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Lipogenesis mitigates dysregulated sarcoplasmic reticulum calcium uptake in muscular dystrophy

Christopher W. Paran^{a,b,c}, Kai Zou^{a,c}, Patrick J. Ferrara^{a,c}, Haowei Song^d, John Turk^d, and Katsuhiko Funai^{a,b,c}

^aDepartment of Kinesiology, East Carolina University, 115 Heart Drive, Greenville, North Carolina 27834, USA

^bDepartment of Physiology, East Carolina University, 115 Heart Drive, Greenville, North Carolina 27834, USA

^cEast Carolina Diabetes and Obesity Institute, East Carolina University, 115 Heart Drive, Greenville, North Carolina 27834, USA

^dDivision of Endocrinology, Metabolism and Lipid Research, Washington University School of Medicine, 660 South Euclid Avenue, St. Louis, Missouri 63110, USA

Abstract

Muscular dystrophy is accompanied by a reduction in activity of sarco/endoplasmic reticulum Ca^{2+} -ATPase (SERCA) that contributes to abnormal Ca^{2+} homeostasis in sarco/endoplasmic reticulum (SR/ER). Recent findings suggest that skeletal muscle fatty acid synthase (FAS) modulates SERCA activity and muscle function via its effects on SR membrane phospholipids. In this study, we examined muscle's lipid metabolism in *mdx* mice, a mouse model for Duchenne muscular dystrophy (DMD). *De novo* lipogenesis was ~50% reduced in *mdx* muscles compared to wildtype (WT) muscles. Gene expressions of lipogenic and other ER lipid-modifying enzymes were found to be differentially expressed between wildtype (WT) and *mdx* muscles. A comprehensive examination of muscles' SR phospholipidome revealed elevated phosphatidylcholine (PC) and PC/phosphatidylethanolamine (PE) ratio in *mdx* compared to WT mice. Studies in primary myocytes suggested that defects in key lipogenic enzymes including FAS, stearoyl-CoA desaturase-1 (SCD1), and Lipin1 are likely contributing to reduced SERCA activity in *mdx* mice. Triple transgenic expression of FAS, SCD1 and Lipin1 (3TG) in *mdx* myocytes partly rescued SERCA activity, which coincided with an increase in SR PE that normalized PC/PE ratio. These findings implicate a defect in lipogenesis to be a contributing factor for SERCA dysfunction in muscular dystrophy. Restoration of muscle's lipogenic pathway appears to mitigate SERCA function through its effects on SR membrane composition.

Address correspondence to: Katsuhiko Funai, East Carolina Diabetes and Obesity Institute, East Carolina University, 115 Heart Drive, Rm 4107, Greenville, North Carolina 27834, USA. Phone: (252)737-4684, Fax: (252) 747-4689, funaik@ecu.edu.

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Keywords

Lipogenesis; Muscular dystrophy; Sarcoplasmic reticulum; Membrane phospholipid; Calcium

1. Introduction

Duchenne muscular dystrophy (DMD) is a severe muscle wasting disorder caused by a mutation in the dystrophin muscular dystrophy (*dmd*) gene. Intracellular Ca^{2+} homeostasis is impaired in dystrophic muscles, activating inflammatory and degenerative pathways. Mechanisms for aberrant Ca^{2+} metabolism in dystrophic muscles include compromised function of sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) [1, 2], as well as other Ca^{2+} ion channels [3, 4] and membrane tears [5, 6]. Ectopic expression of SERCA in muscular dystrophy can ameliorate muscle dysfunction and degeneration [7].

SERCA is a membrane spanning enzyme that translocates Ca^{2+} ions from the cytosol into the SR lumen [8, 9]. The function of SERCA is known to be affected by properties of membrane phospholipids in which they reside [10, 11]. Previously, we identified the role of skeletal muscle fatty acid synthase (FAS) in regulating Ca^{2+} metabolism [12]. Skeletal muscle-specific deletion of FAS led to a concomitant reduction in SR phosphatidylethanolamine (PE) and SERCA's Ca^{2+} uptake activity, resulting in pronounced muscle weakness. A number of studies provide evidence for phospholipid compositional changes in skeletal muscle of DMD patients [13, 14] and in mouse models of muscular dystrophy [15–17]. Defects in some other components of skeletal muscle lipogenesis are known to promote myopathy [18–23].

Dystrophic *mdx* mice carry a spontaneous C-to-T transition in the *dmd* gene, resulting in a premature termination codon [24]. For a reason not fully understood, *mdx* mice do not manifest a full spectrum of symptoms associated with DMD until older age except in the diaphragm muscle [25]. Thus we examined whether lipogenic machinery and SR phospholipid composition is altered in diaphragm muscles of *mdx* mice. We hypothesized that defective skeletal muscle fatty acid synthesis might contribute to aberrant SERCA activity in muscular dystrophy.

2. Material and methods

2.1 Animals

Male wildtype (WT: C57BL/6J, JAX stock number: 000664) and *mdx* (*mdx*-4Cv, JAX stock number: 002378) mice were purchased from Jackson laboratory, fed standard chow, and were studied at three months of age. On the day of experiment mice were fasted four hours prior to being anesthetized with mouse cocktail (80 mg/kg ketamine and 10 mg/kg xylazine). Diaphragm muscles were harvested and were frozen in liquid nitrogen for subsequent analyses of *de novo* lipogenesis, quantitative PCR, SERCA Ca^{2+} uptake, and SR lipidomics. Hindlimb muscles were used for primary muscle cell isolation. All protocols were approved by the East Carolina University Animal Studies Committee.

2.2 De novo lipogenesis

Rates for *de novo* synthesis of fatty acids were measured as previously described [26]. Hypotonic lysed samples were mixed with assay buffer (0.14 M potassium phosphate buffer, 1.4 mM EDTA, 1.4 mM DTT, 0.24 mM NADPH, 0.1 mM acetyl-CoA), and the rate of NADPH oxidation was monitored at 340 nm at baseline and again after adding 10 μ L of 0.85 mg/ml malonyl-CoA. The substrate-dependent rate was determined by subtracting the baseline NADPH oxidation rate from the rate after addition of malonyl-CoA.

2.3 Quantitative PCR

Total RNA was extracted from diaphragm samples with TRIzol reagent (Life Technologies, Grand Island, NY). 1 μ g of total RNA was reverse transcribed using IScriptTM cDNA synthesis kit (BioRad, Hercules, CA) or miScript II RT kit (Qiagen, Venlo, Netherlands). Quantitative PCR was performed with a ViiATM 7 Real-Time PCR System (Life Technologies, Grand Island, NY) using SYBR[®] Green reagent (Life Technologies, Grand Island, NY) or miScript SYBR[®] Green PCR kit (Qiagen, Venlo, Netherlands). Pre-validated primers (qprimerdepot) spanning exon-exon boundaries or miRNA-specific primers (Qiagen, Venlo, Netherlands) were used for amplifications. Assays were performed in duplicate and results were normalized to ribosomal protein L32 mRNA levels (which were unaffected between WT and *mdx* muscles), and all values were divided by the calculated average of the control group.

2.4 SR isolation

SR was isolated by differential centrifugation as previously verified [12]. Diaphragm muscles were homogenized in hypotonic buffer (250 mM sucrose, 10 mM NaHCO₃, 5 mM NaN₃, 0.1 mM PMSF and 1X HaltTM protease and phosphatase inhibitor cocktail [Thermo Fisher, San Jose, CA]) at 4°C using homogenization tubes with motor-teflon pestles. Homogenates were rotated for 1 h at 4°C, and subjected to differential centrifugations at 20,000g for 30 min. Supernatants were collected for further centrifugation at 179,000g for 120 min (4°C). Pellets of this fraction were resuspended in hypotonic buffer.

2.5 Targeted lipidomics

SR phospholipidomics were conducted as previously described [12, 27]. Isolated SR fractions were reconstituted in 1.8 mL ddH₂O. Four mL of extraction buffer [2:2 (v/v) chloroform/methanol] was added in the presence of internal standards: 5 μ g 14:0-PC ([M+Li]⁺ *m/z* 684.58) or 5 μ g 14:0-PE ([M-H]⁻ *m/z* 678.62). After vortex-mixing and centrifugation (800g), the organic (lower) layer was collected, concentrated to dryness under nitrogen, and reconstituted in 200 μ L methanol. A 20 μ L aliquot was removed, diluted with 180 μ L methanol containing 0.6% LiCl, and analyzed by direct injection ESI-MS on a Thermo Vantage triple-quadrupole mass spectrometer in positive mode with different neutral loss scans for the analysis of PC (neutral loss of 183) species. Another aliquot of 20 μ L was diluted in 200 μ L methanol and analyzed in negative mode for PE and phosphatidylserine (PS) species (product ions of 196 and 153). The relative intensity ratio of each individual species to its internal standard was calculated, and then read against a standard curve to obtain its absolute quantity. The final result was reported as nmol/mg protein.

2.6 SERCA-dependent Ca^{2+} uptake assay

Isolated SR fractions from samples were subjected to the measurement of SERCA-dependent Ca^{2+} uptake [12, 28]. The Ca^{2+} transport assay was carried out in reaction mixture containing 100 mM KCl, 30 mM imidazole-histidine (pH 6.8), 5 mM MgCl_2 , 5 mM ATP, 5 mM $(\text{COOK})_2$, 5 mM NaN_3 , and 50 μM CaCl_2 (1 $\mu\text{Ci}/\mu\text{mol}$ of ^{45}Ca) CaCl_2 , American Radiolabeled Chemicals, Inc., St. Louis, MO). The reaction was started by the addition of SR fractions containing 150 μg protein for 15 min in a 37°C water bath, stopped by the addition of 0.15 M KCl and 1 mM LaCl_3 , and filtered through a 0.2 μm HT Tuffryn membrane (PALL Corporation, Port Washington, NY). SERCA-independent Ca^{2+} transport was quantified in the presence of 10 μM thapsigargin.

2.7 Primary myocytes culture

The hindlimb muscles were used for primary muscle cell isolation as previously described [29]. Briefly, muscles were minced with razor blades into small fragments, rinsed with sterile PBS, and then digested with a final concentration of 0.1% pronase (EMD Millipore, Billerica, MA) in high glucose DMEM for 1 h in the culture incubator. The cell suspension was centrifuged at 1,000g for 5 min, and the pellet was resuspended in 10% FBS in DMEM and filtered through a 70 μm nylon mesh (BD Falcon, Franklin Lakes, NJ). The cell suspension was centrifuged again at 1,000g for 5 min, resuspended in growth media (Ham's F10, 20% FBS and 5 ng/ml basic FGF [Life Technologies, Grand Island, NY]), and pre-plated to remove fibroblasts. Cells were maintained in growth media until genetic manipulation or differentiation.

Knockdowns of FAS, SCD1, Lipin1, adipose triglyceride lipase (ATGL), hormone-sensitive lipase (HSL), lipoprotein lipase (LPL), and acyl-CoA oxidase (ACO) were performed with lentivirus pLKO.1 system as previously described [12]. Lentiviruses were produced by transfecting HEK293T cells with 2.66 μg of psPAX2, 0.75 μg of pMD2.G and 3 μg of shRNA using Lipofectamine 2000. Similarly, FAS was overexpressed using retrovirus pBABE-Puro system as previously described [30], by transfecting HEK293T cells with 3 μg of FAS plasmid and 3 μg of ψA helper plasmid. For both the lentivirus and retrovirus systems, viral media were collected 48 h after 293T transfection and filtered through 0.45 μm syringe. Polybrene was added and the media were used to treat fully confluent primary myoblasts. After 48 h of infection, cells were selected with puromycin for another 48 h. For expression of SCD1, Lipin1, choline/ethanolamine phosphotransferase 1 (CEPT1), perilipin 2 (Plin2), phosphatidylserine synthase 2 (PSS2), and 3TG (FAS retrovirus infection with SCD1 and Lipin1), myoblasts were directly transfected with plasmid expressing these genes under a cytomegalovirus (CMV) promoter. After infection/transfection of myoblasts, media were changed to differentiation media (DMEM, 2% horse serum) and studied on D4 of differentiation.

In another set of experiments, differentiated WT or *mdx* myotubes were treated for 24 h with fatty acid free BSA or 100 μM of palmitic acid (PA), oleic acid (OA), eicosapentaenoic acid (EPA), or docosahexaenoic acid (DHA) in growth media.

2.8 Rates for PC and PE synthesis

Rates for PC and PE synthesis were measured as previously described [31]. Primary myotubes were incubated in media containing [^3H]-choline or [^{14}C]-ethanolamine for two hours at 37 °C. Cells were quickly harvested, hypotonically lysed, quantified for protein, and sample volume with equal amount of protein content were used for lipid extraction. Extracted lipids were separated by thin-layer chromatography in chloroform/methanol/acetic acid/ H_2O (80/30/1/4), visualized with iodine, and spots corresponding to PC and PE standards were cut out for scintillation counting. Disintegrations per minute (DPM) counts were normalized to mg of protein.

2.9 Statistical analyses

Values are expressed as means $\pm$ standard error of the mean. Statistical comparisons were performed using an unpaired two-tailed Student's t-test (for two-group analyses), one-way-ANOVA or two-way ANOVA with Tukey's post hoc test (for multiple comparisons).

3. Results

3.1 Examination of skeletal muscle *de novo* lipogenesis from *mdx* mice

Mice that lack capacity for skeletal muscle *de novo* lipogenesis exhibit muscle weakness [12]. Conversely, we found lower rates of muscle *de novo* lipogenesis (Figure 1A) and FAS mRNA level (Figure 1B) in diaphragm muscles of *mdx* mice compared to WT. FAS gene expression can be transcriptionally regulated by sterol regulatory element binding protein-1c (SREBP-1c), upstream stimulatory factor (USF), or liver X receptor- α (LXR α). Compared to WT diaphragms, *mdx* diaphragms had reduced levels for LXR α mRNA but no effects on SREBP-1c or USF mRNAs (Figure 1C). As miR-1 and miR-206 are both known to suppress LXR α expression in liver, we measured the levels of these miRNA in WT and *mdx* diaphragms [32]. Consistent with a previous report that miR-206 mRNA is increased in *mdx* diaphragms [33], miR206, but not miR-1, was increased in *mdx* diaphragms (Figure 1D).

3.2 Muscular dystrophy promotes altered muscle SR phospholipidome

Targeted phospholipidomics of diaphragm's SR enriched fraction revealed that multiple species of PC (Figure 2A: 16:0/16:0, 16:1/18:1, 16:0/18:1, 16:0/20:4, 18:1/18:2, 18:1/18:1, 18:0/18:1, and 18:0/20:4), two species of PE (Figure 2B: 16:0/18:1 and 18:0/18:1), and a species of PS (Figure 2C: 18:1/18:1) were more abundant in *mdx* than in WT muscles. Together, total PC abundance was ~50% greater in *mdx* vs. WT, whereas no statistically significant differences were present for total PE or PS abundance between the groups (Figure 2D). As a result, SR PC/PE ratio was significantly greater in *mdx* diaphragms compared to WT controls (Figure 2E).

In order to gain a more comprehensive understanding of changes in lipid metabolism in *mdx* muscles, we examined mRNA levels of genes involved in fatty acid/triglyceride synthesis (SCD1, DGAT1, DGAT2, Lipin1), PC or PE biosynthesis (Pcyt1, Pcyt1b, Pcyt2, Chkb, ChPT1, CEPT1, PEMT, PSD, PSS1, PSS2), lipolysis (ATGL and HSL), lipid import (CD36 and LPL), oxidation (CPT1b, MCAD, LCAD, and ACO), or storage (Plin2). Among these, ten genes were found to be differentially abundant between WT and *mdx* diaphragms

(Figure 2F, log₁₀ scale). Notably, FAS and SCD1 were ~7-fold lower in *mdx* muscles compared to WT controls. Sorting these genes based on the subcellular localizations of the proteins they encode for revealed that eight out of ten were localized to ER (FAS, SCD1, Lipin1, CEPT1), or ER-associated organelles such as lipid droplets that are derived from ER (HSL, ATGL, and Plin2) and mitochondria-associated membranes that traffic molecules between ER and mitochondria (PSS2) [34]. Differential mRNA abundance of ER-localized enzymes involved in triglyceride and phospholipid biosynthesis suggested that they might be responsible for altered skeletal muscle SR phospholipid composition in *mdx* mice.

3.3 Defective *de novo* lipogenesis is sufficient to promote reduction in SERCA-dependent Ca²⁺ uptake

We previously reported that a defect in skeletal muscle *de novo* lipogenesis alone was sufficient to promote reductions in SR PE content, SERCA-dependent Ca²⁺ uptake, and muscle strength [12]. Thus we investigated whether alteration in lipid enzymes in *mdx* muscles might contribute to altered SERCA activity. To systematically study the roles of the ten genes that were differentially expressed between WT and *mdx* muscles, we studied primary muscle cells isolated from WT and *mdx* mice. Diaphragm muscles from *mdx* mice exhibited reduced SERCA-dependent Ca²⁺ uptake compared to WT muscles (Figure 3A), and these findings were translated to our *in vitro* model of differentiated primary myotubes from WT and *mdx* mice (Figure 3B). *De novo* lipogenesis was also lower in *mdx* myotubes compared to WT controls (Figure 3C).

As there were ten lipid-modifying genes whose mRNA levels were different between WT and *mdx* muscles, we investigated the effects of reproducing these changes to SERCA function in C2C12 cells (Figure 3D–G). Consistent with our previous findings in C2C12 myotubes and muscle-specific FAS knockout mice [12], FAS knockdown in WT myotubes resulted in a substantial reduction in SERCA-dependent Ca²⁺ uptake (Figure 3D, 2nd bar). Although mRNA level for SCD1 was reduced ~7-fold in *mdx* muscles, lentivirus-mediated knockdown of SCD1 had no effect on SERCA (Figure 3D, 3rd bar). In contrast, Lipin1 knockdown resulted in a reduction of SERCA Ca²⁺ uptake (Figure 3D, 4th bar), reminiscent of Lipin1 mutation in humans that are known to promote muscle weakness [20]. Although CEPT1 and PSS2 were more highly expressed in *mdx* muscles than in WT, ectopic expression of CEPT1 (Figure 3F, 2nd bar) or PSS2 (Figure 3F, 4th bar) in WT myotubes had no effect on SERCA.

3.4 Alteration of lipolytic enzymes does not alter SERCA-dependent Ca²⁺ uptake

Out of the ten genes that were differentially expressed between WT and *mdx* muscles, three of them (ATGL, HSL, and Plin2) were enzymes involved in triglyceride breakdown at the level of lipid droplets [35]. However, ATGL knockdown (Figure 3D, 5th bar), HSL knockdown (Figure 3D, 6th bar), or Plin2 expression (Figure 3F, 3rd bar) did not alter SERCA activity. Extracellular lipase LPL and peroxisomal oxidase ACO were reduced in *mdx* muscles compared to WT controls, but LPL knockdown (Figure 3D, 7th bar) or ACO knockdown (Figure 3D, 8th bar) did not affect SERCA.

3.5 Restoration of muscle's lipogenic machinery rescues SERCA function in *mdx* myocytes

We investigated whether restoring the lipogenic machinery of *mdx* muscles would rescue reduced SERCA-dependent Ca^{2+} uptake (Figure 4A–C). FAS expression in myotubes from *mdx* mice resulted in a significant increase in SERCA's Ca^{2+} uptake, but only ~30% of SERCA's function was restored (Figure 4A, 1st–3rd bar). Expression of SCD1 or Lipin1 in *mdx* myotubes did not result in a significant increase in SERCA function (Figure 4A, 4th and 5th bar), but there was a small trend for increase. Thus, we examined the effects of combined overexpression of FAS, SCD1, and Lipin1 on *mdx* cells. Simultaneous expression of FAS and SCD1, or FAS and Lipin1, did not rescue SERCA function to a greater extent than FAS-only expression (Figure 4A, 6th and 7th bar). However, combined expression of the three lipogenic genes FAS, SCD1, and Lipin1 (triple transgenic, or 3TG) had an additive effect, restoring ~60% of SERCA's function in *mdx* myotubes (Figure 4A, 8th bar, *Mdx* + 3TG). FAS or 3TG expression also fully restored the rate of *de novo* lipogenesis in the *Mdx* myotubes (Figure 4C). FAS, SCD1, Lipin1, or 3TG expression in WT myotubes had no effect on SERCA-dependent Ca^{2+} uptake (Figure 4D–E). This is not surprising as SERCA pumps Ca^{2+} up against the 10^4 gradient, and elevated lipogenesis alone would be expected to do very little to increase WT SERCA function that already operates near maximum capacity.

3.6 Exogenous fatty acid supplementation does not ameliorate SERCA-dependent Ca^{2+} uptake in *mdx* myocytes

In skeletal muscle, FAS is primarily localized in SR where it affects the phospholipid composition [12]. Lipid molecules are insoluble to water making their transport thermodynamically unfavorable. Thus PA produced by subcellular-localized FAS likely has specific function distinct from PA available exogenously. Nevertheless, we examined the possibility that fatty acid treatment might rescue lower SERCA function in *mdx* muscles. Treatment of *mdx* cells with PA, OA, EPA, or DHA had no effect on SERCA-dependent Ca^{2+} uptake (Figure 4F). None of the fatty acids had an effect on skeletal muscle *de novo* lipogenesis (Figure 4G).

3.7 Normalization of SR phospholipid composition likely explains elevated SERCA function in triple-transgenic *mdx* myocytes

We hypothesized that the restoration of SERCA-function in *mdx* + 3TG myotubes was likely promoted by changes in SR phospholipid composition. To test this possibility, we conducted a large scale *in vitro* study to acquire sufficient amount of SR from WT, *mdx*, and *mdx* + 3TG myotubes. Isolated SR fractions were used for targeted phospholipidomic analyses, similar to ones conducted in WT and *mdx* diaphragm samples (Figure 2).

Phospholipids' acyl-chain distribution differed slightly between diaphragm SR and myotubes SR (Figure 2 and 5), which has been shown previously [12]. Nevertheless, differences observed between WT and *mdx* diaphragm SR were very similar to those observed between the SR of WT and *mdx* myotubes. Several species of PC (16:0/16:1, 16:0/16:0, 16:1/18:1, 16:0/18:1, 16:0/20:4, 18:0/18:1, and 16:0/22:6) were more abundant in SR of *mdx* myotubes compared to WT controls (Figure 5A). One species of PE (18:0/18:1)

was more abundant in *mdx* vs. WT (Figure 5B), but no PS species were more abundant in *mdx* vs. WT (Figure 5C), though 18:1/18:1-PS was borderline significant ($p = 0.052$). In sum, *mdx* myotubes contained more SR PC and to lesser extent more SR PE compared to WT controls (Figure 5D), leading to greater SR PC/PE ratio in *mdx* compared to WT (Figure 5E).

3TG expression of FAS, SCD1, and Lipin1 in *mdx* myotubes had a robust effect on SR phospholipidome. Interestingly, 3TG had no effect on SR PC content (Figure 5A, *mdx* vs. *mdx* + 3TG). In contrast, several species of PE (16:0/18:2, 16:0/18:1, 16:0p/20:4, 16:0/22:4, 18:0p/22:4, and 16:0/22:6) were more abundant in SR of *mdx* + 3TG group compared to *mdx* (Figure 5B). One species of PS (18:0/18:1) was more abundant in SR of *mdx* + 3TG compared to *mdx* (Figure 5C). Together, total PE, but not PC nor PS, was greater in *mdx* + 3TG group than in *mdx* group (Figure 5D). In turn, the 3TG-induced increase in PE normalized the PC/PE ratio to the WT level in *mdx* + 3TG group (Figure 5E).

3.8 Restoration of lipogenic machinery increases PE synthesis in *mdx* myotubes

The rates for PC and PE syntheses were examined in WT, *mdx*, *mdx* + FAS, and *mdx* + 3TG myotubes. PC and PE synthesis rates were not different between *mdx* and WT myotubes (Figure 5F) despite higher SR PC and PE content in *mdx* vs. WT myotubes (Figure 5D). FAS or 3TG expression did not alter PC synthesis rate in *mdx* myotubes (Figure 5F), consistent with similar PC content in *mdx* and *mdx* + 3TG myotubes (Figure 5D). In contrast, FAS or 3TG expression had a dose-dependent effect on PE synthesis rate (Figure 5G), consistent with greater SR PC content in *mdx* + 3TG myotubes (Figure 5D). Taken together, increased PE synthesis explains the elevated PE content in *mdx* + 3TG myotubes, but increased PC and PE content in *mdx* myotubes appear to come from alternative sources. Thus, a defect in FAS, SCD1 and Lipin1 does not entirely explain the altered SR phospholipidome in *mdx* myotubes.

4. Discussion

Aberrant Ca^{2+} homeostasis contributes to progressive muscle wasting in muscular dystrophy. A reduction in SERCA function likely promotes dysregulated Ca^{2+} metabolism, and restoration of SERCA function can ameliorate the degenerative effects of muscular dystrophy [7]. In this study we tested a hypothesis that a defect in muscle's lipogenic machinery might contribute to altered SERCA function in *mdx* mice. Compared to WT controls, *mdx* diaphragm muscles had substantial reduction in expression of genes involved in lipogenesis including FAS, SCD1, and Lipin1 that coincided with a disrupted phospholipid composition of the SR membrane bilayer. Induction of FAS or Lipin1 knockdown was sufficient to promote lower SERCA-dependent Ca^{2+} uptake in WT myocytes, and restoration of FAS, SCD1, and Lipin1 had cumulative effects on rescuing compromised SERCA function in *mdx* myocytes. These findings suggest that reduction in muscle's lipogenic machinery might contribute to activation of inflammatory and degenerative pathways in muscular dystrophy.

We previously found that mice with skeletal muscle-specific defect in *de novo* lipogenesis (muscle-specific FAS knockout mice) exhibit muscle weakness that was likely attributable

to compromised SERCA activity [12]. In skeletal muscle, FAS is localized to the SR where it appears to contribute to syntheses of PE. Absence of FAS disrupted phospholipid composition of the SR membrane, leading to reduction in SERCA-dependent Ca^{2+} uptake. Conversely, we identified reduced lipogenesis and increased SR PC/PE ratio in muscles from *mdx* mice. As absence of muscle FAS is sufficient to promote the same change in SR PC/PE, we interpret these findings to suggest that altered SR phospholipid composition in *mdx* muscles are at least partly due to changes in skeletal muscle's lipogenic machinery. This is supported by our finding that reconstitution of lipogenic machinery restored SR PC/PE ratio and SERCA function. However, decreased lipogenesis could not fully explain the altered SR phospholipids in *mdx* muscles. SR PC and PE content were greater, not lower, in *mdx* vs. WT myotubes. Rate of lipogenesis, PC, or PE synthesis could not explain altered SR phospholipid composition in *mdx* myotubes. Thus, other metabolic pathways appear to also contribute to elevated SR phospholipid content in *mdx* muscles. One possibility is that there is an elevated influx of phospholipids in *mdx* muscles. Another possibility is that alternative pathways for PE or PC biosynthesis (PS decarboxylation, PE methylation or lyso-PE/PC acylation) [36] become elevated in *mdx* muscles. The method used to measure PE and PC only accounts for those synthesized through Kennedy's pathway (CDP-ethanolamine and CDP-choline pathways). Reports suggest that complex metabolic changes in membrane phospholipids occur in muscular dystrophy. Phospholipase A2 (iPLA2) appears to modulate muscle PC composition and calcium entry through increasing lyso-phosphatidylcholine in *mdx* muscles [16]. Introduction of exogenous nitric oxide donors into *mdx* muscles restores membrane lipid composition [17]. Caveolin-3, which is involved in membrane endocytosis and function of ion channels, is increased in *mdx* mice and in DMD patients, and overexpression of this enzyme appears to produce a dystrophic phenotype [22, 23, 37].

In liver, FAS gene expression can be regulated by transcriptional factors SREBP-1c, USF or LXR α [32, 38], but it is unknown what factors regulate FAS in skeletal muscle. In *mdx* muscles, reduction in LXR α mRNA level coincided with a lower FAS mRNA level, suggesting that LXR α might be the important regulator of FAS in skeletal muscle. LXR α expression is known to be regulated by miR-1 and miR-206 [32]. We quantified the abundance of these miRNAs in WT and *mdx* diaphragms and found that miR-206, but not miR-1, was elevated in *mdx* vs. WT muscles. Thus, these findings suggest that *de novo* lipogenesis is reduced in *mdx* muscles potentially due to elevated miR-206 that represses LXR α -dependent transcription of FAS.

How does alteration in SR phospholipid composition affect SERCA activity? In liver, alteration in ER phospholipid composition also affects SERCA activity [39]. In artificial micelles, elevating the proportion of PE in the membrane bilayer directly increases SERCA activity [10]. The exact biophysical mechanism for how PE increases the apparent Ca^{2+} affinity of SERCA is not completely understood. The SERCA activation could be a result of direct protein-lipid interactions [40], or indirect effects through bilayer properties. PE does not appear to alter lipid chain dynamics, thickness, or SERCA rotational correlation time [41, 42]. PE may function as hydrogen bond donors with sites on SERCA [43]. Indeed, PE has been implicated in SERCA crystal structures [44].

Gene expression analyses of *mdx* diaphragms also showed reductions in enzymes of lipolysis (HSL, ATGL, LPL) and peroxisomal lipid oxidation (ACO). Recapitulation of these changes in WT myocytes did not alter SERCA function (Figure 3D and F), but we cannot rule out a possibility that overexpression of these enzymes on top of the 3TG myocytes might further restore SERCA activity. However, these experiments were not feasible as we could not consistently express more than three genes in our primary myocytes cultures. It's plausible that lipolysis or peroxisomal lipid-processing produces specific lipid species that affect SR membrane properties, in turn contributing to altered Ca^{2+} homeostasis. It is also possible that phospholipids in other organelles are affected in *mdx* muscles, and that these changes affect cellular Ca^{2+} flux in these organelles.

Exogenous fatty acid supplementation did not increase SERCA activity in *mdx* myotubes (Figure 4C). These findings reinforce the idea that endogenously produced fatty-acids are likely functionally distinct from fatty acids imported from circulation or intracellular triglyceride lipolysis [45–47]. We incubated myotubes in OA, EPA, or DHA in addition to PA because of data suggesting that saturated fatty acids, monounsaturated fatty acids, and polyunsaturated fatty acids may differentially affect muscle functions in muscular dystrophy [48, 49]. Neither OA, EPA, nor DHA had an effect on SERCA-dependent Ca^{2+} uptake.

5. Conclusion

In summary, we report altered skeletal muscle lipid metabolism in *mdx* mice, a mouse model for DMD. Specifically, these changes included reductions in mRNA levels of key enzymes in muscle lipogenesis that coincided with altered SR phospholipid composition and reduced SERCA activity. Restoration of lipogenic enzymes FAS, SCD1, and Lipin1 in *mdx* myocytes rescued reduced SERCA-dependent Ca^{2+} uptake, suggesting that dysregulated lipogenesis might contribute to aberrant Ca^{2+} metabolism in muscular dystrophy. Exogenous fatty acid supplementation does not compensate for the lack of endogenously-produced lipids. Restoration of lipogenic pathways might provide an approach to improve function in muscular dystrophy, but such metabolic interventions would likely require a more global understanding of a complex metabolic shift that occurs in these muscles. This was highlighted by our observation that reduction in muscle lipogenesis did not fully explain the changes in the SR phospholipidome that occurred in *mdx* mice. It would also be important to identify whether similar metabolic changes occur in other models of muscular dystrophy and muscular dystrophy in humans.

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Abbreviations

3TG	triple transgenic expression of FAS, SCD1, and Lipin1
ACO	acyl-CoA oxidase

ATGL	adipose triglyceride lipase
CEPT1	choline/ethanolamine phosphotransferase 1
CMV	cytomegalovirus
DHA	docosahexaenoic acid
DMD	Duchenne muscular dystrophy
<i>dmd</i>	dystrophin muscular dystrophy
EPA	eicosapentaenoic acid
FAS	fatty acid synthase
GOI	gene of interest
HSL	hormone-sensitive lipase
LPL	lipoprotein lipase
LXRα	liver X receptor- α
OA	oleic acid
PA	palmitic acid
PC	phosphatidylcholine
PE	phosphatidylethanolamine
Plin2	perilipin 2
PS	phosphatidylserine
PSS2	phosphatidylserine synthase 2
SCD1	stearoyl-CoA desaturase-1
SERCA	sarco/endoplasmic reticulum Ca ²⁺ -ATPase
SREBP-1c	Sterol Regulatory Element Binding Protein-1c
SR/ER	sarco/endoplasmic reticulum
USF	Upstream Stimulatory Factor
WT	wildtype

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Highlights

- *De novo* lipogenesis is reduced in muscles from *mdx* mice
- Reduction in muscle lipogenesis coincided with altered sarcoplasmic reticulum (SR) phospholipids that are known to affect sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) function
- Reconstitution of *mdx* myotubes with lipogenic enzymes FAS, SCD1, and Lipin1 partially rescued SERCA function
- Improved SERCA function in *mdx* myotubes were likely due to normalization of SR phospholipid composition

Figure 1. Skeletal muscle *de novo* lipogenesis is downregulated in *mdx* mice

A: *De novo* lipogenesis in WT and *mdx* diaphragms. B: FAS mRNA levels in WT and *mdx* diaphragms. C: SREBP-1c, USF and LXRα mRNA levels in WT and *mdx* diaphragms. D: miR-1 and miR-206 levels in WT and *mdx* diaphragms. Filled bars: WT, empty bars: *mdx*.

*: $p < 0.05$. $n = 6$ /group. Data are shown mean $\pm$ SEM.

Figure 2. Skeletal muscle SR phospholipidome in WT and *mdx* diaphragm muscles

A: Individual SR PC species. B: Individual SR PE species. C: Individual SR PS species. D: Total PC, PE, and PS in SR. E: SR PC/PE ratio. Filled bars: WT, empty bars: *mdx*. F: Differential mRNA levels of a panel of genes involved in lipid metabolism between WT and *mdx* diaphragms. Twenty-four lipid-modifying genes including those involved in fatty acid, triglyceride, and phospholipid biosynthesis were assessed by quantitative PCR. Bars extending towards left indicate genes that were more highly expressed in WT muscles, whereas bars extending towards right indicate genes that were more highly expressed in *mdx* muscles. Data are shown in $\log_{10}$ scale. Left: genes were categorized according to the subcellular localizations of their respective enzymes. MAMs: mitochondria-associated membranes *: $p < 0.05$. $n = 6/\text{group}$. Data are shown mean $\pm$ SEM.

Figure 3. Defects in muscle's lipogenic machinery are sufficient to promote a reduction in SERCA activity

A: SERCA-dependent Ca^{2+} uptake in diaphragm from WT and *mdx* mice (n = 8/group). B: SERCA-dependent Ca^{2+} uptake in primary myotubes from WT and *mdx* mice (n = 8/group). C: De novo lipogenesis in primary myotubes from WT and *mdx* mice (n = 6/group). D: SERCA-dependent Ca^{2+} uptake in lentivirus-treated WT myotubes (n = 8/group). WT myoblasts were treated with lentiviruses coding scrambled (sc) or shRNA for FAS, SCD1, Lipin1, ATGL, HSL, LPL, or ACO and were differentiated into myotubes. E: Verification of shRNA knockdown by qPCR (n = 6/group). F: SERCA-dependent Ca^{2+} uptake in transfected WT myotubes (n = 8/group). WT myoblasts were transfected with pCMV6 vectors encoding for CEPT1, Plin2 or PSS2 and were differentiated into myotubes. G:

Verification of WT myotubes transfection by qPCR (n = 6/group). GOI: gene of interest. Filled bars: WT, sc or Ctrl samples. Empty bars: *mdx*, shRNA or pCMV-GOI samples. *: p < 0.05 from WT or sc control. Data are shown mean $\pm$ SEM.

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Figure 4. Restoration of muscle's lipogenic machinery rescues SERCA function in *mdx* myotubes

A: SERCA-dependent Ca^{2+} uptake in *mdx* myotubes with ectopic expression of various lipogenic enzymes (n = 8/group). *Mdx* myoblasts were infected with retrovirus encoding for Ctrl (empty vector) or FAS. The Ctrl and FAS infected cells were then transfected with empty vector, SCD1, Lipin1, or SCD1 and Lipin1 (3TG). WT myoblasts infected with Ctrl retrovirus was used as a reference point. All cells were differentiated into myotubes prior to the assay. Filled bars: WT controls, empty bars: *mdx* controls, light gray bars: single transgenic, gray bars: double transgenic, dark gray bars: triple transgenic. *: p < 0.05 from *Mdx* + Ctrl group. †: p < 0.05 from *Mdx* + FAS group. B: Verification of *mdx* myotubes gene expressions by qPCR (n = 6/group). Empty bars: FAS levels, gray bars: SCD1 levels, filled bars: Lipin1 levels. C: *De novo* lipogenesis in WT + Ctrl, *Mdx* + Ctrl, *Mdx* + FAS, and *Mdx* + 3TG myotubes (n = 6/group). Filled bars: WT + Ctrl, empty bars: *Mdx* + Ctrl, light gray bars: *Mdx* + FAS, dark gray bars: *Mdx* + 3TG. *: p < 0.05 from WT + Ctrl group. †: p < 0.05 from *Mdx* + Ctrl group. D: SERCA-dependent Ca^{2+} uptake in WT myotubes with ectopic expression of various lipogenic enzymes (n = 6/group). WT myoblasts were infected

with Ctrl or FAS retrovirus, followed by transfection with empty vector, SCD1, Lipin1 or both. All cells were differentiated into myotubes prior to the assay. Filled bar: WT + Ctrl, light gray bars: WT with single transgenic, dark gray bar: WT + 3TG. E: Verification of WT myotubes gene expression by qPCR (n = 6/group). Empty bars: FAS levels, gray bars: SCD1 levels, filled bars: Lipin1 levels. F: SERCA-dependent Ca^{2+} uptake in fatty-acid treated *Mdx* myotubes (n = 8/group). *Mdx* myotubes were treated with BSA control or BSA-conjugated PA, OA, EPA, or DHA (100 μM). WT myotubes treated with BSA control were used as a reference point. All cells were differentiated prior to fatty-acid treatments. G. *De novo* lipogenesis in fatty-acid treated *Mdx* myotubes (n = 8/group). Filled bar: BSA control, empty bars: fatty-acid treated cells. Data are shown mean $\pm$ SEM.