Effects of muscular dystrophy, exercise and blocking activin receptor IIB ligands on the unfolded protein response and oxidative stress

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Abstract

Protein homeostasis in cells, proteostasis, is maintained through several integrated processes and pathways and its dysregulation may mediate pathology in many diseases including Duchenne muscular dystrophy (DMD). Oxidative stress, heat shock proteins, endoplasmic reticulum (ER) stress and its response, i.e. unfolded protein response (UPR), play key roles in proteostasis but their involvement in the pathology of DMD are largely unknown. Moreover, exercise and activin receptor IIB blocking are two strategies that may be beneficial to DMD muscle, but studies to examine their effects on these proteostasis pathways are lacking. Therefore, these pathways were examined in the muscle of mdx mice, a model of DMD, under basal conditions and in response to seven weeks of voluntary exercise and/or activin receptor IIB ligand blocking using soluble activin receptor-Fc (sAcvR2B-Fc) administration. In conjunction with reduced muscle strength, mdx muscle displayed greater levels of UPR/ER-pathway indicators including greater protein levels of IRE1α, PERK and Atf6b mRNA. Downstream to IRE1α and PERK, spliced Xbp1 mRNA and phosphorylation of eIF2α were also increased. Most of the cytoplasmic and ER chaperones and mitochondrial UPR markers were unchanged in mdx muscle. Oxidized glutathione was greater in mdx and was associated with increases in lysine acetylated proteome and phosphorylated sirtuin 1. Exercise increased oxidative stress when performed independently or combined with sAcvR2B-Fc administration. Although neither exercise nor sAcvR2B-Fc administration imparted a clear effect on ER stress/UPR pathways or heat shock proteins, sAcvR2B-Fc administration increased protein expression levels of GRP78/BiP, a triggering factor for ER stress/UPR activation and TxNIP, a redox-regulator of ER stress-induced inflammation. In conclusion, the ER stress and UPR are increased in mdx muscle. However, these processes are not distinctly improved by voluntary exercise or blocking activin receptor IIB ligands and thus do not appear to be optimal therapeutic choices for improving proteostasis in DMD.

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1. Introduction

Duchenne muscular dystrophy (DMD) is a disease characterized by progressive wasting of skeletal muscle [1]. The absence of functional dystrophin is a major reason for perturbations to cellular changes including abnormal Ca\(^{2+}\) homeostasis, inflammatory cell infiltration, fibrosis, necrosis, regeneration [2] and in turn protein homeostasis (proteostasis) in DMD and its animal model, mdx mice. The restoration of dystrophin expression in all of the muscles and for all of the different mutations is currently unattainable [3]. Therefore, other strategies are being developed that may complement dystrophin restoration approaches.

Oxidative stress is a disruption of thiol redox circuits that results in impaired cell signaling and dysfunctional redox-control [4,5]. It is linked to several pathological processes including dysfunction of proteostasis and the accumulation of misfolded proteins in the lumen of the endoplasmic reticulum (ER), resulting in ER stress [6]. Notably, secondary consequences of dystrophin deficiency include the loss of skeletal muscle calcium homeostasis and hypoxia [7,8] as well as deficiency in nitric oxide synthase NOS [2] that can trigger oxidative stress [9,10] and in theory, ER stress. Furthermore, accumulation of improperly folded dystrophin in mdx mice [11] may also cause ER stress. The unfolded protein response in ER (UPR\(_{\text{ER}}\)) resolves ER stress and consists of several branches of signaling pathways aimed to recover proteostasis by increasing the protein folding machinery (chaperones), suppressing the overall translation of proteins and increasing the ER associated protein degradation (ERAD) [12]. Additionally, mitochondrial UPR (UPR\(_{\text{mito}}\)) [13] and cytoplasmic chaperones including heat shock proteins (HSP) [14] prevent accumulation of unfolded or incorrectly folded proteins. When ER stress is too severe or chronic, or the UPR and HSP responses are impaired and unable to cope with the protein-folding defects needed to maintain proteostasis, pro-apoptotic signaling pathways are activated in the cell [12]. Indeed, a recent study showed that glucose regulated protein 78 (GRP78/BiP), which is a triggering factor for ER stress/UPR activation, was associated with ER-related apoptosis signaling in human DMD muscle and/or mdx mice [15]. A more thorough understanding of these processes in muscular dystrophy would provide further insight into the role these factors may play in mediating the disease pathology in order to develop new therapeutic tools.

Type IIb activin receptor (AcvR2B) ligands myostatin and activins inhibit muscle hypertrophy [16,17]. Blockade of AcvR2B ligands can be achieved, e.g. by using the soluble ligand binding domain of type IIb activin receptor fused to the Fc domain (sAcvR2B-Fc) to effectively increase muscle size [18-21]. Blocking these proteins using various strategies has been shown to attenuate dystrophic pathology of the mdx mouse in some [18,22], but not in all studies [20,23]. However, the effect of AcvR2B ligand blocking on ER stress and UPR in dystrophic muscle is currently unknown.

Muscular dystrophy is associated with a reduced skeletal muscle oxidative capacity [24]. Exercise improves muscle oxidative capacity in mdx mice [25,26], which as an adaptation could increase resistance to the dystrophic pathology [7]. Exercise training may decrease markers of oxidative stress in mdx mice, but this response may depend on the dose, type, intensity and duration of exercise, and possibly the disease status [9,10]. Decreased levels of oxidative stress would be beneficial since ER stress and oxidative stress can work in a positive feed-forward loop in a manner that disrupts cell function and induces pro-apoptotic signaling [6,27]. Therefore, the performance of regular, tolerable exercise alone or in combination with other therapeutic tools may positively modulate pathways involved in proteostasis that could alleviate skeletal muscle pathologies.

The overall purpose of this experiment was twofold. One purpose was to investigate for the first time the effects of muscular dystrophy on oxidative stress concurrently with ER stress, UPR and HSP defense. The second purpose was to examine these same physiological states in response to AcvR2B ligand blocking and voluntary exercise training as these interventions may elicit beneficial effects on mdx muscle by altering muscle proteostasis.

2. Materials and methods

2.1. Animals

Six- to seven-week-old male mdx mice and C57Bl/10ScSnJ controls originating from the same strain (Jackson Laboratories, Bar Harbor, Maine, USA) were used in the experiments. The mice were housed under standard conditions (i.e., 22 °C, 12 h light/dark cycle) and had free access to tap water and food pellets (R36; 4% fat, 55.7% carbohydrate, 18.5% protein, 3 kcal/g, Labmor, Stockholm Sweden).

The treatment of animals was in strict accordance with the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes. The protocol was approved by the National Animal Experiment Board ( Permit Number: ESLH-2009-08528/Ym-23).

2.2. Experimental design

Two experimental designs were used in this investigation: 1) effect of the mdx phenotype on ER stress/UPR and oxidative stress; and 2) exercise and blocking activin receptor ligands on the same parameters in mdx mice. In the first experiment, mdx mice (n = 8) and wild type mice from the same strain (C57Bl/10ScSnJ) (n = 5) were compared. In the second experiment, 7-week old mdx mice were randomly divided into four groups in a 2 x 2 design (n = 8 animals/group): 1) sedentary control injected with PBS (vehicle); 2) running wheel and injection with PBS; 3) sedentary injected with sAcvR2B-Fc; and 4) running wheel and injection with sAcvR2B-Fc. sAcvR2B-Fc (5-mg/kg) or PBS was injected intraperitoneally once per week for seven weeks with or without voluntary wheel running exercise. To allow treatments to take effect, the mice were prevented from exercising by locking the running wheels for two days at the start of the experiment. In order to study only long-term effects of exercise the mice did not have access to running wheels on the last two days of the experiment. During the experiments all the conditions were standardized. At ~14 weeks of age all the mice were euthanized by cervical dislocation and muscle samples were collected. Forelimb grip strength was measured the day before the sacrifice using the protocols of TREAT-nmd (web-link: http://www.treat-nmd.eu/downloads/file/sops/dmd/MDX/DMD_M.2.2.001.pdf). The measurements were conducted five times with the highest score (absolute force) taken as the final result.

2.3. sAcvR2B-Fc production

The recombinant fusion protein was produced and purified in house as described previously [19]. The protein is similar, but not identical with that originally generated by Se-Jin Lee [21]. In short, the fusion protein contains the ectodomain (ecd) of human sAcvR2B and a human IgG1 Fc domain. The protein was expressed in Chinese hamster ovary (CHO) cells grown in suspension culture.

2.4. Voluntary wheel running

The mice were housed in cages where they had free access to custom-made running wheels (diameter 24 cm, width 8 cm) 24 h/
day. Sedentary animals were housed in similar cages without the running wheel.

2.5. Muscle Immunohistochemistry

Gastrocnemius muscle cross-sections were cut using a cryomicrotome and stained for membrane (caveolin 3 (ab2912, Abcam, UK), dilution 1:100) and captured with Olympus BX-50 fluorescent microscopy. 10x magnification was used and the average fiber number in randomly selected fields of high quality was 342.8 ± 30.5 fibers per section. Image analysis of fiber CSA was performed with specific software (ImageJ, U.S. National Institutes of Health, Bethesda, MD, USA).

2.6. Muscle sampling and homogenization

The gastrocnemius was immediately removed from the hind limb, weighed, and frozen. The muscle was pulverized and one-half of the powder was allocated for protein analysis and one-half to RNA isolation. The powder for protein analysis (except enzyme antibodies) was homogenized in ice-cold buffer with protease inhibitors (Pierce Biotechnology, Rockford, IL, USA) and total protein measured using the bicinchoninic acid protein assay (Pierce Biotechnology) with an automated KoneLab analyzer (Thermo Scientific, Vantaa, Finland).

2.7. Western immunoblot analyses

Western blots were conducted in two laboratories and thus two slightly different protocols are provided below. Unless otherwise stated, all chemicals and reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA) and were of analytic grade or the highest grade available.

2.7.1. PDI, IRE-1α, PERK, p-eIF2α/eIF2α, Sirtuins, AMPK, lysine acetylation

Muscle homogenates mixed in Laemmli sample buffer were heated at 95 °C to denature proteins and processed as described previously [19,25]. In short, protein was separated by SDS-PAGE and transferred to a PVDF membrane, blocked and incubated overnight at 4 °C and then washed and incubated with secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for 1 h followed by washing. Proteins were visualized by ECL (SuperSignal West femto maximum sensitivity substrate, Pierce Biotechnology, Rockford, IL, USA) and quantified using a ChemiDoc XR+ device with Quantity One software (version 4.6.3. Bio-Rad Laboratories, Hercules, CA, USA). The uniformity of the protein loading was confirmed by staining the membrane with Ponceau S and by re-probing the membrane with an antibody against GAPDH (Abcam, Cambridge, UK).

2.7.2. Heat shock proteins, GRP78, TRX and TxNIP

After SDS-PAGE, proteins were transferred to a nitrocellulose membrane (Millipore, Bedford, Mass., USA) as previously described [28]. The membranes were blocked and after that treated with antibodies overnight at 4 °C. Immunoblots were visualized using an Odyssey Imaging System (Li-Cor Biosciences Inc., Lincoln, NB, USA) and quantified using Odyssey Software.

2.7.3. Protein carbonyls

Protein carbonyls were derivatized using 2,4-dinitrophenyl hydrazine immediately before electrophoresis as previously described [29,30]. Protein extracts (20 µg protein/lane) were electrophoresed on 12.5% SDS-PAGE. A rat monoclonal primary antibody raised against 2,4-dinitrophenol (Zymed Laboratories, San Francisco, CA, USA) was incubated overnight at 4 °C. After secondary antibody incubation, the immunoblots were visualized using an Odyssey Imaging System (Li-Cor Biosciences Inc., Lincoln, NB, USA) and quantified using Odyssey Software.

2.7.4. Primary antibodies

The antibodies for inositol-requiring enzyme 1α (IRE1α, #3294), protein disulfide isomerase (PDI, #3501), lysine acetylated proteins (#9441), protein kinase R-like ER protein kinase (PERK, #3192), eukaryotic initiation factor 2 subunit α (eIF2α, #5324) and its phosphorylated form at ser51 and phosphorylated sirtuin 1 at ser46 in mouse (#3398) and AMPK total (#2603) and phosphorylated at Thr172 (#4188) were purchased from Cell Signaling Technology. Antibodies against GAPDH (ab48585), sirtuin 1 (ab28170), 3 (ab118334) and 6 (ab62739) were from Abcam. Antibodies that recognize the inducible forms of heat shock protein 70 (HSP70, SPA-S10), heat shock protein 60 (HSP60, SPA-806), heat shock protein 90 (HSP90, SPA-835), heat shock protein 25 (HSP25, SPA-801), heat shock protein 47 (HSP47, SPA-470) and glucose-regulated protein 78 (GRP78, SPA-826) and 75 (GRP75, SPS-825) were purchased from Enzo Life Sciences Inc., (Farmingdale, NY, USA). Antibody against thioredoxin (ATRX-6) was purchased from IMCO Corp (Stockholm, Sweden), TxNIP (thioredoxin-interacting protein, VDUP-1, # K0205-3) from MBL (Medical and Biological laboratories Co. Ltd, Nagoya, Japan) and actin (A-2066) and HSP10 (SAB4501465) from Sigma-Aldrich (St. Louis, MO, USA).

2.8. Assays for glutathione levels and antioxidant enzyme activity

2.8.1. GSSG/TGSH

Gastrocnemius muscle was homogenized on ice in brief bursts by an Ultra-Turax homogenizer (Janke and Kunkel, Germany) in a 1:10 (w:v) dilution of ice-cold 5.0% metaphosphorous acid. Resultant homogenates were centrifuged at 10,000 g for 15 min at 4 °C, and the supernatant was stored at −70 °C. On the day of measurement the supernatant was diluted with distilled water and TGSH was measured spectrophotometrically by a GSSG reductase recycling method as described earlier [31]. The rate of change in absorbance at 412 nm was monitored with double-beam spectrophotometer at room temperature and tissue concentrations were estimated by linear regressions from the standard curve.

2.8.2. GPX, GRD, GST and TPOR

Total glutathione peroxidase (GPX) activity was determined with cumene hydroperoxide as substrate. Glutathione reductase (GRD) activity was determined in the presence of 50 mM Tris-HCl buffer with 1 mM EDTA, 2 mM NADPH, and 20 mM GSSH by monitoring the decrease in absorbance per minute due to the oxidation of NADPH at 340 nm. GST activity was also assayed at 340 nm with 1,2-dichloro-4-nitrobenzene as substrate. Activities of muscle GPX, GRD, and GST were determined from the cytosolic supernatant spectrophotometrically, as described previously [32]. Thiol-Protein Oxidoreductase Activity (TPOR) to demonstrate PDI linked reaction is monitored by spectrophotometer at 340 nm.

2.9. RNA and DNA isolation and cDNA synthesis

Gastrocnemius muscle was pulverized and homogenized in liquid nitrogen after which 65–75 mg of muscle powder was placed into Trizol reagent (Invitrogen, Carlsbad, CA, USA). Total RNA was extracted according to the manufacturer’s guidelines and analyzed in duplicate using a Nanodrop ND-1000 (Thermo Fisher Scientific Inc., Waltham MA, USA). Any possible remaining DNA was further
were conducted using pre-designed Bio-Rad PrimePCR CID0022729) and caseinolytic peptidase (EBP homologous protein (Chop/Ddit3, assay ID qMmu-746) (Invitrogen, USA). qPCR for C/ClpP, qMmuCID0005629) was conducted under standard PCR conditions as recommended by the manufacturer (Applied Biosystems, Foster City, CA, USA). The spliced variant of X-box binding protein 1 (sXbp1) was analyzed using SYBR green primers: forward: TGCTGAGTCCG-3AAGTTTTTGC, reverse: ACTGTGAAGTCTCCCTACTGAGAG with product length of 251 bp (496–746) (Invitrogen, USA). qPCR for C/EBP homologous protein (Chop/Ddit3, assay ID qMmu-CID0020314), activating transcription factor 6b (ATF6b, qMmuCID0022729) and caseinolytic peptidase (ClpP, qMmuCID0005629) were conducted using pre-designed Bio-Rad PrimePCR™ SYBR® Green Assays. The gene expressions were normalized to the expression of X-box binding protein 1 (sXbp1) analyzed using SYBR Supermix (Bio-Rad Laboratories) and CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories). Real-time qRT-PCR was conducted under standard PCR conditions as recommended by the manufacturer (Applied Biosystems, Foster City, CA, USA) in triplicate. The spliced variant of X-box binding protein 1 (sXbp1) was analyzed using SYBR green primers: forward: TGCTGAGTCCG-CACCGAGTG and reverse: CTGATGAGGCTCCCCACTGACAGA with product length of 251 bp (496–746) (Invitrogen, USA), qPCR for C/EBP homologous protein (Chop/Ddit3, assay ID qMmu-CID0020314), activating transcription factor 6b (ATF6b, qMmuCID0022729) and caseinolytic peptidase (ClpP, qMmuCID0005629) were conducted using pre-designed Bio-Rad PrimePCR™ SYBR® Green Assays. The gene expressions were normalized to the expression of stably acted Gapdh (Taqman probe: Mm99999915_g1). The analysis was conducted using the delta delta Ct (ΔΔCt) method and quantification was performed in the exponential amplification phase.  

2.11. Real-time qRT-PCR

Three μg of total RNA were reverse transcribed to synthesize cDNA using a High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA). The mRNA expression levels were quantified with Real-time qPCR according to standard procedures using IQ SYBR Supermix (Bio-Rad Laboratories) and CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories). Real-time qRT-PCR was conducted under standard PCR conditions as recommended by the manufacturer (Applied Biosystems, Foster City, CA, USA) in triplicate. The spliced variant of X-box binding protein 1 (sXbp1) was analyzed using SYBR green primers: forward: TGCTGAGTCCG-CACCGAGTG and reverse: CTGATGAGGCTCCCCACTGACAGA with product length of 251 bp (496–746) (Invitrogen, USA), qPCR for C/EBP homologous protein (Chop/Ddit3, assay ID qMmu-CID0020314), activating transcription factor 6b (ATF6b, qMmuCID0022729) and caseinolytic peptidase (ClpP, qMmuCID0005629) were conducted using pre-designed Bio-Rad PrimePCR™ SYBR® Green Assays. The gene expressions were normalized to the expression of stably acted Gapdh (Taqman probe: Mm99999915_g1). The analysis was conducted using the delta delta Ct (ΔΔCt) method and quantification was performed in the exponential amplification phase.

2.12. Data analysis

All data, with the exception of microarray, were evaluated by analysis of variance (ANOVA) followed by Tukey’s post-hoc test (treatments) or t-test (mdx vs. control). The level of significance in these analyses was set at P < 0.05. Data are expressed as means ± SE. Correlations were analyzed using Pearson’s Product Moment Coefficient. The list of differentially expressed genes generated between the mdx and wild type sample groups from the microarray data were detected utilizing linear modeling and empirical Bayes methods of the Limma package of R. The resulting raw p-values were adjusted using the Benjamini and Hochberg method. The genes with an adjusted P ≤ 0.05 and absolute fold change higher than 1.5 were considered to be differentially expressed. Enrichment of functionally related genes in three different gene set collections was first performed using a non-biased method by Gene Set Enrichment Analysis software (GSEA; Version 2.0). For this analysis, ranking lists representing different ratio combinations of the normalized data were created by averaging the group results for each gene. The enriched gene sets were ranked based on their fold-change ratios. The collection used was the Canonical Pathways collection (1452 gene sets, C2: CP, version 3.0) (http://www.broadinstitute.org/gsea/msigdb/collections.jsp). The number of permutations by gene set was set to 1000 and gene sets with at least five, and no more than 500 genes were taken into account in each analysis. Each analysis was carried out at least five times and all the results were averaged into a single value. The statistical significance was calculated using false discovery rate (FDR). The level of significance was set at FDR < 0.05.

Further, the differentially expressed genes were uploaded to DAVID functional annotation tool [36] to analyze the effects of the phenotype. The enrichment analysis was run against the default gene groups including gene ontologies and KEGG-pathways. The Disulfide bond was highly enriched within the DAVID results, and thus the genes within this pathway with most striking regulation, i.e. having an absolute fold change ≥ 3 and P < 0.05, were more carefully studied with hierarchical clustering. The expression values of these genes were analyzed with hierarchical clustering and illustrated with a heatmap using MATLAB. Within clustering, the Euclidean distance and Ward’s minimum variance criterion linkage method was used.

3. Results

3.1. Reported skeletal muscle characteristics

This study is a follow-up to work that was previously published [25,26]. In brief, muscles of mdx mice typically contained centrally nucleated fibers and a large content of fibrosis and proliferative cells other than muscle fibers. sAcvR2B-Fc administration increased the mass of all muscles weighed. Wheel running exercise performed by the mdx mice enhanced markers of muscle aerobic capacity (e.g. citrate synthase and SDH activities as well as gene sets of aerobic metabolism in microarray) to levels similar to, or higher than those observed in healthy mice.

3.2. Effects of mdx phenotype on muscle morphology and grip strength

No difference in mean fiber cross-sectional area (CSA) was observed in mdx mice when compared to wildtype mice (P = 0.70, Fig. 1A), but fiber size distribution tended to show differences (Fig. 1B). Statistically, this could be observed as a greater percentage of very small (< 600 μm²) or small (< 1000 μm²) fibers in mdx mice (P < 0.05), which represents newly regenerating fibers (Fig. 1C). Along with the higher percentage of very small fibers, lower grip strength presented as absolute values (Fig. 1D) or normalized to body weight (not shown) was also observed among mdx mice (P < 0.05).

3.3. Effects of mdx phenotype on redox balance

Recent studies showed increased oxidative stress as one candidate mechanism that may mediate the pathology of dystrophin deficiency [9,10]. Dystrophic mdx mice at the age of ~14 weeks showed significantly greater levels of oxidized glutathione (GSSG) (P < 0.05) compared to healthy wild type animals (Fig. 2A–C). No effect of muscular dystrophy was seen on protein content of reductase-active proteins, TxnIP and TRX (Fig. 2D).

Next, a gene clustering analysis was conducted from microarray
data with a gene set enrichment analysis (GSEA) that is designed to find physiologically meaningful results from large gene sets with <20% average changes in individual gene expression [37]. A trend for decreased pathways of glutathione (FDR=0.07) metabolism was observed in mdx mice compared to healthy mice (Fig. S1 and Table S1), which further supports the presence of altered redox balance in mdx mice.

In addition, anti-oxidative enzyme activities related to redox balance and glutathione metabolism (glutathione reductase (GRD), glutathione S-transferase (GST), glutathione peroxidase (GPX) and...
Fig. 3. The effects of muscular dystrophy on ER stress, UPR activation, sirtuins and acetylated proteins. A) Most UPR/ER stress markers (PDI, PERK, p-eIF2α, eIF2α, and IRE1α proteins were upregulated (P < 0.05-0.001) in muscle from mdx mice. B) Spliced Xbp1 (sXbp1) and Atf6 mRNA. C) Hierarchical clustering of the gene-wise standardized logarithmic expression values of the genes within disulfide bond pathway that were up-regulated (FC ≥ 3 and P < 0.05) in dystrophic mdx muscle vs. wildtype muscle. D) No major differences were noticed in most of the HSPs between mdx and wt mice except mitochondrial proteins HSP10 and GRP75 which were increased in mdx mice (P < 0.05). E) Higher p-SIRT1, but lower total SIRT1 in mdx mice (P < 0.001), but no changes in other sirtuins (P > 0.05). F) Increased acetylated lysine residues in mdx mice (P < 0.001). Data are mean ± s.e.m; n=8 for mdx and n=5 for wt.
thiol protein oxidoreductase (TPOR)) were measured in order to assess antioxidant capacity, the oxidative protein folding and protein disulfide isomerase (PDI) activity in mdx mice (Fig. S2). There were, however, no significant differences in any of the four measured enzyme activities. Similarly, no effect of the mdx phenotype on protein carbonyls, a marker of oxidative damage to proteins, was observed (Fig. 2E).

3.4. Greater ER stress and UPRER is observed in mdx muscle, without systematic changes in HSP proteins or UPRmt.

In order to achieve a more comprehensive view of the muscle cellular stress state of mdx mice, ER stress and UPRmt were analyzed. Regardless of no significant effect of muscular dystrophy on GRP78/BiP (Fig. 3A), all three canonical branches of the UPRmt and their downstream responses were upregulated in mdx mice. More specifically, higher protein content of PDI (P < 0.001) and PERK (P < 0.001) were detected in mdx muscle. In addition, downstream to PERK, phosphorylated elf2α at serine 51 (P < 0.001), and total elf2α protein (P < 0.001) as well as p-elf2α per total elf2α (P < 0.05) were significantly increased in mdx mice (Fig. 3A). Another UPR pathway component IRE1α (P < 0.05; Fig. 3A) and downstream to it spliced Xbp1 (Xsbp1) mRNA (P < 0.05) were also increased in mdx mice (Fig. 3B). In addition, mRNA levels of the third UPR sensor Atf6β were also upregulated in mdx muscles in microarray (1.4-fold, adjusted P < 0.001) and this was confirmed by qPCR (Fig. 3B). In addition, the third-most upregulated gene set in the microarray DAVID analysis was disulphide bond (fold enrichment 2.13, FDR = 4 × 10−24; Fig. 3C), a set of genes related to protein folding and thus to ER stress, since ER is one of the major sites for disulphide bond formation. To further understand the role of UPR proteostasis in ER, apoptotic pathway components downstream to PERK, Atf4-Chop-Gadd34 were quantified with microarray and qPCR. In microarray Atf4 (-1.3-fold), Chop (-1.3-fold and qPCR NS, Fig. S3) and Gadd34 (1.4-fold) were unaltered or marginally decreased (P < 0.05). This suggests that UPR through PERK-elf2α cascade and translational halt can override PERK-ATF4-CHOP-GADD34 apoptosis pathway at least at age of week 14 in mdx mice.

In addition, several HSPs were measured because of their key role as chaperones that assist with the proper folding of polypeptides into functional proteins and prevent the aggregation of misfolded proteins mainly in the cytosol compartment, but also in other cellular organelles including mitochondria. However, unlike responses of the UPRmt our results did not display significantly altered levels of HSP-proteins (Fig. 3D). Next, UPR in mitochondria, i.e., UPRmt and mitochondrial chaperones were examined. In microarray only marginal (1.1–1.3 fold) changes were observed in the UPRmt markers Hsp60, Clp, Jnk2, Timm17a, Hsp10, Yme1l1, Cebpb, Jun, Hsp74 and Pmpcb (data available at GEO: http://www.ncbi.nlm.nih.gov/geo/; accession no. GSE52766) of which Clp (Fig. S3) and HSP60 (Fig. 3D) were confirmed with qPCR or western blotting, respectively. However, the protein levels of UPRmt marker HSP10 and mitochondrial chaperone GRP75 were slightly increased in mdx mice (Fig. 3D).

3.5. mdx muscle shows alteration of p-SIRT1/SIRT1 and increased total lysine acetylated proteins

Phosphorylated SIRT1 and AMPK (p-SIRT1 and p-AMPK), and protein levels of SIRT1, SIRT3, SIRT6 and AMPK were measured due to their redox, heat shock response and UPRmt properties and possible effects on muscle dystrophy [7,38]. Increased p-SIRT1 and decreased total SIRT1 were observed in mdx (Fig. 3E) mice leading to increased ratio of phosphorylated SIRT1 to total SIRT1 (P < 0.001). No effect on total sirtuins 3 or 6 (Fig. 3E) or phosphorylated AMPK (Fig. S4) was observed. In addition, overall proteome lysine residue acetylation analysis revealed that mdx mice had higher levels of acetylated protein lysine residues (P < 0.001; Fig. 3F).

3.6. Effects of exercise and blocking AcvR2B ligands on muscle morphology and grip strength

Blockade of AcvR2B ligands using sAcvR2B-Fc or placebo (PBS) was administered for seven weeks with or without voluntary running exercise in young male mdx mice. Increased muscle mass by sAcvR2B-Fc, as reported earlier [25,26], did not translate into greater gastrocnemius muscle fiber size or grip strength either presented as absolute values (Fig. S5) or normalized to body weight (not shown). In addition, no effect of exercise was noticed on grip strength, but exercise shifted the CSA distribution further towards smaller fibers (Fig. 5S), along with smaller gastrocnemius mass after running as previously published [25]. No statistically significant difference was noted in the percentage mean count of small fibers (< 600 μm²) by running vs. no running (2 × 2 ANOVA P > 0.29) and thus the decreasing effect of exercise was throughout the fiber size range.

3.7. Redox balance and oxidative stress are affected by running and blocking AcvR2B ligands

Oxidized glutathione (GSSG and GSSG/GSH ratio) in gastrocnemius muscle was increased by running (2 × 2 ANOVA running effect P < 0.05; Fig. 4A–C). No difference in reduced glutathione (GSH) was observed between the treatments. In addition, microarray data showed that combination of sAcvR2B-Fc and exercise significantly increased gene sets of glutathione metabolism (FDR < 0.01) with a smaller increasing effect of running or sAcvR2B-Fc alone (Fig. S1 and Table S1). Thiol-Protein Oxidoreductase Activity (TPOR), which reflects the major PDI function, showed a sAcvR2B-Fc and running interaction effect in gastrocnemius muscle (P < 0.05) (Fig. 4D). There was no consistent effect of the interventions on the enzyme activities of GRD, GST or GPX (Fig. S6).

Exercise increased protein carbonyl levels (2 × 2 ANOVA running effect P < 0.05; Fig. 4E). Furthermore, the exercise response also interacted with administration of sAcvR2B-Fc (2 × 2 ANOVA sAcvR2-Fc and running interaction effect, P < 0.05; Fig. 4E). The interaction effect was further seen in a post hoc test as running increased protein carbonyls only in the group administered with sAcvR2-Fc (sAcvR2-Fc + running vs. sAcvR2-Fc alone, P < 0.05; Fig. 4E).

3.8. sAcvR2B–Fc increased GRP78/BiP and TxNIP, but neither treatment altered UPR markers or HSPs

sAcvR2B–Fc increased a marker of ER stress/UPR, GRP78/BiP (2 × 2 ANOVA sAcvR2B-Fc administration effect, P < 0.05) (Fig. 5A). TxNIP, an endogenous inhibitor of antioxidant TRX and a protein induced in ER stress [39] was also increased due to sAcvR2B–Fc (2 × 2 ANOVA drug effect, P < 0.05; Fig. 5B). Supporting the link between ER stress and TxNIP protein, these two proteins also strongly correlated with each other in mdx mice (r=0.876, P < 0.001) (Fig. S9). No effect of the treatments was found on TRX protein (Fig. 5C).

No systematic effect of sAcvR2B–Fc was seen in ER stress or UPRmt markers (PDI, IRE1α, PERK, p-elf2α at ser135 and elf2α total and elf2α protein levels or Xbp1 mRNA splicing levels (Fig. 6A–E). However, exercise tended to increase PDI and IRE1α (2 × 2 ANOVA running-effect, P=0.07–P=0.08; Fig. 6A–B). According to microarray data, there was no effect of interventions on mRNA expression in ER stress or UPRmt related genes (Grp78, Perk, elf2α and...
Ire1α, Atf4, Chop, Gadd34, Pdi, Xbp1, and Atf6; P > 0.85, data not shown). There were also no systematic effects of exercise or sAcvR2B-Fc administration alone or in combination on HSPs

Fig. 4. The effects of exercise and sAcvR2B-Fc on redox markers and oxidative stress in mdx muscle. (A–C) Oxidized glutathione (GSSG), reduced glutathione (GSH), ratio of oxidized glutathione (GSSG) and reduced glutathione (GSSG/GSH) levels, (D) TPOR activity and (E) protein carbonyls after the 7-week intervention period. PBS = PBS injected sedentary (n = 8), AcvR = sAcvR2B-Fc administered sedentary (n = 8), PBS Run = PBS injected voluntary wheel running (n = 7) and AcvR Run = sAcvR2B-Fc administered voluntary wheel running (n = 7). Data are mean ± s.e.m. 2 × 2 ANOVA (main and interaction effects) results are shown as text above the bars and possible post hoc (Tukey’s test) differences between individual groups.

Fig. 5. The effects of exercise and sAcvR2B-Fc on ER chaperones and thioredoxin and its regulation in mdx muscle. (A) GRP78/BiP, (B) TxAIP and (C) TRX protein content after the 7-week intervention period. PBS = PBS injected sedentary (n = 8), AcvR = sAcvR2B-Fc administered sedentary (n = 8), PBS Run = PBS injected voluntary wheel running (n = 7) and AcvR Run = sAcvR2B-Fc administered voluntary wheel running (n = 7). Data are mean ± s.e.m. 2 × 2 ANOVA (main and interaction effects) results are shown as text above the bars. There were no post hoc (Tukey’s test) differences between individual groups.

However, exercise tended to increase HSP70 (P < 0.07) (Fig. 7B) and decreased HSP90 protein (2 × 2 ANOVA running effect, P < 0.05, Fig. 7C). There were also no effects of treatments on
UPR_{mt} markers such as HSP60 protein and in microarray Clpp, JNK2, Timm17a, HSP10, Yme1l1, Cebpb, Jun, HSP74 and Pmpcb mRNA (P > 0.85, data not shown).

3.9. AMPK, Sirtuin1 and lysine acetylated proteins are affected by the treatments

No effect of exercise or sAcvR2B-Fc on pAMPKα at Thr^{172}, total AMPK or p-AMPK/AMPK was observed, but p-AMPK/AMPK showed a running x drug interaction effect (2 × 2 ANOVA, P < 0.05; Fig. S7). The phosphorylated sirtuin 1 (p-SIRT1 at Ser^{46}) showed both sAcvR2B-Fc administration (2 × 2 ANOVA, P < 0.05) and running x sAcvR2B-Fc administration interaction effects (2 × 2 ANOVA, P < 0.05) (Fig. S7). This was due to a fact that sAcvR2B-Fc increased p-SIRT1 in the exercised mice (P < 0.05) and not in sedentary mice (Fig. S7). The ratio of p-SIRT1 to total SIRT1 (p-SIRT1/
SIRT1) was in line with the p-SIRT1 result (not shown). There were no significant differences in the protein expression levels of SIRT3 or SIRT6 between the treatments (Fig. S7). Running increased levels of protein acetylts at lysine residues (2 × 2 ANOVA, \( P < 0.05 \); Fig. S8).

3.10. Associations between the measured variables

A computationally determined network was created between different variables. For this purpose, biologically similar variables were merged. Katiska/Himmeli software using in GNU Octave program environment (http://www.finndiane.fi/software/katiska/) was used for analysis [40]. Clearly, different HSPs were connected and a link to UPR/ER stress was also evident (Fig. 8A). UPR/ER stress was also linked to acetylated proteins and protein carbonyls to mitochondria count (Fig. 8A). Of individual associations, TxNIP correlated strongly and positively with HSPs in \( mdx \) mice (\( P < 0.01 \)) (Fig. S9). Especially interesting was the strong correlation between TxNIP and GRP78 as mentioned earlier (Fig. S9). Carbonyls correlated positively and strongly in the sAcvR2B-Fc injected mice with activity index (\( r = 0.87, P < 0.001 \)) that was assessed by measuring the whole activity level of mice in the cage (running and non-running) (Fig. S9), a measure reported earlier [26]. Fig. 8B summarizes the main effects of muscular dystrophy on ER stress/UPR/chaperones in the present manuscript.

4. Discussion

The present study provides an extensive analysis of the effects of muscular dystrophy on various types of metabolic stress and two candidate therapeutic strategies to treat dystrophin deficient...
skeletal muscle. Of these complex processes we focused specifically on proteostasis pathways of ER stress and the UPR, which consists of three canonical branches (PERK, IRE1α and ATF6).

Notably, most of the measured components of the three canonical branches (IRE1α protein level, spliced Xbp1 mRNA, PERK, p-eIF2α and total eIF2α, and Atf6 mRNA) in skeletal muscle were greater in...
Moreover, HSP72 provides protection against ER stress by demonstrating a clear increase in UPRER in dystrophic posttranslational modifications, increased acetylation may compete with other posttranslational modifications [49]. Collectively, these results demonstrate a clear increase in UPRER in dystrophic mdx mice that is accompanied by increases in some indices of oxidative stress and redox regulation including GSSG levels and lysine acetylated proteins. This is interesting because protein hyper-acetylation can be associated with many pathological states [48]. Moreover, decreased markers of oxidative stress were not significantly affected by the mdx phenotype, nor were levels changed in response to exercise or sAcvR2B-Fc treatment in mdx mice. A lack of a well-established effect of regular exercise on increasing HSP levels in mdx muscle suggests the presence of an ameliorated stress protein response and disrupted proteostasis in dystrophic muscle. This assumption is in agreement with previous studies showing that the various disease models which may interfere with protein synthesis also blunt HSP responses to physical exercise [54].

An objective of this study was to also address whether two different, but often investigated treatment options including physical exercise and/or blocking myostatin/activins would improve the status of these pathways in skeletal muscle of mdx mice. Administration of sAcvR2B-Fc to block myostatin/activins increased protein expression levels of GRP78 and TxNIP, independent of exercise. GRP78 is an ER-located molecular chaperone belonging to the HSP70 family that is involved in many ER-related cellular processes including sensing ER stress, inducing UPR, the translocation of newly synthesized polypeptides across the ER membrane, facilitation of protein folding, targeting misfolded proteins for degradation and regulation of calcium homeostasis [for a review see [50]]. Although markers of UPRER were unchanged by sAcvR2B-Fc, an increase in GRP78 expression is consistent with a recent study wherein overexpression of decorin, an inhibitor of myostatin [51], increased ER-marker genes including GRP78 in duck muscle cells [52]. Conversely, TxNIP inhibits the thioredoxin system, which is a key antioxidant system that protects cells from oxidative stress through its disulfide reductase activity [53]. It was previously shown that ER stress increases TxNIP levels through induction of IRE1α activity [39]. Interestingly, in the present study GRP78 was strongly associated with TxNIP, a mediator of ER stress-induced inflammation and a protein linking oxidative stress, ER stress and UPR pathways [39]. GRP78 is significantly upregulated after 2 weeks of sAcvR2B-Fc administration, but not yet after single injection (1–2 days) (Hentila et al. unpublished data) when protein synthesis is acutely already increased [19]. This suggests that the level of disruption to ER homeostasis through increased amounts of newly synthesized proteins by blocking AcvR2B ligands may not be severe enough to trigger a UPRER response. HSP levels were not significantly affected by the mdx phenotype, nor were levels changed in response to exercise or sAcvR2B-Fc treatment in mdx mice. A lack of a well-established effect of regular exercise on increasing HSP levels in mdx muscle suggests the presence of an ameliorated stress protein response and disrupted proteostasis in dystrophic muscle. This assumption is in agreement with previous studies showing that the various disease models which may interfere with protein synthesis also blunt HSP responses to physical exercise [54].

Protein carbonyls can be used as an outcome marker of oxidative damage to skeletal muscle proteins. Voluntary running in combination with sAcvR2B-Fc increased protein carbonyl levels. In addition, protein carbonyl levels showed a strong, positive correlation with activity index in the sAcvR2B-Fc injected mice. These results were corroborated by observations showing that voluntary running also increased GSSG, which is also correlated with protein carbonyl levels in sAcvR2B injected mice (Fig. S8). Even if exercise training has been shown to increase the endogenous anti-oxidative capacity in healthy organisms [4], no consistent effects of exercise on the endogenous antioxidant markers (except glutathione metabolism gene set in microarray data) were observed in the present study. Thus, it seems that running alone, and particularly in combination with sAcvR2B-Fc was not completely tolerable and shifted the redox-balance of the functional dystrophin deficient skeletal muscle to more oxidizing direction. Previously, increased oxidative stress was reported after 4 weeks of running in mdx mice [55]. However, decreased markers of oxidative stress in mdx mice has also been reported after 8 weeks of very low-intensity treadmill running [9] and after 4 weeks of low intensity swimming [10]. Differences in the age of animals, time point of animal euthanization following the last bout of exercise, exercise mode, intensity and volume, and in the basal redox status of mdx mice may explain these results.

In addition to elevating protein carbonyls, running further increased lysine acetylated proteins in mdx mice. Based on these
findings it is tempting to suggest that the lack of functional dystrophin protein in skeletal muscle cells of *mdx* mice leads to hyperacetylation of proteins that becomes exacerbated when additional stress (e.g., wheel running) is added. However, the significance of this observation to the pathophysiology of DMD can only be speculated and needs to be studied further in the future. Running independently, and in combination with sAcvR2B-Fc did not exert significant effects on protein folding gene set, PDI protein expression, or other ER stress or UPR markers. Thus, low intensity voluntary running independently or combined with AcvR2B ligand blocking does not increase ER stress or UPR in *mdx* mice despite the elevated oxidative stress.

Increased muscle mass by sAcvR2B-Fc was not the result of a statistically significant increase in muscle fiber size, nor was it associated with grip strength. Our muscle strength finding using sAcvR2B-Fc is in agreement with others [20,23], and muscle fiber size by some [23], but not all studies [20]. The reason for this lack of change is unknown, but may be explained by changes in proteostasis in the present study and eventually in muscle quality shown previously in long-term AcvR2 ligand blocking experiment [23] and in myostatin knock-out mice [56], possibly together with altered calcium signaling [57].

Exercise shifted the muscle fiber distribution towards smaller fibers, which may explain why strength endurance was not improved by exercise, although aerobic capacity per volume of muscle mass was increased as reported earlier [25]. Exercise has well-known positive effects on health and also voluntary wheel running can improve skeletal muscle function of *mdx* mice at least in some muscles [58,59]. However, some studies have even shown that certain markers are negatively altered by exercise in *mdx* mice, including e.g. calcium homeostasis if the intensity of the exercise is high [55,60]. Clearly, more research is needed on the dose and type of exercise in dystrophic muscle. Thus, although positive effects of exercise on markers of oxidative capacity was reported earlier [25,26], it can be speculated that partially due to increased oxidative stress, no positive effects was seen in muscle endurance or in muscle histology.

Sirtuins are proteins involved in many important processes, including aerobic metabolism, redox regulation as well as UPR [61,62]. Increased phosphorylation of SIRT1 at ser46 and decreased total SIRT1 was observed in *mdx* mice when compared to wild-type mice. Moreover, voluntary wheel running independently, and in combination with sAcvR2B-Fc increased the phosphorylation of SIRT1. This coincided with increased protein carbonyls and oxidized glutathione. Previously, Chalkiaidi et al. [38] showed no difference in SIRT1 protein and mRNA expression between wild-type and *mdx* mice (age 10–12 weeks). This is in contrast with our results and Hourde et al. [58] that reported lower mRNA expression levels of SIRT1 in *mdx* mice that are increased following 4 months of voluntary wheel running. The effects of the altered phosphorylation of SIRT1 at Ser46/47 on the activity of the protein are unclear. However, the interventions that increase the enzymatic activity of SIRT1 were suggested to be beneficial for muscular dystrophies [38,63]. On the other hand, even if the increased phosphorylation of SIRT1 at ser46 caused by exercise alone or in combination with sAcvR2B-Fc would improve redox regulation, the outcome of the running combined with the sAcvR2B-Fc was a greater amount of protein carbonyls and increased level of oxidized glutathione that are indicators of elevated levels of oxidative stress and damage.

5. Conclusions

Lack of functional dystrophin protein resulted in altered redox regulation, activation of UPR response in ER and increased lysine acetylated proteome in 14-week-old *mdx* mice. These processes may contribute to the pathophysiology of Duchenne muscular dystrophy. However, mitochondrial UPR or heat shock response/adaptation were not consistently altered, thus the muscle proteostasis of *mdx* mice at the age of 14 weeks is not totally dysfunctional. Apart from the observation that sAcvR2B-Fc administration increased protein expression levels of GRP78/Bip and TxiNP, running and activin receptor IIB ligand blocking did not exert a systematic effect on ER stress, UPR or heat shock proteins. A marginal oxidative stress resulting from voluntary wheel running alone or in combination with sAcvR2B-Fc administration does not further compromise the proteostasis of *mdx* mice but has rather neutral effect. Thus, these treatments may be utilized in conjunction with more direct dystrophin restoration approaches in an effort to restore dysfunctional proteostasis in muscular dystrophy.

Competing interests

No conflicts of interest, financial or otherwise, are declared by the authors.

Author contributions

JH, HK and OR designed the original study, while the present study were mainly designed by JH, JH and MA. JH and BMO carried out the in vivo experiments. MS assisted in the in vivo experiments and isolated muscles. JH and MA drafted the manuscript with the help from JH and KCD. JH, JH, MA, AK and BMO carried out the analyses. JH, UMK, HK, KGP and RA were involved in the microarray analysis. OR prepared sAcvR2B-Fc used in the study. All authors read and approved the final manuscript.

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Data availability


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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.freeradbiomed.2016.08.017.


