Lipid Accumulation in Dysferlin-Deficient Muscles

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Dysferlin is a membrane-associated protein involved in vesicle trafficking and fusion. Defects in dysferlin result in limb-girdle muscular dystrophy type 2B and Miyoshi myopathy in humans and myopathy in A/J dysferlin/C0 mice, but the pathomechanism of the myopathy is not understood. Oil Red O staining showed many lipid droplets within the psoas and quadriceps muscles of dysferlin-deficient A/J dysferlin/C0 mice aged 8 and 12 months, and lipid droplets were also conspicuous within human myofibers from patients with dysferlinopathy (but not other myopathies). Electron microscopy of 8-month-old A/J dysferlin/C0 psoas muscles confirmed lipid droplets within myofibers and showed disturbed architecture of myofibers. In addition, the presence of many adipocytes was confirmed, and a possible role for dysferlin in adipocytes is suggested. Increased expression of mRNA for a gene involved in early lipogenesis, CCAAT/enhancer binding protein-δ, in 3-month-old A/J dysferlin/C0 quadriceps (before marked histopathology is evident), indicates early induction of lipogenesis/adipogenesis within dysferlin-deficient muscles. Similar results were seen for dysferlin-deficient BLAJ mice. These novel observations of conspicuous intermyofibrillar lipid and progressive adipocyte replacement in dysferlin-deficient muscles present a new focus for investigating the mechanisms that result in the progressive decline of muscle function in dysferlinopathies. (Am J Pathol 2014, 184: 1668–1676; http://dx.doi.org/10.1016/j.ajpath.2014.02.005)

Muscular dystrophies represent a large group of inherited human muscle diseases that result from a diversity of gene defects that include many membrane-associated proteins such as dysferlin, caveolin-3, dystrophin, and members of the dystroglycan/sarcoglycan complex, as well as other proteins such as laminins (reviewed in Saini-Chohan et al1 and Bushby2). This study is focused on dysferlinopathies that result from mutations in the dysferlin gene that was identified in 1998.3,4 These dysferlinopathies are a clinically heterogeneous group of disorders usually with onset in late teens and slow progression. They include limb-girdle muscular dystrophy type 2B and Miyoshi myopathy, with weakness mainly in proximal limb-girdle muscles or distal muscles, respectively.4–8

Dysferlin is a member of a large ferlin family of transmembrane proteins that are involved in protein vesicle trafficking and fusion,9 with dysferlin initially attracting attention because of its role in the rescaling of experimentally damaged sarcolemma.10–12 However, the extent to which such damage might normally occur in dysferlin-deficient muscles in vivo is unknown; although membrane rescaling has proved a useful experimental tool, this may not be the primary cause of the dystropathology.13 More recently, the localization of dysferlin in intracellular membranes such as T-tubules and sarcoplasmic reticulum14 with a potentially important role in calcium homeostasis related to excitation-contraction coupling has attracted attention.15–17 Dysferlin has high expression in skeletal myofibers but is also present in many other tissues3 and cells, including macrophages18 and endothelium.19 The precise role that dysferlin plays in mature myofibers in vivo remains unclear. A striking feature of the onset of dysferlinopathies in humans is that growing children and adolescents are often asymptomatic, although many are active in sports,20 with the disease manifesting within 1 to 2 years of cessation of growth, indicating a lesser role for dysferlin in such growing, elongating myofibers.22 This is also

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supported by the late onset of dystropathology in a wide range of mouse models for dysferlinopathies, ranging from dysferlin-deficient or nulls to engineered-specific gene defects.\textsuperscript{23–26} Many initial studies were performed in dysferlin-deficient SJL/J (Swiss) mice and then A/J (A/J\textsuperscript{Dysf\textsuperscript{−/−}}) mice and more recently BLAJ mice (B6.A-Dysf\textsuperscript{GJenJ}), whereby the A/J\textsuperscript{Dysf\textsuperscript{−/−}} mutation was introduced into the C57Bl/6J background strain. The disease is generally mild in dysferlin-deficient mice, and all mice show a late onset, with minimal symptoms before 3 months of postnatal age, histological evidence of disease by 8 and 12 months in some muscles (especially psoas and also quadriceps), and more pronounced pathological changes by 19 months.\textsuperscript{27}

In a recent analysis of the disease progression in A/J\textsuperscript{Dysf\textsuperscript{−/−}} mice we observed abnormally high levels of fatty tissue that replaced up to 20% to 40% of myofibers in the severely affected psoas muscle (and to a lesser extent in quadriceps femoris), compared with normal A/J controls, at 8, 12, and 19 months of age.\textsuperscript{27} We had previously observed this conspicuous fatty tissue in rectus femoris (quadriceps) muscles of 12-month-old dysferlin-deficient SJL/J mice in which fat can replace up to approximately 20% of myofibers (J. Torrisi and M. D. Grounds, unpublished data); the lipid replacement in old quadriceps of SJL/J mice has also been reported by others.\textsuperscript{25} It is unlikely that fat cell replacement of dysferlin-deficient myofibers is a consequence of the incidence of myonecrosis (and impaired regeneration), because the level of myonecrosis, classically identified by fragmentation of myofiber sarcoplasm with inflammatory cell infiltration, was relatively low (<1% of the total muscle area) in A/J\textsuperscript{Dysf\textsuperscript{−/−}} mice aged up to 19 months.\textsuperscript{27} It is noted that such fatty replacement of limb muscles is not seen in mdx mice (that lack dystrophin and are a model of Duchenne muscular dystrophy), even though they have much higher levels of myonecrosis throughout life (up to approximately 80% of myofibers damaged in young mice and approximately 8% in adult mice). Dysferlin-deficient muscles exhibit excellent myogenesis and readily form new muscle in response to experimental muscle damage in vivo\textsuperscript{28–30}; however, macrophages and other cells are also affected by dysferlin deficiency,\textsuperscript{15} and it appears that there may be an impaired resolution of the inflammatory response associated with regeneration in some models of injury.\textsuperscript{20,31} The present study investigates the nature of this high fatty tissue replacement of myofibers and the steatosis within them, in severely affected dysferlin-deficient muscles\textsuperscript{27} of A/J\textsuperscript{Dysf\textsuperscript{−/−}} and BLAJ mice and in patients with dysferlinopathy, using a range of techniques, including Oil Red O and BODIPY staining and electron microscopy (EM).

Materials and Methods

Sources of Mouse and Human Tissues

The A/J dysferlin-deficient A/J\textsuperscript{Dysf\textsuperscript{−/−}} mice were generously provided by Dr. Sandra Cooper (Children’s Hospital at Westmead, Sydney, Australia) (as for Terrill et al\textsuperscript{27}). Additional inbred strains of mice, including control normal A/J and C57Bl/6J (sometime referred to as C57), and dysferlin-deficient BLAJ mice (B6.A-Dysf\textsuperscript{GJenJ}) were obtained from the Animal Resources Centre, Murdoch, Western Australia. Where required, mice were maintained at the Preclinical Animal Facility at the University of Western Australia on a 12-hour light/dark cycle, under standard conditions (at 23°C), with free access to meat-free rat and mouse diet (Specialty Feeds, Glen Forrest, Australia) and drinking water. All experiments were conducted in accordance with the guidelines of the National Health and Medical Research Council, Australia, and were approved by the Animal Ethics Committee of the University of Western Australia.

The human biopsies and blood from patients with dysferlinopathy and other myopathies were obtained for diagnostic purposes in Berlin, and informed consent was given by all patients that stained sections and blood can be analyzed for scientific purposes: a summary of all patients is provided in Supplemental Table S1. For dysferlinopathies, muscles were examined from four patients: 1 (male, aged 40 years), 2 (male, aged 52 years), 3 (female, aged 44 years), and 4 (female, aged 32 years, with severe disease), and blood from two patients, 3 and 5 (female, aged 18 years), was used for standard lipid analysis. Information on four of five patients has been published (patients 1, 2, and 3 in Wenzel et al\textsuperscript{32} and patient 5 in Diers et al\textsuperscript{33}).

Sampling and Processing of Mouse Tissues

Most analyses were done with the affected psoas and quadriceps muscles from female dysferlin-deficient A/J\textsuperscript{Dysf\textsuperscript{−/−}} and wild-type (control) A/J mice aged 3 to 12 months. Many of the tissues were from the same A/J\textsuperscript{Dysf\textsuperscript{−/−}} and A/J mice as used in a previous study of histological quantification.\textsuperscript{27} Some other muscles such as gastrocnemius were also examined. In addition, muscles from male A/J\textsuperscript{Dysf\textsuperscript{−/−}} and A/J mice, from male and female dysferlin-deficient BLAJ mice, and normal control C57Bl/6J mice, plus from female dysferlin-deficient SJL/J mice, were used to confirm observations for sex and age and for different strains. The range of mice and analyses used are indicated in Supplemental Table S2.

All mice were sacrificed by cervical dislocation while under terminal anesthesia (2% v/v Atane isoflurane; Bomic Animal Health Pty Limited, Hornsby, Australia). For RNA extraction muscles were snap-frozen in liquid nitrogen. For histological analyses muscles were either frozen in isopentane cooled in liquid nitrogen or fixed in 4% paraformaldehyde and embedded in paraffin. For transmission EM, muscles were placed into 2.5% glutaraldehyde in 0.05 mol/L cacodylate buffer (pH 7.4) at room temperature for 24 hours. Samples were postfixed in 1% osmium tetroxide, dehydrated in graded solutions of ethanol, infiltrated, and embedded in Araldite. Semithin sections were stained in 1% toluidine blue in 5% borax and were examined by light microscopy to select the samples for EM.
analysis. For these blocks, 50 nm ultra-thin sections were cut, mounted on 200 mesh thin-bar copper grids, double stained in uranyl acetate and lead citrate, and examined with a JEOL 1400 Transmission Electron Microscope at an accelerating voltage of 100 kV.

**Oil Red O, BODIPY, and NADH Histochemical Staining**

Lipid was visualized on frozen muscle (8 μm sections) with the use of Oil Red O histochemical staining. In brief, transverse and longitudinal sections were air dried and fixed in 10% formalin. After 2 minutes of incubation in propylene glycol, sections were stained in 0.5% Oil Red O in propylene glycol for 10 minutes at 60°C. Sections were differentiated in 85% propylene glycol, rinsed in distilled water, and stained in hematoxylin for 30 seconds. A similar staining protocol was used for the frozen human muscle biopsies.

BODIPY 493/503 (Invitrogen, Carlsbad, CA) is a non-polar fluorophore that binds to neutral lipids and is used to identify and quantify lipids, including lipid droplets, in tissues with the use of fluorescent microscopy.34,35 After staining by using a standard protocol,35 images were captured with a fluoroscent Nikon Eclipse Ti microscope equipped with a Roper Industries CoolSNAP-HQ2 camera, a 450- to 490-nm excitation filter, a 515-nm emission barrier filter, and Nikon NIS-Elements software version 3.0. Quantification was performed with ImageJ software version 1.44 (NIH, Bethesda, MD), where image thresholds were adjusted, allowing the percentage of lipid in the whole muscle cross-sections to be measured.

To identify fast and slow myofiber types, 8-μm frozen muscle sections were stained with a standard protocol for nicotinamide adenine dinucleotide—reduced (NADH). Sections were incubated with a solution that contained equal parts NADH solution (1.6 mg/mL 0.05 mol/L Tris buffer, pH 7.6), and nitro blue tetrazolium solution (2 mg/mL 0.05 mol/L Tris buffer, pH 7.6) for 30 minutes at 37°C. Slides were washed in H2O, before multiple washes in acetone:H2O at increasing concentrations of acetone.

**RNA Extraction and RT-qPCR**

Gene expression was quantified for CCAAT/enhancer binding protein (C/EBP)-δ and peroxisome proliferator activation receptor-γ, because these are associated with induction of lipogenesis and differentiation of adipocytes (reviewed in Shavlakadze et al16). For RNA extraction, muscles were ground in liquid nitrogen, and RNA was extracted with Tri-Reagent (Sigma Chemical Co., St. Louis, MO) treated with RQ1 DNase 1 (Promega, Madison, WI) to remove DNA; reverse transcription was performed on 2 μg of RNA with the use of Moloney Murine Leukemia Virus Reverse Transcriptase (Promega). cDNA was purified with Ultra Clean PCR Clean-up kit (Mo Bio Laboratories, Carlsbad, CA), and quantitative RT-PCR (RT-qPCR) was performed on a Rotor Gene 6000 real-time rotary analyzer by using the QuantiFast SYBR Green RT-qPCR Kit (Qia-gen, Valencia, CA). All primers were purchased from Qia-gen and were mouse CCAAT/enhancer binding protein δ (Cebpd; QT00312809), peroxisome proliferator activation receptor gamma (Pparg; QT00100296), and hypoxanthine guanine phosphoribosyl transferase (Hprt; QT00166768) that was used as a reference gene to normalize mRNA levels of Cebpd and Pparg.

**Results**

**Lipid Accumulation in Dysferlin-Deficient Muscles**

A large area of lipid (approximately 20% to 40% of area) within muscles has previously been described for A/J dys−/− mice,27 and sections from the same A/J dys−/− muscles stained with Oil Red O (Figure 1, A and C) showed many lipid droplets within myofibers and interstitium (probably adipocytes) were conspicuous in psoas (A) and quadriceps (C) muscles. Such lipid droplets were not evident in the psoas (B) nor quadriceps (D) muscles of 12-month-old female A/J (wild-type) mice. Human muscles examined from three patients with dysferlinopathy (E–H) showed striking lipid droplets within many myofibers, with extensive lipid in one severe case (G and H).
extensive lipid compared with age- and sex-matched wild-type A/J muscles (Figure 1, B and D). The lipid was located in adipocytes between the myofibers and also appeared to be present as droplets within many myofibers in selected regions of the muscle. BODIPY fluorescent staining was used to quantitate lipid in a range of female and male muscles, from different ages and strains (A/Jdyx−/−, BLAJ, their respective normal controls, and SJL/J) (Supplemental Table S2), with data for BLAJ (and control) psoas muscles shown in Supplemental Figure S1. There did not appear to be any sex-dependent effect in the various strains, and BODIPY also stained the SJL/J muscles (not shown). There was also broad strain similarity, with approximately 20% lipid present in BLAJ psoas at 12 months which is similar to that previously reported for A/Jdyx−/− psoas muscles at 8 and 12 months. Smearing of lipid can occur when the tissue is cryo-sectioned, making it difficult to determine the precise location of the lipid with the use of Oil Red O (or BODIPY). For this reason, the presence of lipid droplets within myofibers was confirmed by EM (Figure 2).

The proportion of fast and slow myofibers in quadriceps muscles was observed with NADH staining and appeared similar in control and dysferlin-deficient mice up to 12 months of age; shown for A/J mice in Supplemental Figure S2. Equivalent patterns of NADH staining were seen for quadriceps muscles of male BLAJ mice aged 3, 8, and 12 months (data not shown). It was noticed that the myofiber appearance in 12-month-old A/Jdyx−/− and BLAJ muscles was disturbed compared with controls; this is more clearly visible in the high-power images for A/Jdyx−/− muscles (Supplemental Figure S2, D and E).

In all human muscles from patients with dysferlinopathy, strong staining by Oil Red O of lipid within many myofibers was evident (Figure 1, E and F), and lipid was pronounced also in many adipocytes in one severe case, patient 4 (Figure 1, G and H). Lipid droplets are rare in normal healthy human myofibers. Although an increased number of small lipid droplets in oxidative (type 1) myofibers is occasionally seen in patients with metabolic syndrome (the significance is unclear), none of the patients with dysferlinopathy studied here had a metabolic syndrome (S. Spuler, unpublished data).

Examination of a further 16 biopsy specimens stained with Oil Red O from three other myopathies found no evidence of lipid droplets within myofibers (data not shown). These biopsies from 10 males and six females aged 20 to 65 years are summarized in Supplemental Table S1; they were from five patients with dystrophinopathy (adult Becker phenotype), five patients with calpainopathies (limb-girdle muscular dystrophy type 2A), and six patients with myotonic dystrophy type II.

The lipid profiles of blood from two patients with dysferlinopathy were analyzed and were in the normal range (data not shown).

EM of Dysferlin-Deficient Mouse Muscles

The ultrastructure of dysferlin-deficient psoas muscles from three mice (two A/Jdyx−/− and one BLAJ) aged 8 months (Figure 2) showed many features of disturbed muscle architecture within myofibers, plus many large adipocytes (Figure 3). In oxidative myofibers (characterized by thick Z bands and many mitochondria), many lipid droplets were evident between the myofibrils (Figure 2, A–C); the darker staining of some lipid droplets with osmium probably reflects the presence of unsaturated lipid. Most lipid droplets were closely associated with mitochondria that were occasionally enlarged, pleomorphic, and present in high numbers (Figure 2C). Accumulation of lipid droplets was pronounced in slow oxidative myofibers that were often adjacent to fast myofibers that generally appeared normal (Figure 2C). Occasional myofibers were severely disorganized with few intact myofilaments or intact sarcoplasmic organelles, indicating severe sarcoplasmic degeneration (Figure 2D). However, leukocytes were not conspicuous in these areas. Autophagosomes and prominent multivesicular bodies were also observed (Figure 2, E and F) as were distinct lipofuscin granules that were often present near myonuclei (Figure 3C). Also noted were vesicles, blebs (Figure 2F), and cytoplasmic extrusions at the myofiber surface (not shown) that have been well described for human and mouse dysferlinopathies.10,23,37,38

Quantification was done for the EM images of psoas muscles from two A/Jdyx−/− mice and one BLAJ mouse...
aged 8 months. The data are for affected areas of myofibers
with concentrations per unit area of 10 μm²; 18 fields were
analyzed, 7 fields for each A/J²⁵^-/- (A–E) and one BLAJ (F and G) mouse aged 8 months.
The semithin sections are stained with toluidine blue and indicate the
accumulation of adipocytes (A and B). Transmission EM clearly shows the
boundary between adipocyte (a) and myofibers (C, E, F, and G) with
basement membranes of both cells evident with intervening collagen fibrils
(asterisk). Cholesterol clefts were present within some adipocytes (F, white arrows).
Many large lipid droplets were seen within the cytoplasm of
adipocytes (C and D, white arrows); the high-power view (D) also shows
many vesicles near the cell membrane (black arrows) and an autophago-
some (asterisk). Micropinocytic vesicles are also evident in the thin
cytoplasm near the surface of the adipocyte (G, white arrows).

Expression of mRNA for C/EBP-δ in Dysferlin-Deficient and Control Mouse Muscles

Levels of C/EBP-δ mRNA were increased by 1.7-fold in
quadriceps muscles from female A/J²⁵^-/- mice aged 3
months, compared with control A/J mice (Figure 4). The
reason for doing the molecular analyses on 3-month-old
muscles was because we specifically wanted to look for early
changes in gene expression that might lead to lipid deposi-
tion, before pronounced pathology was present. A significant
increase was also observed in 3-month-old male BLAJ
mice that had about 1.5-fold more C/EBP-δ mRNA than control
C57Bl/6J mice. No significant changes were seen for
expression of peroxisome proliferator activation receptor-γ
mRNA between any of these muscles (data not shown).

Discussion

Our study identifies an abnormally high incidence of lipid
droplets within dysferlin-deficient myofibers in both
A/J²⁵^-/- and BLAJ mice and humans, along with areas of
increased numbers of interstitial adipocytes, in dysferlin-
deficient mice; these extend the recent observation of lipid
replacement in A/J²⁵^-/- mouse muscles. Our observations
suggest that frozen sections of human dysferlinopathy
muscle routinely stained with Oil Red O should be more
widely examined. Marked fatty replacement of human
muscles with dysferlinopathy has been reported with the use
of magnetic resonance imaging or computer tomography.
For example, complete replacement by fatty degeneration of lumbar and lower thoracic erector spinae muscles was described in one case, and marked fatty replacement of paraspinal muscles was reported in another case. Others have concluded that fatty replacement is the rule at advanced stages of dysferlinopathy whereby fibrofatty tissue can replace >70% of muscle tissue; importantly, magnetic resonance imaging abnormalities are evident before symptoms (reviewed in Amato and Brown). However, such lipid accumulation in dysferlin-deficient muscles does not seem to have attracted much attention, and the nature and mechanism of this high-fat accumulation in dysferlinopathies has not previously been investigated.

Our observations include high intermyofibrillar lipids in dysferlin-deficient human and mouse muscles and in psoas muscles of A/J^dys^fbs^−/− mice aged 8 months, severely disturbed sarcoplasmic organization with some loss of sarcomeric architecture, increased numbers and size of mitochondria with altered morphology, and autophagosomes filled with vesicles and lipid. This occurred almost entirely in oxidative myofibers, usually in the absence of any evidence of myonecrosis. It is noted that at 3 months of age, lipofuscin granules were already elevated approximately 2.5-fold in A/J^dys^fbs^−/− psoas muscles before manifestation of pronounced pathological changes, indicating an early metabolic disturbance within these muscles.

The mechanism(s) responsible for the high lipid within dysferlin-deficient muscles and the accumulation of adipocytes outside myofibers requires intensive investigation. Within the myofibers, steatosis reflects some imbalance in normal lipid metabolism, because of increased entry of fatty acids into the myofibers, inefficient oxidation of fatty acids by mitochondria, and/or excessive lipid production. The significant elevation of mRNA for C/EBP-β in quadriceps muscles of A/J^dys^fbs^−/− and also BLAJ mice aged 3 months indicates a striking early induction of the lipogenic/adipogenic program, well before these muscles manifest histopathology or have conspicuous numbers of identifiable (differentrated) adipocytes. Elevated C/EBP-β mRNA may prove to be a useful biomarker for early-stage dysferlinopathy. It is not clear if induction of this lipogenic gene program is occurring in myogenic or other cells; it might be due to transdifferentiation of myonuclei (with a metabolic switch into a lipogenic rather than a myogenic program), conversion of a (myogenic) satellite cell into an adipogenic precursor, or, more likely, activation of pre-adipocytes or conversion of mesenchymal stem cells in the intersitium. These scenarios are briefly discussed below but clearly need to be thoroughly investigated in vivo. Studies in tissue culture over many years have demonstrated a ready conversion of myogenic stem cells/myoblasts from cell lines or from skeletal muscle, into an adipogenic phenotype; however, the idea of fate-switch between muscle and fat cells remains controversial. Recent studies indicate that cells extracted from skeletal muscle that readily convert to adipogenic cells are not derived from the satellite cells per se; although satellite cells under adipogenesis-inducing conditions can accumulate cytoplasmic lipid, they maintain myogenic protein expression and do not fully execute the adipogenic differentiation program. Evidence also suggests that certain endothelial cell populations can give rise to adipocytes. Although it has been demonstrated that the condition of the myofiber (eg, damage) can affect fat accumulation, it is challenging to determine precisely what cells are contributing to lipogenesis/adipogenesis in vivo, especially under pathological conditions, and this is likely to vary between different muscles.

In the absence of dysferlin, the adipocytes also show structural changes, with many cytoplasmic lipids, altered mitochondria, prominent autophagosomal bodies, small vesicles near the cell membrane, and some fragmentation of external lamina that are reminiscent of the changes described for dysferlin-deficient myofibers. These observations suggest that dysferlin may normally play an important role in adipocytes; this has not been described previously but was indicated by a quantitative proteomic analysis of adipocyte plasma membranes that reported dysferlin was present. Lipid droplet size is important, and diverse sizes are observed in different tissues under pathophysiological conditions. The involvement of aberrant adipocytes in the pathogenesis of dysferlinopathy does not seem to have been considered previously.

To date many studies into dysferlinopathies have focused on defining the genetics and molecular biology of dysferlin and related ferlin molecules, their localization and interactions with a wealth of other proteins, and their roles in studies of myogenesesis and resealing of membranes in myotubes and myofibers ex vivo. Fewer studies have investigated the consequences of dysferlin deficiency in mature adult muscles in vivo with respect to metabolism, and none appear to have recognized the significance of the lipid changes. Analyzing the molecular basis for the lipid accumulation is a major challenge that falls far beyond the scope of this study that is focused on presenting and describing the novel lipid phenotype. However, it is pertinent to make some brief comment about the wealth of possibilities that might contribute to this elevated lipid in dysferlinopathies.

The field of lipids and their regulation within muscle and adipose tissue is highly complex and is the focus of much research into adiposity, obesity, insulin resistance, and metabolic disorders such as diabetes, with lipid disturbances also reported in a range of myopathies; these complexities involve lipid trafficking within the cell and mitochondrial metabolism and are comprehensively discussed in recent reviews. Other molecules that are associated with dysferlin, such as caveolins, are involved in lipid regulation, and mitsugumin 53 (also called TRIM72) is involved in insulin resistance and metabolic disorders. Defects in dysferlin are likely to have a wide range of consequences, including disturbance to the dynamics of many membrane-associated molecules such as receptors, in the sarcolemma and other cellular compartments, as has been reported for
myoferlin. The lack of dysferlin has different consequences in slow twitch oxidative and glycolytic myofibers, and this needs to be considered because there are many important differences between fast and slow myofibers (discussed in Agbulut et al57).

Inflammation can also affect lipid metabolism, and this involves a further complexity of interacting factors, including endoplasmic reticulum stress, Toll-like receptors, eicosanoids, and fetuin. Disturbance of endoplasmic reticulum/sarcoplasmic reticulum function is known as endoplasmic reticulum stress and is associated with metabolic diseases and various myopathies, including dysferlinopathies.62 There are many interactions between endoplasmic reticulum stress, inflammation, autophagy, and mitochondrial changes. Inflammation is an important feature of dysferlinopathies, with cross talk between the inflammasome and myofibers, altered function and enhanced phagocytosis of dysferlin-deficient macrophages, increased expression of complement by dysferlin-deficient myofibers, and susceptibility of such myofibers to complement attack. Thus, in the context of the high-lipid content of dysferlin-deficient myofibers, one must consider not only the complex molecular interactions within and between myofibers and adipocytes but also the role of other dysferlin-deficient cells, especially macrophages and other components of the inflammatory response.

The novel observations of large numbers of lipid droplets within dysferlin-deficient human and mouse myofibers, with increasing numbers of large adipocytes in the interstitium, combined with the very early induction of the lipogenic factor C/EBP-δ in young adult mouse muscles before the onset of marked histopathology, suggest a fundamental metabolic disturbance in dysferlin-deficient muscles. Such major disruption of normal cell and biochemical/metabolic homeostasis of myofibers will, inevitably, impair muscle function. The precise relationship between the changes within myofibers and outside myofibers with the large number of adipocytes is unclear. The strong association of these lipid-related changes with slow oxidative myofibers emphasizes that the myofiber type needs to be more carefully considered in future investigations into the in vivo molecular basis for dysferlinopathies. These studies demonstrate the value of using the A/J dysf+/− and BLAJ mice as models for human dysferlinopathies. They also present the possibility of identifying new diagnostic and therapeutic targets for dysferlinopathy. This focus on lipid provides new directions for investigating the mechanisms that result in the progressive decline in function of dysferlin-deficient muscles.

Acknowledgments

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Supplemental Data

Supplemental material for this article can be found at http://dx.doi.org/10.1016/j.ajpath.2014.02.005.

References

Lipid in Dysferlin-Deficient Muscles

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Supplementary Figure S1: Lipid content in psoas muscles from female and male BLAJ and control C57Bl/6J (C57Bl) mice, using fluorescent BODIPY staining. Images show BODIPY staining in 8-month-old female and male A/J dys−/− mice. Quantification of lipid is expressed as percentage of total muscle CSA. $P < 0.05$ for difference between all BLAJ (8 and 12 months) and C57Bl (8 months) muscles; †$P < 0.05$ for difference between 8 and 12 months BLAJ muscles. There is no difference between females and males for all ages and strains. Scale bar = 200 μm. CSA, cross-sectional area.
Supplementary Figure S2: NADH staining to identify patterns of myofiber types in quadriceps muscle sections from 12-month-old wild-type control A/J (A and C) and dysferlin-deficient A/Jdys−/− (B, D, and E) mice. Low-power images (A and B) show the overall distribution and number of fast (pale and large) and slow (dark and small) as well as intermediate myofibers; similar patterns of myofiber types are present in both strains. The high-power images show disturbed appearance of myofibers (D and E, asterisk) in two sections from the same A/Jdys−/− muscle; some myofibers have lost the granular appearance, indicating that they are degenerating (E, asterisk). Scale bars: 200 μm (A and B); 50 μm (C–E).
Supplemental Table S1. Summary of patients with a range of myopathies used in this study.

<table>
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<th>Diagnosis</th>
<th>Number</th>
<th>Male</th>
<th>Female</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>N (age)</td>
<td>N (age)</td>
</tr>
<tr>
<td>Dysferlinopathy</td>
<td>5</td>
<td>2 (40, 52)</td>
<td>3 (18*, 32, 44)</td>
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<tr>
<td>Dystrophinopathy</td>
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<td>4 (29, 39, 52, 60)</td>
<td>1 (symptomatic carrier, 45)</td>
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<td>LGMD2A</td>
<td>5</td>
<td>3 (20, 27, 35)</td>
<td>2 (22, 29)</td>
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<tr>
<td>Myotonic dystrophy II</td>
<td>6</td>
<td>3 (30, 44, 54)</td>
<td>3 (28, 45, 65)</td>
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</table>

All human muscle biopsies were stained with Oil Red O and examined.

*This patient was used for blood analysis only, as muscles were not biopsied
Supplemental Table S2. Indication of dysferlin-deficient A/J, BLAJ, and SJL/J male (M) and female (F) mice at different ages, and their control strains, used for the range of analyses.

<table>
<thead>
<tr>
<th>Mouse strain</th>
<th>Age (m)</th>
<th>Sex</th>
<th>Histology</th>
<th>ORO (lipid)</th>
<th>BODIPY (lipid)</th>
<th>Semi-thins and TEM (lipid)</th>
<th>RT-qPCR (mRNA)</th>
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<td>A/J&lt;sup&gt;dy&lt;/sup&gt;-/</td>
<td>3, 8, 12</td>
<td>M and F</td>
<td>3-19m F</td>
<td>3-19m F</td>
<td>8-12m</td>
<td>3m, 8m F psoas</td>
<td>3, 8, 12m F</td>
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<tr>
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<td>M and F</td>
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<td>3m, 8m F psoas</td>
<td>3, 8, 12m F</td>
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<tr>
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<td>M and F</td>
<td>3-12m F</td>
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<td>3m, 8m F psoas</td>
<td>3m M</td>
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<td>3, 8</td>
<td>F</td>
<td>3, 8 F</td>
<td>8m F</td>
<td>8m F</td>
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*There is no wildtype control strain available for SJL/J mice.

M-Male; F- Female