Changes in Muscle Metabolism are Associated with Phenotypic Variability in Golden Retriever Muscular Dystrophy

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Duchenne muscular dystrophy (DMD) is an X-chromosome-linked disorder and the most common monogenic disease in people. Affected boys are diagnosed at a young age, become non-ambulatory by their early teens, and succumb to cardiorespiratory failure by their thirties. Despite being a monogenic condition resulting from mutations in the DMD gene, affected boys have noteworthy phenotypic variability. Efforts have identified genetic modifiers that could modify disease progression and be pharmacologic targets. Dogs affected with golden retriever muscular dystrophy (GRMD) have absent dystrophin and demonstrate phenotypic variability at the functional, histopathological, and molecular level. Our laboratory is particularly interested in muscle metabolism changes in dystrophin-deficient muscle. We identified several metabolic alterations, including myofiber type switching from fast (type II) to slow (type I), reduced glycolytic enzyme expression, reduced and morphologically abnormal mitochondria, and differential AMP-kinase phosphorylation (activation) between hypertrophied and wasted muscle. We hypothesize that muscle metabolism changes are, in part, responsible for phenotypic variability in GRMD. Pharmacological therapies aimed at modulating muscle metabolism can be tested in GRMD dogs for efficacy.

INTRODUCTION

Duchenne muscular dystrophy (DMD) occurs in approximately 1 in 5,000 boys, resulting from out-of-frame mutations in the DMD gene that lead to loss of dystrophin protein and its glycoprotein complex [1]. Disruption of these scaffolding and signaling molecules leads to myofiber instability and progressive muscle wasting and weakness, with patients losing ambulation by their early teens and succumbing to the condition by their thirties. A milder, allelic form of dystrophinopathy, Becker muscular dystrophy (BMD), results from in-frame DMD gene

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†Abbreviations: AMPK, AMP kinase; CS, cranial sartorius muscle; DMD, Duchenne muscular dystrophy; GRMD, golden retriever muscular dystrophy; LDE, long digital extensor muscle; MHC, myosin heavy chain; PCR, polymerase chain reaction; SPP1, secreted phosphoprotein 1 or osteopontin; VL, vastus lateralis muscle.

Keywords: Duchenne, golden retriever muscular dystrophy, dystrophin, muscle, metabolism, phenotype, AMPK

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mutations that allow production of a truncated, partially functional protein [2,3]. Becker patients usually live and ambulate for several additional decades [4]. In addition to the pathologic changes directly caused by dystrophin deficiency, so-called genetic modifiers and secondary effects contribute to further phenotypic variation. Studies in the DMD animal models, X-linked muscular dystrophy (mdx) mouse [5] and golden retriever muscular dystrophy (GRMD) dog [6], have played an important role in defining the potential basis for this variability. Based on gene and protein studies, reduced glycolytic and oxidative enzyme expression and activity, a so-called “metabolic crisis,” contributes to differential involvement of both individuals and muscles [7-11]. These metabolic changes may be more pronounced in DMD and GRMD versus the mdx mouse, perhaps contributing to the more severe phenotype of dystrophin deficient boys and dogs. As such, the GRMD canine model appears to be a suitable animal model for muscle metabolism changes and response to respective therapy.

In this study, we evaluated the metabolic changes that occur in the GRMD dog. We hypothesized that GRMD muscle undergoes similar metabolic changes seen in DMD and mdx, including fiber type switching, reduced glycolytic and oxidative metabolism, defective mitochondria, and molecular aberrations. We show for the first time a molecular compensatory mechanism involving AMP-kinase (AMPK), which may drive hypertrophy versus wasting with regards to muscle metabolism.

METHODS

Animals. The dogs were used and cared for according to principles outlined in the National Research Council’s Guide for the Care and Use of Laboratory Animals. They were maintained at North Carolina State University, University of Missouri, University of North Carolina, and then at Texas A&M University. Historical data for each dog in the colony were stored in Breeder’s Assistant (Tenset Technologies, Ltd; United Kingdom), including genotype, date of birth, date of death, and any other defining information.

Surgical Muscle Biopsies. Biopsy samples from the cranial sartorius (CS), long digital extensor (LDE), and vastus lateralis (VL) muscles were collected surgically, snap frozen in liquid nitrogen-cooled SUVA34A (a Freon analog; DuPont Fluorochemicals, Wilmington, DE), and subsequently archived at -80°C until analysis.

Functional Outcome Measures. Surgically measured CS muscle circumference, a directly measure of muscle size was performed, as previously described [8].

Light Microscopy. Muscle sample processing, hematoxylin, and eosin (H&E) staining, and light microscopic analysis were performed, also as previously described [8].

Immunofluorescence Microscopy. Muscle samples were stored at -80°C prior to processing. For immunofluorescence, serial muscle cryosections were cut at 7 μm, thawed on Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA), rehydrated and permeabilized in physiological buffered saline (PBS) containing 0.2 percent fish skin gelatin (FSG) and 0.1 percent Triton X-100 for 10 minutes at room temperature (~20°C). Samples were washed two times with PBS. Sections were fixed in cold, 100 percent acetone for 10 minutes and then washed three times with PBS (5 minutes each). Samples were blocked for 1 hour at ~20°C with 5 percent normal goat serum, PBS+0.3 percent Triton X-100. Primary antibody incubation occurred overnight (~17 hours) at 4°C with the following antibodies: mouse monoclonal myosin heavy chain (fast twitch) (NCL-MHCF; Leica Biosystems, Buffalo Grove, IL) and mouse monoclonal myosin heavy chain (slow twitch) (NCL-MHCS; Leica Biosystems, Buffalo Grove, IL). Sections were then washed two times in PBS-FSG-Triton (5 minutes each) and then once with PBS-FSG. Secondary antibody incubation was for 1 hour at ~20°C with goat anti-mouse Alexa Fluor® 594 (A11005; Thermo Fisher, Waltham, MA). Samples were washed two times with PBS-FSG-Triton and then incubated with DAPI (for DNA) for 5 minutes at 20°C. Prolong® Gold Anti-fade reagent (Life Technologies, Carlsbad, CA) was placed on the sections followed by coverslips. Images of the sections were viewed on a Nikon Eclipse 80i microscope and collected for analysis with NIS-Elements Basic Research software (Laboratory Imaging, Version 3.22.14). Further
imaging processing was performed in ImageJ software (National Institute of Health, Version 1.48).

**Transmission Electron Microscopy (TEM)**. Cranial sartorius and VL muscle samples were collected at necropsy from 6- to 12-month old GRMD and normal dogs and routinely prepared for TEM. Samples were fixed immediately in 3 percent glutaraldehyde + 2 percent paraformaldehyde buffered with 0.1 M sodium cacodylate (pH 7.4) for 1 hour. Individual muscle fascicles were dissected from each muscle sample and placed in fresh fixative overnight at 4°C. Muscle fascicles were then removed from the fixative and washed with 0.1 M sodium cacodylate solution. Following fixation, tissues were treated with 2 percent OsO$_4$ + 1.5 percent K Ferrocyanide in 0.1 M sodium cacodylate for 5 hours at room temperature. Next, tissues were washed three times with water for 5 minutes each and then incubated with 2 percent uranyl acetate in distilled water for 3 hours. Following uranyl acetate treatment, the tissue was washed overnight in water at room temperature and three times with water prior to dehydration. Tissues were dehydrated using a graded series of absolute ethanol solutions, finishing with absolute acetone, and then incubated overnight with 1/3 EMbed 812 Resin (Electron Microscopy Sciences, Hatfield, PA) and 2/3 absolute acetone at room temperature. Next, samples were incubated with 2/3 Resin and 1/3 absolute acetone at room temperature for 8 hours and then overnight in 100 percent Resin. Finally, tissues were placed in molds, embedded in Resin, and baked at 60°C for ~72 hours. Thin sections (80 nm) were placed on formvar-coated mesh grids and copper mesh grids for staining with uranyl acetate and lead citrate. Images were collected from an FEI Transmission Electron Microscope in the Image Analysis Laboratory at Texas A&M University and digitally captured for analysis. Further imaging processing was performed in ImageJ.

**Protein Extraction and Western Blot Analysis.** Stain-free BioRad gels (12 percent, 1.5mm) were prepared using the TGX Stain-Free Fast Cast Acrylamide Starter Kit. Muscle tissue from normal and GRMD CS and LDE samples at 6 months of age were homogenized and quantified, as previously described [12]. One hundred μg of protein was mixed with Laemmli two times electrophoresis buffer until the total volume reached (20 μL). The samples were heated at 95°C for 4 minutes, and then each sample was loaded into the gel along with 10 μL of Novex Sharp prestained ladder (Invitrogen; Carlsbad, CA). Duplicate samples were loaded into two separate gels, which were electrophoresed for 35 minutes at 200 V and then a picture was taken using BioRad ImageLab 4.1 and Gel Doc EZ (Hercules, CA). The proteins were transferred to two separate PVDF membranes for 1 h at 100 V. The membranes were blocked in 5 percent bovine serum albumin (BSA) and 0.1 percent tween TBST at 4°C overnight. Membranes were then vigorously washed three times with 0.1 percent tween in TBST for 5 minutes at room temperature. AMPKα-rabbit antibody (detects total AMPKα1 and α2 subunits; Cell Signaling Technology #2532; Danvers, MA), at a dilution of 1:1,000 in 5 percent BSA/TBST, was applied to one membrane. PhosphoAMPKα-rabbit antibody (detects both phosphorylated AMPKα1 and α2 subunits at threonine 172; Cell Signaling Technology #2535) at a dilution of 1:500 in 5 percent BSA/TBST, was applied to the other membrane. Membranes incubated overnight at 4°C and then vigorously washed three times with 0.1 percent tween in TBST for 5 minutes at room temperature. Membranes were treated with Jackson ImmunoResearch (West Grove, PA) goat anti-rabbit IgG horseradish peroxidase secondary antibody at a dilution of 1:5,000 in 5 percent BSA/TBST, and finally, exposed to radiographic film. Blots were quantified with BioRad ImageLab 4.1

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**Figure 2.** GRMD dogs showed significant variation in phenotype. A) Normal dog (Winthrop) at 6 months of age. B) Mildly affected GRMD dog (Lunes) at 6 months of age. Note the mild muscle atrophy of the pelvic limbs (black arrows) and mild hyperextension of the carpal joints. C) Severely affected GRMD dog (Kermit) at 6 months. Note the prominent joint angle changes and postural deficits, particularly at the carpal, tibiotarsal, stifle, and hip joints, which were due to severe muscle weakness/wasting and joint contractures.
Phenotypic variability in dystrophin deficiency is associated with genetic modifiers. Over and above phenotypic variation due to in-frame vs. out-of-frame mutations, resulting in variable levels of dystrophin expression, the secondary effects of dystrophin deficiency can lead to striking functional differences in DMD boys. For instance, time to wheelchair can vary markedly from 7 to 17 years of age in the era of glucocorticoid therapy with some boys exhibiting severe muscle wasting, weakness, and postural deficits, while other boys have a milder phenotype (Figure 1) [13]. With regards to animal models, dystrophin-deficient GRMD dogs and DMD boys share pathogenetic mechanisms. Affected dogs also show phenotypic variability, including muscle weakness and postural abnormalities, despite having the same DMD gene mutation (Figure 2) [8].

Phenotypic variability is observed among different dystrophin deficient muscles. In DMD, GRMD, and mdx, histopathological variability occurs at the individual muscle level. Most muscles, such as the VL head of the quadriceps, undergo progressive dystrophy, with initial necrosis followed by immune cell infiltration and fibrosis (Figure 3B) [8]. On the other hand, the sartorius muscle of DMD boys is spared. Similarly, after a period of early necrosis, the CS of GRMD dogs regenerates and may undergo paradoxical hypertrophy by 6 months of age. (Figure 3C) [14,15].

*Dystrophin-deficient skeletal muscle undergoes myofiber type switching.* In further evaluating the molecular signatures associated with phenotypic variability, we evaluated the well characterized switching of fiber types, as detected by fiber-type-specific myosin heavy chain (MHC) staining [16]. Indeed, we detected reduced fast twitch (type II) and increased slow twitch (type I) staining in both hypertrophied GRMD CS and wasted VL muscles compared to normal (Figure 4).

**AMP-kinase (AMPK) modifies the dystrophic process at the molecular level.** Several studies in the mdx mouse have demonstrated a beneficial effect of AMPK activation [17-19]. In an analogous way, we found that AMPKα phosphorylation (activation) was increased in the spared/hypertrophied GRMD CS muscle compared to normal dogs (Figures 5A and 5B), while GRMD and normal levels were comparable in the LDE muscle (wasted and measuring half the size of normal in GRMD dogs) [14]. Interestingly, we observed a positive correlation between phosphoAMPKα and surgically measured CS muscle size in GRMD dogs at 6 months of age (Figure 5C).

**Dystrophin-deficient muscle has reduced glycolytic and oxidative capacity.** Regardless of the molecular pathway(s) responsible, dystrophic muscle is under a higher metabolic demand due to a change in glycolytic and oxidative metabolism. The Hoffman laboratory performed genome-wide mRNA expression profiling on DMD muscle and found reduced glycolytic and oxidative enzyme expression compared to normal, suggesting a secondary metabolic defect [7]. Using proteomic profiling via mass spectrometry [8], we also detected similar changes in glycolytic enzyme expression in the GRMD CS muscle at 6 months of age, specifically a reduction in phosphoglucomutase 1, 6-phosphofructokinase, and glucose-6-phos-
allele, which was associated with a more severe phenotype in DMD [22,23]. Given that the genotype seemed to alter longitudinal, functional changes in DMD (i.e. a faster deterioration of ambulatory function was observed with the minor allele) and also increase eccentric contraction damage by affecting Sp1 binding sites [24], we recommended OPN be considered as a covariate in DMD clinical trials [25]. A draft guidance for drug development in DMD, initiated by patient advocates and submitted to the FDA, suggested that genetic modifiers should be used in post-hoc analyses of clinical trial data [26]. Flanigan and colleagues described an additional DMD modifier, a coding haplotype in \textit{LTPB4}, which segregated with age at loss of ambulation [27]. A more recent exome-chip based association study found that a single nucleotide polymorphism in the \textit{CD40} gene, encoding a T helper costimulatory protein, affected function in DMD [28]. Definition of complex molecular mechanisms that explain the effects of modifier polymorphisms should improve understanding of disease pathophysiology and allow modifier proteins to be manipulated pharmacologically. As an example, pharmacological blockade of LTBP4 was suggested as a promising therapeutic strategy for fibrotic disorders such as DMD [29]. Genome-wide mRNA/microRNA expression (Affymetrix chip) and proteomic profiling (mass spectrometry) in GRMD dogs from our colony

**DISCUSSION**

DMD boys and GRMD dogs have progressive muscle weakness, but phenotypic variability is observed among individuals and within different muscle groups [2,8]. Although some phenotypic variation can be explained by the amount of dystrophin expression in muscle [2-4], genes outside the \textit{DMD} gene may modify the disease process. Giacopelli et al. described a polymorphism in the promoter of the \textit{SPP1} gene, encoding the cytokine osteopontin (OPN), which reduced OPN expression at baseline [21]. Pegoraro et al. described the same, minor
species barriers and influence the DMD phenotype was provided by identification of the \( \text{LTBP4} \) locus in a delta-sarcoglycan deficient mouse strain, carrying a 36 bp insertion/deletion [35], before a different modifier haplotype was identified in the homologous human gene [27].

In DMD and its animal models, phenotypic variability occurs at the histopathological level. In the GRMD dog, this differential muscle involvement is thought to arise at least partially from the opposing of flexors versus extensor muscles, with the former being affected early in life, as the animal crawls, and the latter spared [36]. As the individual begins to ambulate, extensors become more affected. An alternative, not mutually exclusive explanation might be that specific muscles have peculiar fiber type composition, innervation, and gene expression patterns. This is intriguing from a therapeutic standpoint; identifying molecular signatures associated with muscle sparing/hypertrophy could provide molecular targets for therapeutic development [8,37].

In further evaluating the molecular signatures associated with muscle hypertrophy versus wasting in dystrophin deficient, changes in muscle metabolism are of great interest to our lab. As such, evaluating myofiber type composition would be a logical first step. Type II (fast-twitch) fibers seem to be preferentially affected (and reduced in number) in dystrophin-deficient muscle, most likely due to reduced oxidative-phosphorylative and increased glycolytic capacity of these fiber types. On the other hand, type I (slow twitch) fibers have increased oxidative capacity, are relatively spared and even increased in number compared to type II, suggesting a metabolic switch from glycolytic to oxidative metabolism [16]. We showed in this study that GRMD dogs undergo fiber switching from fast to slow, but no obvious differences between hypertrophied and wasted muscle. In the GRMD (CXMD) Japanese colony, perpetuated from a descendent of our colony, researchers also showed type II to I fiber type switching in dystrophic muscle [38].

Figure 5. The hypertrophied CS muscle had increased phosphorylated (P) AMPKα compared to normal. A) Note the variability in western blot expression of P-AMPKα in GRMD dog samples. B) P-AMPKα was normalized to Total AMPKα protein and increased compared to normal samples. * = p < 0.05. C) P-AMPKα was positively correlated with CS circumference.
Activation may have a protective and even beneficial effect in dystrophic muscle. Further studies are underway to activate AMPK in the GRMD dog and evaluate changes in phenotype and downstream molecular signatures.

The accumulating data outline the increased metabolic demand that occurs secondarily to dystrophin deficiency. Chen and colleagues evaluated microarray profiles of DMD muscle and found reduced glycolytic and oxidative metabolism genes [7]. In *mdx* mice, there was a reduction in lactate dehydrogenase (marker for glycolytic enzyme) activity [9]. We also showed in this study several glycolytic enzymes reduced at the protein level in GRMD muscle, as detected by proteomic profiling and mass spectrometry [8]. Terrill and colleagues revealed this glycolytic/oxidative phosphorylative enzyme reduction was due to protein thiol oxidation [41]. A quantitative proteomic profile of GRMD versus normal muscle confirmed a defect of metabolic proteins, many regulated by PGC-1α [42].

In further evaluating metabolic capacity, we evaluated mitochondria localization in GRMD muscles. The ATP producing organelles are normally dispersed throughout myofibers and interface with the sarcolemmal membrane [43]. A recent study by Percival and colleagues showed a reduction in mitochondria in *mdx* mice and suggested the metabolic defect was directly related to dystrophin deficiency [20]. We also observed reduced and swollen mitochondria in both dystrophin-deficient hypertrophied and wasted muscle. A recent study evaluated muscle damage induced by cardiotoxin injection and showed similar histopathological, ultrastructural, and metabolic profiles compared to dystrophic muscle [44]. Indeed, the reduced glycolytic enzyme expression and mitochondria observed in our GRMD dogs may be due to the inflammation and healthy animals, cross-innervation of muscles with alpha motor neurons of an opposing nerve resulted in fiber type switching [39]. In dystrophic muscle, the molecular signatures responsible for the fast to slow switch are mediated, in part, by the folliculin interacting protein-1 (fnip-1) – AMPK pathway [40].

In the context of phenotypic variability at the muscle level, we showed that phosphorylated AMPKα1- and -α2 are increased in hypertrophied muscle. Moreover, P-AMPK was directly correlated with surgically measured CS muscle size, suggesting the energy sensor kinase may promote muscle hypertrophy. These data are supported by the fact that the wasted LDE, a muscle measuring half its normal size in GRMD dogs [14], showed similar phosphoAMPKα expression compared to normal muscle. Pharmacological activation of the kinase was associated with neuronal nitric oxide synthase (nNOS) activation and increased nitric oxide production in *mdx* mouse cardiomyocytes [17]. The authors suggested that the mechanical stretch-mediated AMPK phosphorylation of nNOS was dependent on an intact DGC; pharmacological activation of AMPK in dystrophin-deficient *mdx* muscle restored this function [17]. Indeed, another study in *mdx* mice showed that administration of the AMPK activator, metformin, increased downstream expression of several muscle proteins, including peroxisome proliferator-activated receptor γ Co-activator 1α and the dystrophin homologue utrophin [18]. Providing further evidence of the importance of AMPKα1, *mdx* mice treated with the AMPK agonist, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) had improved function, reduced creatine kinase levels, and increased oxidative metabolism proteins [19]. The previous *mdx* studies and the GRMD data presented here suggest that AMPKα activation may have a protective and even beneficial effect in dystrophic muscle. Further studies are underway to activate AMPK in the GRMD dog and evaluate changes in phenotype and downstream molecular signatures.

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**Figure 6. Glycolytic enzymes were reduced in hypertrophied GRMD CS muscle at 6 months of age compared to normal.** Phosphoglucomutase-1, 6-phosphofructokinase, and glucose-6-phosphate isomerase were reduced at 6 months (M) in GRMD cranial sartorius (CS) (black bars) compared to normal (white bars). There was not a significant change at 4 to 9 weeks (W). N = 3 for each group. Total spectral counts were used to determine relative abundance. * = P < 0.05; ** p < 0.01; *** p < 0.001.
CONCLUSION

The role of genetic modifiers in altering muscle metabolism and its association with phenotypic variability is an area of active investigation. In addition to well-documented changes in the mdx mouse model, we have identified analogous GRMD abnormalities, including fiber type switching, glycolytic enzyme reduction, mitochondrial defects, and AMPKα activation in hypertrophied muscle. Given the similar progressive phenotype observed between GRMD and DMD, affected dogs appear to be a suitable model for the metabolic crisis occurring in dystrophic muscle. Pharmacological approaches to enhance muscle metabolism in dystrophin-deficient individuals should be explored using the GRMD dog for efficacy.

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