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Original article

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.

Abbreviations: AcvR2B, activin receptor IIB; sAcvR2B-Fc, ligand blocking using soluble activin receptor-Fc; AMPK, 5′ adenosine monophosphate-activated protein kinase; CSA, cross-sectional area; DMD, Duchenne muscular dystrophy; *mdx*, DMD mouse model; eIF2α, eukaryotic initiation factor 2 subunit α; ER, endoplasmic reticulum; GPX, glutathione peroxidase; GRD, glutathione reductase; GRP78, glucose regulated protein 78; GSEA, gene set enrichment analysis; GSH, reduced glutathione; GSSG, oxidized glutathione; GST, glutathione S-transferase; HSP, heat shock protein; IRE1α, inositol-requiring enzyme 1α; PDI, protein disulfide isomerase; PERK, protein kinase R-like ER protein kinase; ROS, reactive oxygen species; SDH, succinate dehydrogenase; TPOR, thiol protein oxidoreductase; TRX, thioredoxin; TxNIP, thioredoxin-interacting protein; UPR, unfolded protein response; XBP1, X-box binding protein 1

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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_{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_{mt}) [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, Labfor, 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 2×2 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/