-Sed quadriceps in the MDX-Ex mice, with significant trend to be down-regulated (Figure 4). Similarly to proteomic analysis, WB analysis showed that there was no significant modulation of CA3 levels in WT-Ex mice as compared to WT-Sed (Figure 4).

The WB data of SODC showed that there is a significant reduction of SODC levels in the quadriceps of MDX-Sed mice compared to WT-Sed mice (Figure 4), confirming data obtained using proteomic analysis. These SODC levels found in MDX-Sed mice appeared completely restored to WT-Sed mice levels in MDX-Ex mice. However, while in WB the SODC levels found in MDX-Ex mice appeared completely restored to WT-Sed mice levels, they were only partially but significantly restored in proteomic analysis. WB analysis also confirmed proteomic data showing no significant modulation of SODC in WT-Ex mice as compared to WT-Sed mice (Figure 4).

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DISCUSSION

In the present study, with the proteomic procedure used, we have mapped few proteins as compared to data of previous studies [4,17,19,20] and therefore in the results obtained there is a restricted identification of proteins changed in MDX-Sed and MDX-Ex mice. However, despite the restricted number of proteins examined, we could identify some proteins with changed levels in MDX versus WT mice and their modulation in MDX-Ex versus MDX-Sed mice. In fact, among 17 proteins mapped we identified 8 protein spots that were significantly changed in quadriceps muscle profiling of MDX-Sed mice, as compared to quadriceps of sedentary WT-Sed mice. The proteomic profiling suggests a dystrophy-associated alteration in various proteins of contractile apparatus (MLRS, MYH4, TNNT), in proteins of oxidative stress (CA3, SODC) and ion homeostasis (PRVA). All these altered proteins levels in *mdx* mice confirm previous available data [4,17-19].

Comparative proteomic profiling: MDX-Ex mice versus MDX-Sed mice

The aim of present work was the identification of altered protein levels in MDX-Ex mice as compared to MDX-Sed mice in order to find explanation of strong reduction of degeneration muscle fibers in *mdx* mice induced by low-intensity endurance exercise [9]. Among the examined proteomic profiling, two type of proteins, CA3 and SODC, were significantly modified in MDX-Ex mice as compared to MDX-Sed mice. Indeed, levels of these proteins were already found dramatically changed in quadriceps of MDX-Sed mice, with CA3 up-regulated and SODC down-regulated, when compared to quadriceps of WT-Sed mice. Therefore, the comparative analysis revealed a statistically significant reduction of all four CA3 isoform levels and an increase of SODC levels in MDX-Ex mice as compared to respective protein levels found in MDX-Sed mice. All together these results highlight that exercise may restore to WT-Sed mice values the protein levels of CA3 and SODC found modified in muscle of MDX-Sed mice.

The increased level of CA3 isoforms in quadriceps of MDX-Sed mice and the strong decreased SODC protein levels suggest a state of oxidative stress in dystrophic muscle. On the other hand, the decreased levels of all isoforms of CA3 found in MDX-Ex mice and the parallel increased SODC protein level suggest improved muscle fibers response to oxidative stress. Since oxidative stress may contribute to pathophysiology of muscular dystrophy [21-23], the restored antioxidative response, especially the increased SODC levels, suggests a possible mechanism by which low-intensity endurance exercise may reduce muscle degeneration in *mdx* mice.

Oxidative stress and SODC levels

A plethora of therapeutic strategies have been employed with the purpose of ameliorating dystrophin deficiency, including antioxidant therapeutics that may reduce damage and improve muscle function and quality of life [22,23]. The possibility that oxidative stress may contribute to muscle pathology is supported by experimental evidences that a deficit of antioxidant defense can lead to cellular dysfunction, damage and tissue degeneration [21]. Additionally, an elevated oxidative stress has been also proposed as a contributing mechanism to muscle damage and weakness in dystrophin deficiency [21,22,24,25]. In view of this oxidative damage in dystrophic muscle, our findings showing that low-intensity endurance exercise may significantly recovery SODC levels in muscle of *mdx* mice suggest that a specific program of training could contribute to dystrophic muscle recovery by promoting muscle protection against oxidative stress.

Oxidative stress and CA levels

CA are a family of zinc metalloenzymes which have various tissue distributions and intracellular locations in mammals [26,27]. These enzymes catalyze the interconversion between carbon dioxide and the bicarbonate ion and thus they are involved in crucial physiological processes connected with respiration and transport of CO₂/bicarbonate between metabolizing tissues and lungs. CA3 is largely present in type I fibers of skeletal muscle and in less extent in type IIa and apparently absent in type IIb fibers [28,29].

As first evaluation of mean of the observed increase of all CA3 isoform protein levels in quadriceps of MDX-Sed mice, we considered the possible positive correlation with an increased need of acid-base balance regulation [30] and, based on this point of view, the increased levels of CA3 in *mdx* muscle should result protective against e.g. pH change. However in contrast to this possibility, it has been reported that treatment with CA inhibitors in muscular dystrophy reduces the degeneration/regeneration process [31]. This result suggests that high levels of CA3 could also be responsible for muscle degeneration, as also supported by data showing that CA inhibitors may be used as a therapeutic approach in some pathologies in which CA is increased [26]. According to this prospective, the recovery of normal CA3 levels in MDX-Ex mice could mimic the therapeutic approach with CA inhibitors and therefore may contribute to the reduction of degeneration in dystrophic muscles.

As second evaluation of mean of the CA3 up-regulation in *mdx* muscle we considered the possible protective role against oxidative stress. According to this, CA3 is largely present in slow twitch fibers with high oxidation potential and it has been suggested that CA3 may have a role in scavenging oxygen radicals and thereby protecting cells from oxidative damage [32,33]. Indeed, CA3 in skeletal muscle exists as a reservoir of oxidizable sulfhydryls that can be recruited to counteract acute and chronic oxidative insults [32]. Considering this potential role of CA3, the observed increase level of CA3 in MDX-Sed mice could correlate with elevated oxidative stress levels and therefore contribute to counteract muscle damage in *mdx* mice. Similarly, the reduction of CA3 levels in MDX-Ex mice could also be correlated to reduced oxidative stress by improving antioxidant mechanisms, such as the observed SODC protein level recovery, and consequently less stimulus for CA3 as antioxidant protein. However, to better define a correlation between the reduced levels of CA3 in MDX-Ex mice and the recovery of damaged fibers specific investigations are needed, since the reduction of CA3 levels in MDX-Ex mice could be secondary to muscle recovery itself.

CA3 and SODC levels in WT-Ex mice

Comparative analysis of quadriceps from WT-Sed and WT-Ex mice did not show difference in both CA3 and SODC levels. The result that the low-intensity endurance training did not modify the CA3 levels is in agreement with other report [34], and could be correlated to the chosen low intensity exercise protocol. In addition, although only speculative and therefore suggestion for future specific investigations, since CA3 is high expressed in slow twitch oxidative fibers [28,29], the unchanged CA3 levels in MDX-Ex mice indirectly may suggest a lack of significant shifting of fast versus slow fibers subtype following the low-intensity endurance training. On the other end, a lack of significant fiber type shifting could be also supported by the data that in MDX-Ex mice CA3 levels were found reduced as compared to MDX-Sed mice.

Although previous data have reported that endurance exercise increases SODC levels [35], in this study we did not find change in the SODC levels of WT-Ex mice. This result, as above discussed for CA3 levels, could depend on the low intensity endurance exercise used in our experiments and could correlate with absence or weak oxidative stress [36]. However, since

in MDX-Ex mice, subject to the same training of WT mice, there was a significant recovery of SODC levels, we can't exclude a SODC gene stimulation in addition to recovery of damaged fibers, that we observed in MDX-Ex mice [9].

CONCLUSION

In conclusion, based on data found in the present study, it seems that low-intensity endurance exercise, by modulating some proteins involved in oxidative stress defense, may in part contribute to reduce, as observed in our previous study, cell degeneration in *mdx* muscles [9]. However, further investigations are needed to better define the extension of proteins change in MDX-Ex versus MDX-Sed mice and its correlation with the recovery of damaged fibers in MDX-Ex mice.

AUTHOR CONTRIBUTION:

Simona Fontana performed proteomic experiments. Marco Giallombardo performed western blot experiments. Monica Frinchi prepared all protein samples and performed exercise experiments. Odessa Schillaci contributed to some experiments. Giuseppe Morici: designed exercise experiments. Valentina Di Liberto: analysed data. Riccardo Alessandro contributed to write the manuscript. Giacomo De Leo designed proteomic experiments. Vincenzo Perciavalle contributed to write the manuscript. Natale Belluardo conceived the project. Giuseppa Mudò: wrote the manuscript.

All authors discussed the results and read, revised and approved the final manuscript

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SUMMARY STATEMENT

By proteomic analysis we found an up-regulation of four Carbonic anhydrase-3 isoforms and a down-regulation of SODC in quadriceps of sedentary mdx mice as compared to wild type mice and they levels were significantly restored to wild type values following low-intensity endurance exercise

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Table 1. Over/underexpressed proteins in WT and *mdx* quadriceps

Nr Spot	Protein nome	Abbreviated name	AC number ^a	pI/Mr (kDa)	MDX-Sed vs WT-Sed ^b		MDX-Ex vs MDX Sed ^b	
					Fold change	<i>p</i> -values	Fold change	<i>p</i> -values
1	Myosin 4	MYH4	Q5SX39	5.37/59.1	-4.3	0.027	NDE ^c	-
2	Troponin T	TNNT	Q9QZ47	8.65/33.7	-3.1	0.020	NDE ^c	-
3	Carbonic anhydrase 3	CA3	P16015	6.72/28.1	2.8	0.046	-3.6	0.022
4	Carbonic anhydrase 3	CA3	P16015	6.74/27.6	2.7	0.040	-2.3	0.042
5	Carbonic anhydrase 3	CA3	P16015	6.82/27.8	2.1	0.033	-1.7	0.039
6	Myosin regulatory light chain 2 skeletal muscle isoform	MLRS	P97457	4.78/15.8	-7.9	0.005	NDE ^c	-
7	Superoxide dismutase [Cu-Zn]	SODC	P08228	6.10/14.6	-5.28	0.02	2.10	0.01
8	Parvalbumin alpha	PRVA	P32848	4.84/12.9	-4.7	0.013	NDE ^c	-

^aAccession number in UniProtKB/Swiss-Prot"

^bOnly proteins spots exhibiting at least 1.5-fold changes in %Vol and $p < 0.05$ (calculated by Student's *t*-test) were considered differentially expressed. Differences with $p < 0.05$ were considered significant.

^cNDE: no differential expression.

Figure 1 - Representative silver stained proteomic maps of quadriceps from sedentary wild type mouse. Proteins (60 μ g) were loaded on IPG strips (18 cm, 3.5–10 nonlinear pH gradient). The second dimension was performed on a vertical linear-gradient slab gel (9–16%). The pH-values of the first dimension gel system and molecular mass range (in kDa) of the second dimension are indicated on the top and on the left of the panels, respectively.

Spots labelled with the UniProt Accession Number were identified by MALDI-TOF/TOF-MS. Details of the identification are reported in Supplementary Table 1 – Supporting Information.

Figure 2 – Detection of proteins differentially expressed in WT and MDX quadriceps. Comparative analysis of protein profile of quadriceps from sedentary Wild Type (WT-Sed) and sedentary *mdx* (MDX-Sed) mice highlighted 8 protein spots showing a significant change in expression levels. These spots are marked by arrows and are numbered 1–8. The comparison of protein profile of quadriceps from MDX-Sed and exercised *mdx* (MDX-Ex) mice revealed that levels of spots 3, 4, 5 and 7 were affected by the low intensity endurance exercise. Details of all these protein spots are reported in Table 1.

Figure 3 – Expression pattern of CA3 isoforms and SODC in quadriceps. Expanded view of 2-D gels of quadriceps from sedentary Wild Type (WT-Sed), exercised Wild Type (WT-Ex), sedentary *mdx* (MDX-Sed) and exercised *mdx* (MDX-Ex) mice, in which the modulation of CA3 and SODC in MDX preparations can be observed. In the corresponding histogram, the %Vol values of each CA3 isoforms and SODC are reported. The CA3 isoforms are identified with pI-values and relative molecular masses: (1) pI 6.72/Mr 28122 Da; (2) pI 6.74/Mr 27613 Da and (3) pI 6.82/Mr 27782 Da. The values reported in the graphs are the mean of three independent experiments \pm SD. *t*-test: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

Figure 4 –Western Blot of CA3 isoforms and SODC in quadriceps. Representative immunoblots with expanded views of antibody-decorated protein spots and bands. Immunoblotting was performed after 2DE for CA3 and after 1DE for SODC. β -actin was used as loading control. The blot densities are expressed as folds of control. CA3 isoforms are identified with pI-values and relative molecular masses: (1) pI 6.62/Mr 28122 Da; (2) pI 6.72/Mr 28122Da; (3) 6.74/Mr 27613 Da and (4) pI 6.82/Mr 27782Da. Data are mean \pm SD; * $p < 0.05$; ** $p < 0.01$. Sedentary Wild Type (WT-Sed), exercised Wild Type (WT- Ex), sedentary *mdx* (MDX-Sed) and exercised *mdx* (MDX-Ex) mice.

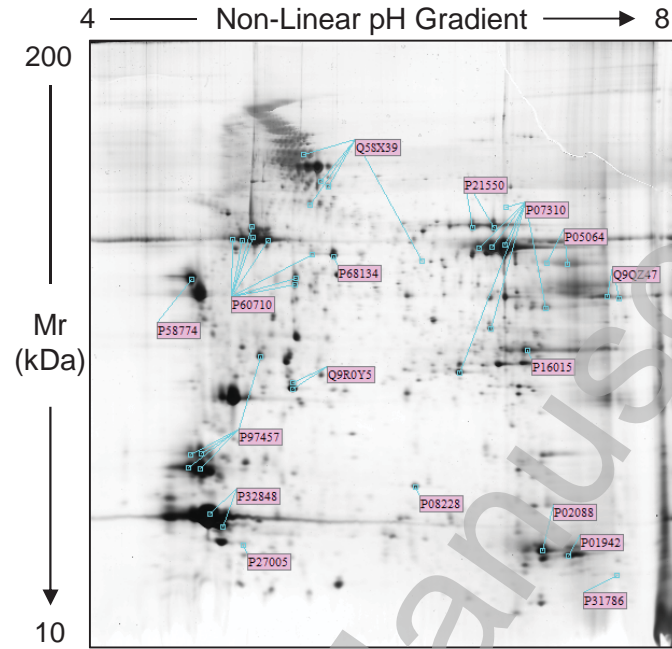


Figure 1

Representative silver stained proteomic maps of quadriceps from sedentary wild type mouse. Proteins (60 μ g) were loaded on IPG strips (18 cm, 3.5–10 nonlinear pH gradient). The second dimension was performed on a vertical linear-gradient slab gel (9–16%). The pH values of the first dimension gel system and molecular mass range (in kDa) of the second dimension are indicated on the top and on the left of the panels, respectively.

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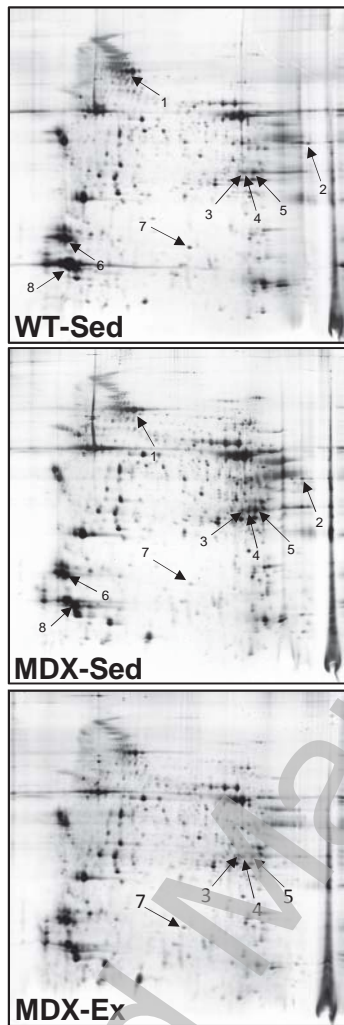


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Detection of proteins differentially expressed in WT and MDX quadriceps. Comparative analysis of protein profile of quadriceps from sedentary Wild Type (WT-Sed) and sedentary *mdx* (MDX-Sed) mice highlighted 8 protein spots showing a significant change in expression levels. These spots are marked by arrows and are numbered 1–8. The comparison of protein profile of quadriceps from MDX-Sed and exercised *mdx* (MDX-Ex) mice revealed that levels of spots 3, 4, 5 and 7 were affected by the low intensity endurance exercise. Details of all these protein spots are reported in Table 1.

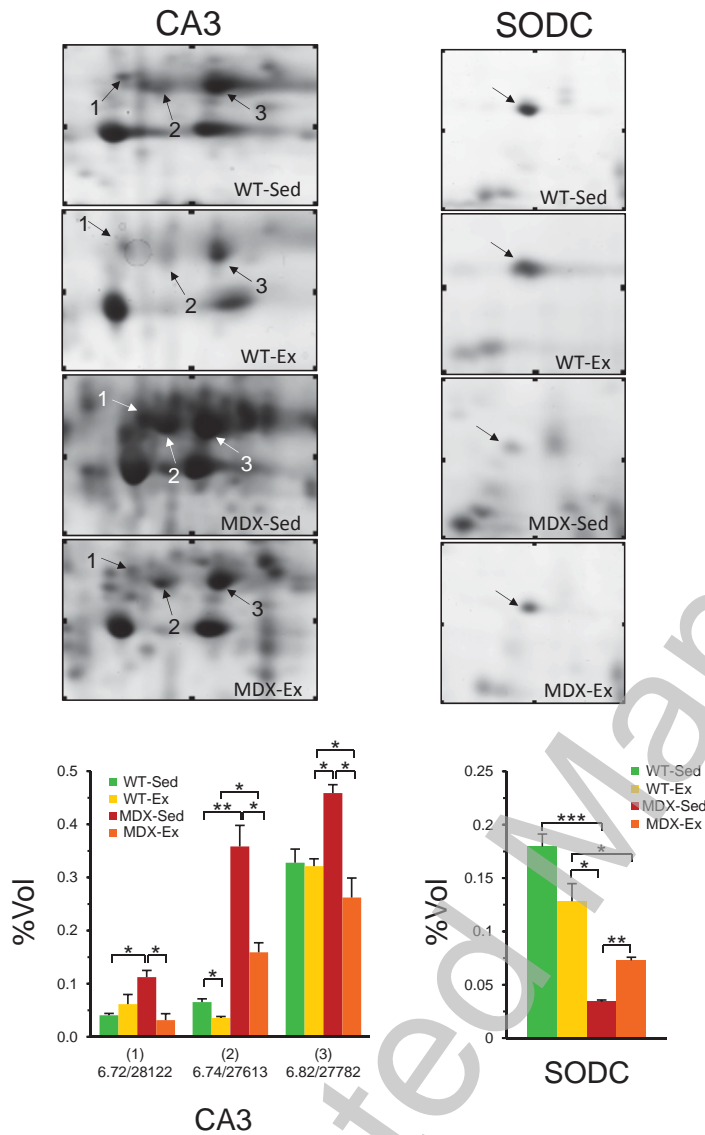


Figure 3 – Expression pattern of CA3 isoforms and SODC in quadriceps. Expanded view of 2-D gels of quadriceps from sedentary Wild Type (WT-Sed), exercised Wild Type (WT-Ex), sedentary mdx (MDX-Sed) and exercised mdx (MDX-Ex) mice, in which the modulation of CA3 and SODC in MDX preparations can be observed. In the corresponding histogram, the %Vol values of each CA3 isoforms and SODC are reported. The CA3 isoforms are identified with pI-values and relative molecular masses: (1) pI 6.72/Mr 28122 Da; (2) pI 6.74/Mr 27613 Da and (3) pI 6.82/Mr 27782 Da. The values reported in the graphs are the mean of three independent experiments \pm SD. t-test: *p<0.05; **p<0.01; ***p < 0.001

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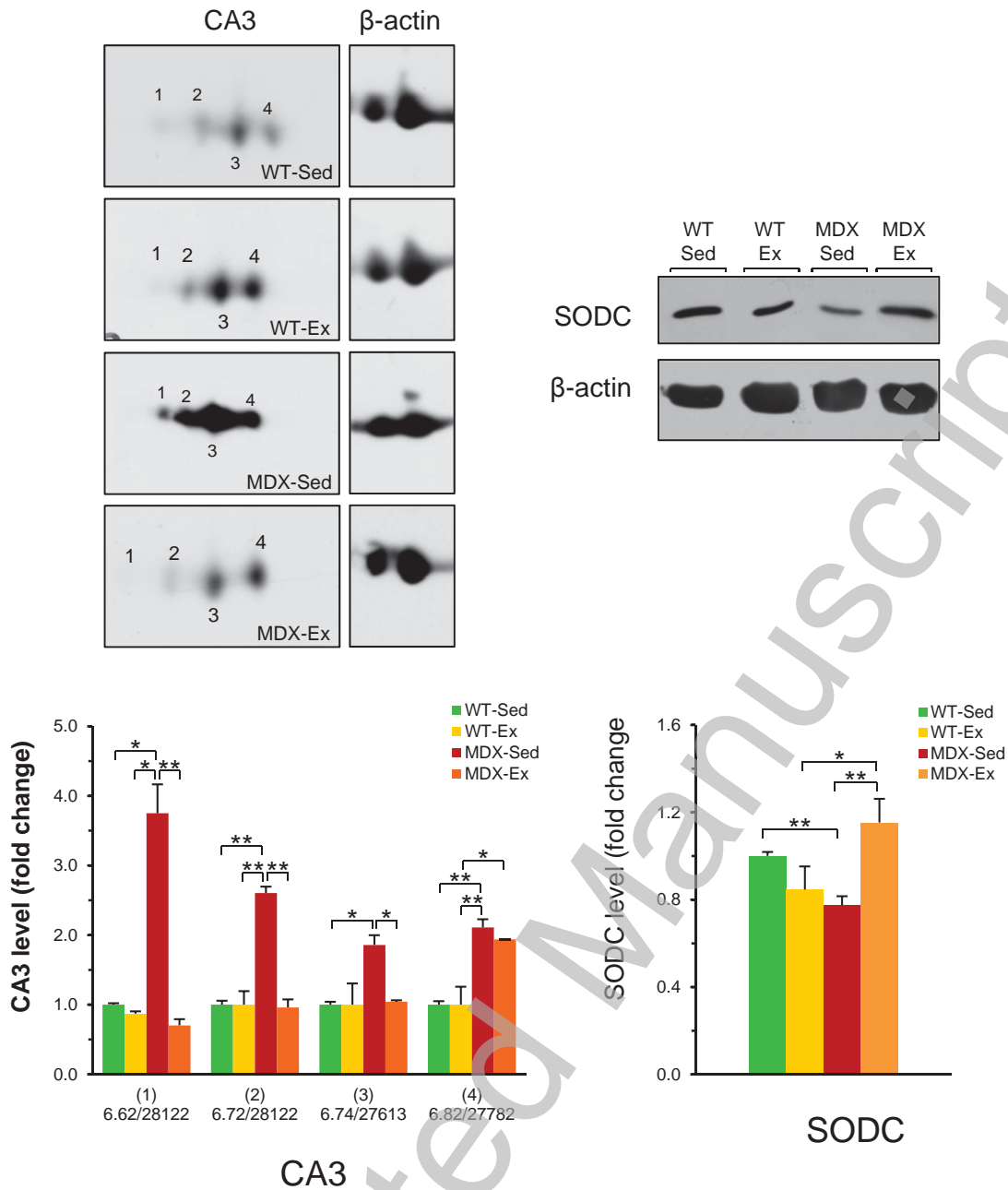


Figure 4

Figure 4 – Western Blot of CA3 isoforms and SODC in quadriceps. Representative immunoblots with expanded views of antibody-decorated protein spots and bands. Immunoblotting was performed after 2DE for CA3 and after 1DE for SODC. β-actin was used as loading control. The blot densities are expressed as folds of control. CA3 isoforms are identified with pI-values and relative molecular masses: (1) pI 6.62/Mr 28122 Da; (2) pI 6.72/Mr 28122Da; (3) 6.74/Mr 27613 Da and (4) pI 6.82/Mr 27782Da. Data are mean ± SD; *p < 0.05; **p < 0.01. Sedentary Wild Type (WT-Sed), exercised Wild Type (WT- Ex), sedentary *mdx* (MDX-Sed) and exercised *mdx* (MDX-Ex) mice.