Oxidative stress and pathology in muscular dystrophies: focus on protein thiol oxidation and dysferlinopathies

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The muscular dystrophies comprise more than 30 clinical disorders that are characterized by progressive skeletal muscle wasting and degeneration. Although the genetic basis for many of these disorders has been identified, the exact mechanism for pathogenesis generally remains unknown. It is considered that disturbed levels of reactive oxygen species (ROS) contribute to the pathology of many muscular dystrophies. Reactive oxygen species and oxidative stress may cause cellular damage by directly and irreversibly damaging macromolecules such as proteins, membrane lipids and DNA; another major cellular consequence of reactive oxygen species is the reversible modification of protein thiol side chains that may affect many aspects of molecular function. Irreversible oxidative damage of protein and lipids has been widely studied in Duchenne muscular dystrophy, and we have recently identified increased protein thiol oxidation in dystrophic muscles of the mdx mouse model for Duchenne muscular dystrophy. This review evaluates the role of elevated oxidative stress in Duchenne muscular dystrophy and other forms of muscular dystrophies, and presents new data that show significantly increased protein thiol oxidation and high levels of lipofuscin (a measure of cumulative oxidative damage) in dysferlin-deficient muscles of A/J mice at various ages. The significance of this elevated oxidative stress and high levels of reversible thiol oxidation, but minimal myofibre necrosis, is discussed in the context of the disease mechanism for dysferlinopathies, and compared with the situation for dystrophin-deficient mdx mice.

Introduction

Overview of oxidative stress

Reactive oxygen species (ROS) are formed during a variety of biological processes for all eukaryotes, and although they are essential for cell signaling, excess generation of ROS may harm biological components. This occurs when the action of endogenous defense mechanisms of the cell, involving various molecules called antioxidants, is outweighed by the generation of ROS, a state called oxidative stress [1]. Oxidative stress

Abbreviations
DMD, Duchenne muscular dystrophy; FLM, BODIPY FL–N-(2-aminoethyl) maleimide; NAC, N-acetylcysteine; ROS, reactive oxygen species; Texas Red, Texas Red C2-maleimide.
is implicated in the pathology of numerous conditions, including aging, inflammatory disorders, cancer, muscle wasting and muscular dystrophies [2–5]. ROS causes cellular damage by directly and irreversibly altering macromolecules such as proteins, membrane lipids and DNA [6], but another (less studied) major cellular consequence of ROS exposure is the reversible modification of protein thiol side chains. Thiols are organic sulfur derivatives, identified by the presence of sulfhydryl residues (–SH) at their active site. Biological thiols include low-molecular-weight free thiols and protein thiols, the functional group of the amino acid cysteine. In the presence of ROS, sulfhydryl residues may undergo reversible modifications, whereby sulfhydryl bonds are broken and disulfides are formed. These thiol modifications are reversible via the action of certain antioxidant molecules that reduce disulfides via thiol/disulfide exchange, including the enzymes thioredoxin and peroxiredoxin, as well as free cysteine and glutathione. Cysteine availability is a rate-limiting step in the synthesis of glutathione, which is a ubiquitously expressed tripeptide that is considered to be the most important cellular antioxidant molecule [7–9].

Oxidation of protein thiols may be crucial to the normal function of a specific protein, and may affect a vast variety of functions, including protein structure, protein–protein interactions, catalysis, electron transfer, ion channel modulation, phosphorylation-dependent signal transduction, post-translational protein modification and transcriptional activation [10,11]. In skeletal muscle, contractile function and force production and the development of fatigue, are directly influenced by the reduction/oxidation (redox) state of protein thiols of contractile proteins. Contractile (myofibrillar) proteins such as troponin, tropomyosin, myosin and actin contain thiol side chains that are sensitive to oxidation, and modifications may alter excitation/contraction coupling and cross-bridge cycling, and therefore modulate muscle contraction [12–24]. Excessive oxidative stress, which occurs in conditions such as chronic inflammation, during strenuous exercise and disease states, may cause muscle weakness [25], and is implicated in the pathology of numerous muscular diseases such as muscular dystrophies.

**Oxidative stress in muscular dystrophies**

The muscular dystrophies comprise more than 30 hereditary clinical disorders that are characterized by progressive skeletal muscle wasting and degeneration. They generally share common histological features, including variation in myofibre size, myofibre degeneration and regeneration, and the replacement of muscle with connective tissue and fat [26]. These conditions vary in many aspects, including prevalence, age of onset, severity, the muscles affected and the underlying gene defect [27]. These disorders are due to mutations in a wide variety of molecules, including extracellular matrix, sarcolemmal, cytoskeletal, cytosolic and nuclear membrane proteins [28,29]. Although the genetic basis of many of these disorders has been identified, the exact mechanism for pathogenesis remains unclear; however, there is evidence that interactions between the primary genetic defect and elevated levels of ROS contribute to the pathology of several muscular dystrophies [30]. Oxidative stress has been investigated extensively in Duchenne muscular dystrophy (DMD), which is discussed in more detail below, and is also clearly involved in other dystrophies and myopathies [30–35].

For example, oxidative stress is strongly implicated in the SEPN1-related myopathies that are due to deficiency in the protein selenoprotein N (SEPN1): these comprise four congenital skeletal muscle disorders that are characterized by severe weakness and wasting of neck and trunk muscles, leading to scoliosis and respiratory insufficiency [31]. Selenoproteins contain selenocysteine and include many proteins involved in the regulation of oxidative stress, including glutathione peroxidases and thioredoxin reductases; selenoprotein N has recently been identified as a key protein in cell protection against oxidative stress and redox-related calcium homeostasis [32]. Another disorder, facioscapulohumeral muscular dystrophy is an autosomal dominant muscle disease that is characterized by progressive weakness and atrophy of facial, shoulder girdle and upper-arm muscles. Its molecular pathogenesis is due to deletions that lead to an increase in function of the DUX4 (double homeobox 4) protein in muscles [33]. DUX4 is a transcription factor that initiates an extensive gene de-regulation cascade, and many genes that are differentially expressed in the muscles of facioscapulohumeral muscular dystrophy patients are involved in oxidative stress responses [33]. Altered levels of ROS and a higher susceptibility to oxidative stress are also a feature of laminopathies, which result from mutations in the LMNA gene, encoding A-type lamins, proteins that are associated with the nuclear membrane [34]. Elevated oxidative stress is also implicated in myopathies due to mutations in the ryanodine receptor RYR1, an essential component of the excitation/contraction coupling apparatus; these include several congenital RYR1-related myopathies that are the most common non-dystrophic muscle diseases of childhood [35].
In our laboratory, we have applied a wide range of quantitative measures to analyze oxidative stress in two forms of muscular dystrophy, using the mdx mouse model for DMD and the dysferlin-deficient A/J mouse model for dysferlinopathies. These two diseases are discussed in detail below.

**Oxidative stress in Duchenne muscular dystrophy**

Duchenne muscular dystrophy (DMD) is a lethal X-chromosome-linked skeletal muscle disease, manifested in children, that is caused by mutations in the dystrophin gene resulting in the absence or decreased function of the membrane-associated protein dystrophin [27,36]. Other mutations in the same gene result in a mildly defective dystrophin protein, with a less severe disease, usually with later onset, called Becker muscular dystrophy [27]. Skeletal and cardiac myofibres lacking functional dystrophin have an increased susceptibility to sarcolemma damage after muscle contraction, which leads to myofibre necrosis; this results in inflammation, myogenesis and new muscle formation to regenerate the tissue [37,38]. However, repeated cycles of damage and inflammation over months and years progress to replacement of muscle by fat and fibrous connective tissues, with severe loss of muscle function, resulting in premature death, often due to respiratory or cardiac failure [36,39]. It has been proposed that growth (as well as muscle size and mechanical loading) increases the severity of dystrophopathy, which is less pronounced in animal models of DMD, such as mdx mice and Golden Retriever dogs [40]. The pathology of DMD also appears to be exacerbated by oxidative stress: precisely why dystrophin deficiency leads to the generation of ROS in skeletal muscle is unclear, although probable reasons include interactions between excessive intracellular calcium and inflammation [5,30,41-44]. It is well documented that elevated cytosolic calcium concentrations increase mitochondrial calcium, which is an effector of ATP synthesis, and an increase in ATP synthesis increases production of ROS by mitochondria, via higher oxygen consumption and enhanced electron flow through the electron transport chain [45-47]. Membrane damage stimulates degranulation of resident mast cells [48] and also releases intracellular contents that activate the immune system of the host, further increasing the inflammatory cell cascade [49,50]. In addition, immune cells such as neutrophils and macrophages generate ROS in order to promote phagocytosis [51-53]. A role for oxidative stress in DMD is supported by a wealth of pre-clinical studies in mdx mice that report benefits such as improved muscle pathology and decreased necrosis for many antioxidant drugs and interventions, such as green tea extracts, resveratrol, coenzyme Q10 and catalase [54-61]. Many of these drugs may have broad-based effects *in vivo*, and the potential translational benefits of these for clinical treatment of DMD remain to be substantiated [62,63]. There is a notorious lack of success of antioxidants in clinical trials [64] that may be due in part to a lack of understanding of the precise nature of oxidative stress involved with the particular pathology [5].

While irreversible oxidative damage of protein and lipids, as measured by the carbonyl and malondiadehyde assays, respectively, has been widely studied and targeted by antioxidant treatment, there has been little information related to protein thiol oxidation in muscular dystrophies. However, this topic has been a focus of recent research in our laboratory. The development of a specific two-tag assay to measure protein thiol oxidation in skeletal muscle tissues has revealed significantly elevated levels of protein thiol oxidation, as well as elevated protein carbonylation, in dystrophic muscle of mdx mice [65-68]. In addition, treatment with the thiol-reducing antioxidant N-acetylcysteine (NAC) reduces the severity of dystrophopathy *in vivo*, as measured by decreased levels of plasma creatine kinase and reduced myonecrosis, and this study specifically demonstrated *in vivo* that NAC reduced the level of oxidized protein thiols in dystrophic muscles [67]. NAC has previously been shown to improve force production by mdx muscles [69] and the pathology of mdx hearts [70], and a recent *in vivo* study further confirmed the beneficial effects of NAC on creatine kinase levels and myonecrosis (using diaphragm muscles), as well as reduced levels of tumor necrosis factor in mdx mice [71]. These combined studies implicate protein thiol oxidation in the dystrophopathy of DMD, and support further evaluation of specific thiol antioxidant drugs for clinical translation.

**Oxidative stress in dysferlinopathies**

Another group of muscular dystrophies of interest, that have been far less well studied, are the dysferlinopathies. These encompass two disorders, limb-girdle muscular dystrophy type 2B and Miyoshi myopathy, which are both rare adult diseases, with weakness in either distal muscles (Miyoshi myopathy) or proximal limb-girdle muscles (limb-girdle muscular dystrophy type 2B) [72,73]. Limb-girdle muscular dystrophy type 2B and Miyoshi myopathy are considered to
result from allelic variations of the same dysferlin gene; however, the reason why mutations to this gene give rise to two different phenotypes is unknown [74]. The dysferlin gene encodes dysferlin, a membrane-associated protein that is involved in membrane vesicle trafficking and fusion, that is localized to transverse tubules and the periphery of myofibers and cardiomyocytes and, to a lesser extent, other tissues such as monocytes, brain and kidney [75–77]. Muscles lacking dysferlin may have problems in the membrane rescaling that follows mechanical injury of contraction [78], and an absence of dysferlin reportedly leads to intracellular calcium dysregulation through membrane modifications and cell signaling dysfunction [78–81]. Dysferlin deficiencies are also associated with excessive inflammation [49,82,83], and, although it has been hypothesized that this leads to an increased presence of ROS, very little experimental work on the subject has been published.

A case study in a single Miyoshi patient identified increased protein and lipid oxidation and protein thiol content in affected muscle, suggesting increased ROS levels in dysferlin-deficient muscle as well as protein thiol alterations [84]; increased levels of antioxidants, including glutathione and catalase, were also observed. A study investigating oxidative damage in DMD, dysferlinopathies and sarcoglycanopathy identified amplified lipid peroxidation and protein oxidation in all three human muscular dystrophies [85]. The study also showed dysregulation of glutathione-recycling antioxidants, such as glutathione reductase and peroxidase, suggesting perturbations in glutathione metabolism in the muscle of patients with these dystrophies. Other studies have used a combination of the antioxidants coenzyme Q10 and resveratrol in SJL/J dysferlin-deficient mice, and reported reduced inflammation and muscle pathology, although these results were not quantified [86,87]. Several strains of mice that lack dysferlin are available, and include SJL/J, A/J and Bla/J (A/J mice bred onto a C57BL/6 background), as well as genetically engineered null strains such as the B6.129–Dysf<sup>tm1Kcam</sup>/J and B10.SJL–Dysf<sup>tm1Awa1</sup>/J mice: all show a late onset but relatively mild pathology compared with the human condition (http://www.jain-foundation.org/our-dysferlin-research-institute/research-tools/mouse-models-dysferlin-deficiency/).

### Elevated protein thiol oxidation in dysferlin-deficient muscles of A/J mice

Here we present new data for protein thiol oxidation, and other measures of oxidative stress, along with severity of histopathology, in a range of muscles from dysferlin-deficient A/J (A/J<sup>Dysf<sup>–/–</sup></sub>) mice, a naturally occurring dysferlin-deficient strain of mice with a retrotranspon insertion in dysferlin intron 4 [88]. These data are compared to those for normal control A/J mice at various ages, and then critically compared with data for mdx mice. The A/J<sup>Dysf<sup>–/–</sup></sub> mice exhibit dystrophic changes, including necrosis and inflammation, that are histologically evident by 12 months of age in proximal muscles, whereas distal muscles appear relatively unaffected even in late stages of the disease [88]. The present study examined both distal and proximal muscles, including the psoas and quadriceps (severely affected) and gastrocnemius, biceps brachii muscles (mildly affected), at four ages: 3, 8, 12 and 19 months. The histology of muscles was assessed, and reversible protein thiol oxidation was quantified using a dual-labeling technique that indicates the percentage of oxidized to total (reduced and oxidized protein thiols) in muscles [66,67], and was further analyzed using a quantitative method that visualizes the localization of oxidized protein thiols on tissue sections. Other measures of oxidative stress included determination of irreversible oxidative damage to proteins using the carbonyl assay [89] and of cumulative damage by quantification of lipofuscin granules [90]. All of these measures were correlated with histopathology. Identifying the targets of oxidative stress that are affected in dysferlin-deficient muscle provides insight into the molecular basis for pathology and may also identify more specific drugs for possible therapeutic intervention.

### Results

#### Preliminary semi-quantification of necrosis, fat content and protein thiol oxidation in severely affected 19-month-old A/J<sup>Dysf<sup>–/–</sup></sub> mice, to select muscles most affected by pathology

A range of muscles from 19-month-old (severely affected) A/J<sup>Dysf<sup>–/–</sup></sub> mice were subjected to semi-quantitative histological analysis and measurement of total protein thiol oxidation. The histology results indicated that all muscles had a low–medium score for necrosis, apart from the biceps, which scored normal–low (Fig. 1A). For fat content (Fig. 1B), both the psoas and quadriceps muscles scored medium–high, whilst the gastrocnemius, biceps and deltoid scored normal–low. Quantification of total protein thiol oxidation of the muscles of 19-month-old mice (Fig. 2) showed that both the psoas and quadriceps muscles had significantly higher levels of protein thiol oxidation, while the gastrocnemius, biceps and deltoid had a low level of oxidation.
As all three parameters were high in psoas and quadriceps muscles, these muscles were considered the most affected, and were selected for quantification of histology and oxidative stress measures in further studies, together with a relatively unaffected muscle (either gastrocnemius or deltoid) as an additional control.

Quantification of areas of necrosis and fatty tissue in 3-, 8-, 12- and 19-month-old A/J\textsuperscript{dysf}/−/− mice

Quantification of myofibre necrosis (Figs 3A and 4) and fat content (Figs 3B and 4) for psoas, quadriceps and gastrocnemius muscles was performed in 3-, 8-, 12- and 19-month-old A/J\textsuperscript{dysf}/−/− mice. These data were compared with age-matched control A/J wild-type mice (except for 19 months old, as control mice of this age were not available); data are not shown for wild-type muscles as all values were very low compared with A/J\textsuperscript{dysf}/−/− mice, as were A/J\textsuperscript{dysf}/−/− gastrocnemius
values. Although necrosis was significantly elevated in the A/J<sup>dy</sup>−/− psoas and quadriceps muscles compared with normal controls, the extent of necrosis was low (<1% of the cross-sectional area), and there were no significant differences in necrosis between these A/J<sup>dy</sup>−/− muscles at any age.

In marked contrast, large areas of the muscles were occupied by fat (20–30% of the tissue), and this was especially prominent in the psoas muscle (Figs 3B and 4). Fat content was significantly higher in the psoas muscle compared with the quadriceps muscle at 8 and 12 months. Fat content was also markedly higher in both muscles at 8, 12 and 19 months compared to 3 months (Fig. 3B).

**Protein thiol oxidation in tissue samples and histological sections**

The percentage of total protein thiol oxidation in muscle tissue samples was quantified in the gastrocnemius (Fig. 5A), psoas (Fig. 5B) and quadriceps (Fig. 5C) muscles of 3-, 8-, 12- and 19-month-old A/J<sup>dy</sup>−/− mice and wild-type controls. At 3 months of age, there was no significant difference between any groups. At 8 months, protein thiol oxidation was significantly higher in the psoas muscle of A/J<sup>dy</sup>−/− compared with control mice, and in the A/J<sup>dy</sup>−/− psoas muscle compared with the gastrocnemius muscle. At 12 months, protein thiol oxidation was significantly higher in the gastrocnemius and quadriceps muscles of A/J<sup>dy</sup>−/− mice, compared with controls. At 19 months, protein thiol oxidation was significantly higher in A/J<sup>dy</sup>−/− psoas and quadriceps muscles compared to the gastrocnemius muscle. Protein thiol oxidation was also significantly higher in the psoas muscle of 19-month-old A/J<sup>dy</sup>−/− mice than in the psoas muscle at all other ages, and the level in the quadriceps muscle of 19-month-old A/J<sup>dy</sup>−/− mice was significantly higher than in the quadriceps muscles of 3-month-old A/J<sup>dy</sup>−/− mice.

We also calculated the amount of reduced, oxidized and total thiols in muscle tissue samples. For simplicity, only the level of reduced, oxidized and total thiols in the quadriceps muscle is presented (Table 1). Total thiols were significantly reduced in 19-month-old quadriceps muscles of A/J<sup>dy</sup>−/− mice compared to all other mice, as were reduced thiols. This was also the case for the psoas muscle of 19-month-old A/J<sup>dy</sup>−/− mice (data not shown). At 19 months of age, reduced thiols...
were significantly lower in the A/J<sup>dysf</sup>-/- psoas muscle compared to the A/J<sup>dysf</sup>-/- gastrocnemius muscle. Even at 8 months of age, the A/J<sup>dysf</sup>-/- mice had significantly lower levels of reduced thiols than mice at 3 months, while 12-month-old A/J<sup>dysf</sup>-/- mice had significantly more oxidized thiols than age-matched wild-type controls.

Protein thiol oxidation was also visualized and quantified on histological tissue sections of 12-month-old A/J<sup>dysf</sup>-/- and wild-type controls (Fig. 6). The total area of protein thiol oxidation was significantly higher in A/J<sup>dysf</sup>-/- tissue compared with control tissue, and was evident both in areas with extensive lipid presence and without (Fig. 6A), being mainly present throughout intact myofibres (Fig. 6B). This pattern of histological localisation was compared with mdx muscles, where pronounced protein thiol oxidation (Fig. 6B) occurs most dramatically in fragmented myofibres associated with areas of necrosis, and there are more oxidized thiols in intact myofibres compared to controls.

**Oxidative damage to proteins**

**Carbonyl content**

As the muscle most affected by oxidative stress (the psoas muscle) was too small to measure protein carbonyl content, the gastrocnemius (Fig. 7A) and quadriceps (Fig. 7B) muscles were analyzed in 3-, 8-, 12- and 19-month-old A/J<sup>dysf</sup>-/- mice and wild-type controls. There was no significant difference in either muscle for protein carbonylation between strains or ages, apart from the level in the quadriceps muscle of 19-month-old A/J<sup>dysf</sup>-/- mice, which was significantly higher than that in the quadriceps muscle of 12-month-old A/J<sup>dysf</sup>-/- mice, and also that in the quadriceps muscle of 3-month-old A/J<sup>dysf</sup>-/- and wild-type control mice.

**Lipofuscin content**

The presence of lipofuscin (also known as ceroid) indicates the extent to which a tissue has been exposed over time to irreversible oxidative stress [91], although decreased degradation through impaired autophagy may also increase the accumulation of lipofuscin [92]. Lipofuscin was evident in the psoas and quadriceps muscles of A/J<sup>dysf</sup>-/- mice as autofluorescent granules at all ages (Fig. 8A). The amount of lipofuscin was significantly higher in the psoas muscles of A/J<sup>dysf</sup>-/- mice at all ages (3, 8 and 12 months) compared with age-matched controls, and was also higher in the psoas muscle of 12-month-old A/J<sup>dysf</sup>-/- mice compared with 3-month-old A/J<sup>dysf</sup>-/- mice (Fig. 8A). Although there was a trend for increased lipofuscin content in
Table 1. Redox state of protein thiols in quadriceps muscle from A/J(dysf−/−) mice and wild-type controls. The asterisk indicates a significant difference from age-matched wild-type controls (P < 0.05). The ampersand (&) indicates a significant difference from 3-month-old A/J(dysf−/−) and wild-type mice (P < 0.05). The dollar symbol ($) indicates a significant difference from A/J(dysf−/−) and wild-type mice at all other ages (P < 0.05). Values are means ± SEM (n = 6).

<table>
<thead>
<tr>
<th>Age</th>
<th>Genotype</th>
<th>Reduced thiols (nmol-mg protein−1)</th>
<th>Oxidized thiols (nmol-mg protein−1)</th>
<th>Total thiols (nmol-mg protein−1)</th>
<th>Percentage oxidized (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 months</td>
<td>Wild-type</td>
<td>36.4 ± 1.8</td>
<td>4.6 ± 0.4</td>
<td>41.0 ± 2.1</td>
<td>8.9 ± 1.1</td>
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<td></td>
<td>A/J(dysf−/−)</td>
<td>38.3 ± 0.7</td>
<td>5.2 ± 0.1</td>
<td>38.2 ± 1.6</td>
<td>10.1 ± 1.1</td>
</tr>
<tr>
<td>8 months</td>
<td>Wild-type</td>
<td>31.7 ± 1.5</td>
<td>5.2 ± 0.5</td>
<td>38.1 ± 1.7</td>
<td>11.5 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>A/J(dysf−/−)</td>
<td>30.6 ± 2.1*</td>
<td>4.8 ± 0.4</td>
<td>35.4 ± 2.2</td>
<td>14.2 ± 1.4</td>
</tr>
<tr>
<td>12 months</td>
<td>Wild-type</td>
<td>36.7 ± 1.0</td>
<td>3.1 ± 0.3</td>
<td>39.8 ± 1.2</td>
<td>7.7 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>A/J(dysf−/−)</td>
<td>33.6 ± 1.7</td>
<td>4.5 ± 0.3*</td>
<td>36.9 ± 1.1</td>
<td>14.3 ± 1.6*</td>
</tr>
<tr>
<td>19 months</td>
<td>A/J(dysf−/−)</td>
<td>24.1 ± 0.9*</td>
<td>4.9 ± 0.3</td>
<td>29.1 ± 0.8*</td>
<td>17.6 ± 1.2*</td>
</tr>
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Discussion

Correlation between pathology and thiol oxidation in affected dysferlin-null muscles

While the level of myonecrosis was very low (< 1%) in dysferlin-deficient muscles at all ages, the replacement of muscle with fat was a very striking feature of the affected muscles (psoas and quadriceps) in A/J(dysf−/−) mice. In diseases such as DMD, the increased adiposity of muscle is a result of progressive replacement of myofibres in response to repeated cycles of myofibre necrosis [39,93], but this does not appear to be the explanation for dysferlin-deficient muscles, in which myofibre necrosis is relatively low: instead it must be due to another pathogenic mechanism. An increase in adiposity in skeletal muscle is associated with modifications to adipogenic genes and cell signaling pathways [94], and this may occur in dysferlin-deficient muscle as a direct cellular consequence of dysferlin deficiency, or indirectly as a secondary consequence of this gene defect. Further experimental work is required to understand the reasons for increased fat content in dysferlin-deficient muscles and why only some muscles are so severely affected.

Oxidative stress may lead to cellular dysfunction through permanent damage to proteins, lipids and DNA, as well as causing reversible modifications to thiol side chains on cysteine residues, leading to alterations in protein function [6]. The protein carbonyl assay is commonly used as an indicator of irreversible oxidative damage to proteins, and increased carbonyl levels have been reported in human muscle lacking dysferlin [84,85]. The fact that protein carbonylation was not significantly increased in the muscles of A/J(dysf−/−) mice (compared to age-matched controls) may reflect the relatively mild phenotype of this mouse model up to 12 months of age, as carbonyl content was significantly increased in the severely affected quadriceps muscles of 19-month-old A/J(dysf−/−) mice compared with younger A/J(dysf−/−) and control mice (Fig. 7).

A more striking result in A/J(dysf−/−) mice was the reversible modification of protein thiol side chains, with significantly elevated protein thiol oxidation in the most severely affected psoas and quadriceps muscles as early as 8 and 12 months, respectively (compared with controls), and a significant further increase by 19 months. This pattern of protein thiol oxidation correlates with disease manifestation in specific muscles and progression with age. Visualization of protein thiol oxidation in tissue sections indicated that affected dysferlin-deficient myofibres had high levels of protein thiol oxidation: this was not evident in the interstitial tissue and was not particularly pronounced in areas of severe pathology where fat was present.

Another measure of oxidative stress that was increased in both dystrophin- and dysferlin-deficient muscle is the accumulation of lipofuscin: lipofuscin levels are already significantly elevated in the psoas muscles of A/J(dysf−/−) mice at 3 months of age and increase further with age and disease progression (Fig. 8). This elevated lipofuscin level at the young age of 3 months (when no striking pathology is evident in A/J(dysf−/−) muscles) appears to be of particular importance as it indicates that there are already significant disturbances to oxidative stress levels and implies an early role for oxidative stress in subsequent disease manifestation.

Is the pattern of oxidative stress similar in DMD and dysferlinopathies?

There is clearly a very different pattern in the time of onset and the nature of pathology for DMD and dysferlinopathies, and it is of interest to compare the
parameters of oxidative stress for the mouse models of these diseases. Myofibre necrosis and oxidative stress measures (protein thiol oxidation, carbonyl content and lipofuscin accumulation) are compared in Table 2 for mdx and A/J<sup>dyf<sup>-/-</sup></sup> mice and their respective control strains (C57BL/10ScSn and A/J) at 3 months, with additional data at 12 months for A/J<sup>dyf<sup>-/-</sup></sup> mice as the disease was evident by this later age. At 3 months, active myofibre necrosis occupies approximately 6% of the muscle area for mdx mice, whereas necrosis is very low in the muscles of A/J<sup>dyf<sup>-/-</sup></sup> mice. This correlates with protein carbonyl oxidation, which is elevated in mdx mice but not in A/J<sup>dyf<sup>-/-</sup></sup> mice at this young age. Increased protein carbonyl content has been reported in both DMD and mdx muscle [65,85,95–97], but whether this is a cause or consequence of the myonecrosis (and associated inflammation) is unclear. Protein carbonylation is also evident in dysferlinopathies after disease manifestation in humans [84,85] and for very old A/J<sup>dyf<sup>-/-</sup></sup> mice at 19 months, with this late onset suggesting that this is more likely to be a consequence rather than a cause of the pathology.

Fig. 6. (A) Protein thiol oxidation on muscle sections from 12-month-old A/J<sup>dyf<sup>-/-</sup></sup> mice and wild-type controls, in areas with and without extensive fat. The asterisk indicates a significant difference from age-matched wild-type control (P < 0.05). Values are means ± SEM (n = 3). (B) Comparison of protein thiol oxidation on muscle tissue sections in dysferlin-null (A/J<sup>dyf<sup>-/-</sup></sup>) and dystrophin-null (mdx) mice, and wild-type controls (A/J and C57BL/10ScSn respectively). Red., reduced thiols; Ox., oxidized thiols. Protein thiol oxidation is confined to intracellular proteins of myofibres in tissue sections of skeletal muscles, and is increased in both A/J<sup>dyf<sup>-/-</sup></sup> and mdx muscle compared with controls.
At 3 months of age, the lipofuscin level was significantly increased (two to threefold) in both mdx and A/Jdysf/C0/C0 muscles, compared with controls; this was unexpected for the A/Jdysf/C0/C0 mice as there is little manifestation of the disease in young adult mice. Lipofuscin levels increase with age, and this was evident for both wild-type and A/Jdysf/C0/C0 mice at 12 months, with much higher levels (approximately twofold) still present in the muscles of A/Jdysf/C0/C0 mice. Elevated lipofuscin levels and accumulation with age have previously been reported for skeletal muscles from DMD patients and mdx mice [98].

Both mdx and A/Jdysf/C0/C0 muscle show an increase (approximately twofold) in protein thiol oxidation compared with controls. This is evident in mdx mice at 3 months (when the disease is already advanced), but not in A/Jdysf/C0/C0 mice at this age (before the onset of muscular dystrophy).

Table 2. Comparison between two forms of muscular dystrophy in terms of extent of necrosis, protein thiol oxidation, carbonyl and lipofuscin content in dyferlin-null (A/Jdysf/C0/C0) and dystrophin-null (mdx) mice, and wild-type controls (A/J and C57BL/10ScSn respectively). n/a, not applicable.

<table>
<thead>
<tr>
<th>Age</th>
<th>Genotype</th>
<th>Percentage necrosis</th>
<th>Percentage protein thiol oxidation</th>
<th>Carbonyl content (nmol/mg protein)</th>
<th>Percentage lipofuscin</th>
</tr>
</thead>
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<td>3 months</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>C57BL/10ScSn</td>
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<td>9.7</td>
<td>3.1</td>
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<tr>
<td></td>
<td>mdx</td>
<td>6.1</td>
<td>16.8</td>
<td>7.8</td>
<td>0.06</td>
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<td></td>
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<td>1.9</td>
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<td>12 months</td>
<td></td>
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<td></td>
</tr>
<tr>
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</table>
of pathology), although it is evident by 12 months in A/Jdyssf−/− mice when the pathology has already manifested. In the mdx muscles, pronounced protein thiol oxidation (Fig. 6B) occurs most dramatically in fragmented myofibres associated with areas of necrosis, and, like the muscles of A/Jdyssf−/− mice, there are also significantly more oxidized thiols inside intact myofibres (also evident as lower levels of reduced protein thiols in Fig. 6B). This suggests that skeletal muscle proteins are undergoing protein thiol oxidation in relatively ‘unaffected’ myofibres of the dystrophic muscles, potentially causing changes in protein function and thus contributing to the resulting pathology and altered muscle performance. There are a wide range of target proteins for thiol oxidation and modulation of function that include many contractile proteins, and we have recently identified significant changes in the thiol oxidation state of such proteins, specifically myosin and tropomyosin in the quadriceps muscle of 3-month-old mdx mice (J. Terrill, M. Grounds, P. Arthur, unpublished data).

The A/Jdyssf−/− mouse is a useful model to study dysferlinopathies, with a strong correlation between the onset and severity of pathology in specific muscles and the incidence of various measures of oxidative stress. The early elevation of protein thiol oxidation in affected muscles (compared with the late increases in carbonylation) suggests a potentially important role for such reversible oxidation of key muscle proteins in the manifestation of pathology in dysferlinopathies, and presents the opportunity to assess the effects of specific thiol-reducing antioxidants such as NAC in the A/Jdyssf−/− mice. In mdx mice, NAC has various benefits and reduces the severity of pathology [67,69,71], providing further evidence for the probable important role of protein thiol oxidation in the pathology of DMD. It is also of interest to identify the specific proteins that are affected by thiol oxidation in the muscles of A/Jdyssf−/− mice to determine their potential role in the mechanism of the disease. The fact that lipoxygenase is already significantly elevated by 3 months of age in the muscles of A/Jdyssf−/− mice, before the disease is obvious, indicates that this is a particularly sensitive measure and strongly supports an early and key role for altered oxidative stress prior to disease manifestation. Further investigations into such aspects of oxidative stress in dysferlinopathies (as have already been initiated for the mdx mouse and DMD) appear warranted.

**Experimental procedures**

All reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified.

**Animal procedures**

A/J control mice were obtained from the Animal Resources Centre (Murdoch, Western Australia). A/Jdyssf−/− mice were obtained from the Institute for Neuroscience and Muscle Research, Children’s Hospital at Westmead, (Sydney, Australia). Mice were transported to the University of Western Australia and maintained on a 12 h light/dark cycle under standard conditions, with free access to food and drinking water. All animal experiments were performed in strict accordance with the guidelines of the National Health and Medical Research Council of Australia code of practice for the care and use of animals for scientific purposes (2004), and the Animal Welfare Act (2002), and were approved by the Animal Ethics Committee at the University of Western Australia.

**Tissue collection, histology and image acquisition**

All mice were killed by complete cervical dislocation while under terminal anesthesia (2% v/v Attane™sflurane, Bomac, Hornsby, NSW, Australia). Muscles were collected from wild-type control mice at 3, 8 and 12 months of age, and from A/Jdyssf−/− mice at 3, 8, 12 and 19 months (19-month-old wild-type controls were omitted as these were not available for analysis), and immediately snap-frozen in liquid nitrogen for biochemical analysis. For histology, one whole upper limb and one lower limb were immersed in 4% paraformaldehyde and fixed for 1 week; samples were then processed for paraffin histology. Transverse muscle sections (5 μm) were cut through the mid-region of each muscle on a Leica microtome, as previously described [99], and sections were stained with haematoxylin and eosin for morphological analysis.

Groups of three control and three A/Jdyssf−/− mice at three ages (3, 8 and 12 months old) were sampled, and muscles were obtained for frozen histology for the analysis of lipofuscin and protein thiol oxidation. The fresh muscles were bisected transversely and longitudinally and mounted on cork squares using tragacanth gum. The muscles were frozen in a slurry of isopentane cooled in liquid nitrogen. Cryostat sections (8 μm) were cut directly onto silinised glass slides, and stored at −20 °C until stained or analyzed.

**Histological image analysis**

Myofibre necrosis was identified as areas of myofibres with fragmented sarcoplasm and/or increased inflammatory cell infiltration. Fat content was identified as areas of many large circular cells unstained by haematoxylin and eosin. Both were assessed semi-quantitatively in all skeletal muscles (psoas, biceps brachii, quadriceps, gastrocnemius, deltoid) from 8-, 12- and 19-month-old A/Jdyssf−/− mice. In brief,
skeletal muscles were prepared for paraffin histology, stained with haematoxylin and eosin [67,68,99], and all muscles (n = 6 per group) were examined by light microscopy at 10× magnification. For the preliminary semi-quantitative analysis, each parameter was given a score of 0–4; where 0 = normal, 1 = < 5%, 2 = 5–10%, 3 = 10–15%, 4 = > 15%. These scores for six samples were averaged to provide an overall score. After the semi-quantitative analysis it was revealed that psaos and quadriceps muscles were the most affected, these muscles were quantitatively assessed for necrosis and fat content using non-overlapping tiled images of transverse muscle sections that provided a picture of the entire muscle cross-section. Digital images were acquired using a Leica Microsystems (Wetzlar, Germany) DM RBE microscope, a Hitachi (Tokyo, Japan) HVC20M digital camera, IMAGE PRO PLUS 4.5.1 software (Media Cybernetics, Rockville, MD, USA) and VEXTA STAGE MOVEMENT software (Oriental Motor Co, Tokyo, Japan). Tiled images were taken at 10× magnification. Muscle morphology was drawn manually by the researcher using IMAGE PRO PLUS 4.5.1 software. The area occupied by necrotic myofibres (i.e. myofibres with fragmented sarcoplasm and/or areas of inflammatory cells) or fat was measured as a percentage of the whole muscle section area. Histological analysis was completed according to the TREAT-NMD recommended standard protocol “Histological Measurements of Dystrophic Muscle – M.1.2_007” (http://www.treat-nmd.eu/research/preclinical/dmd-sops/).

**Carboxylated protein**

Oxidative damage to proteins in muscles was determined by measuring the carbonyl content using 2,4-dinitrophenylhydrazine as previously described [65,100,101]. Frozen muscles were crushed under liquid nitrogen, and protein was extracted using 20% trichloroacetic acid/acetone. The protein pellets were washed in acetone and ethanol, precipitated, dried, re-suspended in 10 mM 2,4-dinitrophenylhydrazine in 2 mM HCl, and incubated for 30 min at room temperature in the dark. Proteins were washed with ethyl acetate/ethanol (1:1) for one hour at room temperature, dissolved in 6 mM guanidine, and absorbance was measured at 370 nm. Protein concentration (mg·mL⁻¹) was determined using the Bio–Rad (Hercules, CA, USA) Bradford protein assay. Carbonyl concentrations are expressed as nmol carbonyl per mg protein.

**Lipofuscin quantification in muscle**

Lipofuscin is composed of autofluorescent granules that accumulate in tissue and are generated as a consequence of irreversible oxidative stress [91]. The granules are visible on unstained frozen tissue sections using fluorescent microscopy. The amount of lipofuscin in frozen muscle sections was measured by the non-subjective boot strap-ping method [90]. Images were captured using a fluorescent Nikon (Tokyo, Japan) Eclipse Ti microscope equipped with a Roper Industries (Sarasota, FL, USA) CoolSNAP-HQ2 camera, a 450–490 nm excitation filter, a 515 nm emission barrier filter, and Nikon NIS-Elements software. A 40× magnification was used, and sections were scanned using an automatic stage control setting that generated a grid structure of images in a set area. For each section scanned by fluorescent microscopy, eight images were selected for lipofuscin analysis: images with tissue edges or obvious artefacts were discarded. Quantification of lipofuscin granules was performed using IMAGEJ version 1.44 (http://imagej.nih.gov/ij/download/), and the green channel of an eight-bit RBG image was analyzed. The area occupied by fluorescent granules was expressed as a percentage of the total image area.

**Protein thiol oxidation**

Reduced and oxidized protein thiol levels were measured using a dual-labeling technique [66–68]. In brief, snap-frozen quadriceps muscle was crushed under liquid nitrogen, and protein was extracted using 20% trichloroacetic acid/acetone. Protein was solubilized in 0.5% SDS/0.5 M Tris at pH 7.3 (SDS buffer), and protein thiols were labeled with the first tag, the fluorescent dye BODIPY FL-N-(2-aminoethyl) maleimide (FLM) (Invitrogen, Mulgrave, VIC, Australia). After removal of the unbound dye using ethanol, protein was re-solubilized in SDS buffer, pH 7, and oxidized thiols were reduced using Tris(2-carboxyethyl)phosphine, before labeling of the resultant unlabeled reduced thiols with a second tag, the fluorescent dye Texas Red C2-maleimide (Texas Red) (Invitrogen). The sample was washed in ethanol and resuspended in SDS buffer. Samples were read using a fluorescent plate reader (Fluostar Optima, BMG Labtech, Otenberg, Germany) with wavelengths of 485 nm for excitation and 520 nm for emission for FLM, and 595 nm for excitation and 610 nm for emission for Texas Red. A standard curve for each dye was generated using ovalbumin, and the results are expressed per mg of protein, quantified using a Detergent Compatible Protein Assay (Bio–Rad).

**Protein thiol oxidation on tissue sections**

Reduced and oxidized protein thiols on frozen tissue sections were measured using an adaptation of the dual-labeling technique described above (T. Iwasaki, J. Terrill, M. Grounds and P. Arthur, unpublished). Serial muscle sections (9 µm) used for detecting reduced thiols were treated immediately in FLM. After washing in NaCl/Pₐ, sections were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer, and immersed in NaCl/Pₐ overnight. For detection of oxidized thiols, frozen sections from each muscle were treated with N-ethylmaleimide to block free thiols. After washing in NaCl/Pₐ to remove unreacted
N-ethylmaleimide, sections were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer. The fixed sections were washed again with NaCl/Pi. Oxidized thiols were reduced with Tris(2-carboxyethyl)phosphine; this was omitted in negative control sections. The reduced thiols were washed twice in NaCl/Pi to remove Tris(2-carboxyethyl)phosphine before labeling with FLM. All sections were mounted with polyvinyl acetate mounting medium for microscopy. Another serial section from each muscle was stained with haematoxylin and eosin for morphometric observation. Fluorescence images were acquired as per lipofuscin quantification. Sections were scanned using an automatic stage control setting that generated a grid structure of images covering a set area. The level of oxidized thiols in muscle sections was estimated by image analysis using IMAGEJ version 1.44. For each section scanned by fluorescence microscopy, three images were selected for all image analysis: images with tissue edges or obvious artefacts were discarded. The selected fluorescence images were used for quantification of the mean fluorescence intensity (arbitrary units) of the section.

Statistics

Significant differences between groups were determined using one-way ANOVA with post hoc tests, and all data are presented as means ± standard error of the mean. Significance was set at $P < 0.05$.

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Protein thiol oxidation in dysferlinopathies

J. R. Terrill et al.


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Protein thiol oxidation in dysferlinopathies


