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SYMPOSIUM REVIEW

Exacerbation of pathology by oxidative stress in respiratory and locomotor muscles with Duchenne muscular dystrophy

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Abstract Duchenne muscular dystrophy (DMD) is the most devastating type of muscular dystrophy, leading to progressive weakness of respiratory (e.g. diaphragm) and locomotor muscles (e.g. gastrocnemius). DMD is caused by X-linked defects in the gene that encodes for dystrophin, a key scaffolding protein of the dystroglycan complex (DCG) within the sarcolemmal cytoskeleton. As a result of a compromised dystroglycan complex, mechanical integrity is impaired and important signalling proteins (e.g. nNOS, caveolin-3) and pathways are disrupted. Disruption of the dystroglycan complex leads to high susceptibility to injury with repeated, eccentric contractions as well as inflammation, resulting in significant damage and necrosis. Chronic damage and repair cycling leads to fibrosis and weakness. While the link between inflammation with damage and weakness in the DMD diaphragm is unresolved, elevated oxidative stress may contribute to damage, weakness and possibly fibrosis. While utilization of non-specific antioxidant interventions has yielded inconsistent results, recent data suggest that NAD(P)H oxidase could play a pivotal role in elevating oxidative stress via integrated changes in caveolin-3 and stretch-activated channels (SACs). Oxidative stress may act as an amplifier, exacerbating disruption of the dystroglycan complex, upregulation of the inflammatory transcription factor NF-κB, and thus functional impairment of force-generating capacity.

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Abbreviations DGC, dystroglycan complex; DMD, Duchenne muscular dystrophy; ECM, extracellular matrix; MMP, matrix metalloproteinase; ROS, reactive oxygen species; SAC, stretch-activated channel; TGF, transforming growth factor; TNF, tumour necrosis factor; TRPC1, transient receptor potential canonical 1.

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Pathophysiological significance

The subsarcolemmal cytoskeleton ensures mechanical resilience, includes proteins that serve as sensors of mechanical stress and strain, regulates satellite cell activation, and governs protein turnover (Rando, 2001). The initial triggers for dozens of muscular dystrophies stem from a mutation of a gene encoding for proteins normally located in the subsarcolemmal cytoskeleton (e.g. dystrophin, ß-dystroglycan, caveolin-3), including the dystroglycan complex (DGC) (Rando, 2001; Tidball & Wehling-Henricks, 2007). Duchenne muscular dystrophy (DMD) is the most common and devastating type of muscular dystrophy with an incidence of 1 in every 3500 males, as a result in a mutation of the dystrophin gene (Nakamura & Takeda, 2011; Spencer & Tidball, 2001). Symptoms of Duchenne muscular dystrophy usually appear between 3 years of age, and by the age of 12 patients are typically no longer able to breathe or walk unassisted (Escolar & Scacheri, 2001). Muscle wasting and weakness are especially critical for muscles of locomotion and respiratory muscles (e.g. diaphragm), with fast-twitch fibres being particularly susceptible to myopathy with DMD (Tkatchenko *et al.* 2000). Decline of diaphragm muscle function is directly related to the clinical need for mechanical ventilation in more advanced stages of DMD, and respiratory muscle failure remains a leading cause of death before the age of 30 (Escolar & Scacheri, 2001). The *mdx* mouse model, which also has a mutation of the dystrophin gene, is a common analogue for human Duchenne muscular dystrophy. The diaphragm muscle in the *mdx* mouse is highly susceptible to oxidative stress and experiences a disease progression similar to human DMD (Tkatchenko *et al.* 2000; Hartel *et al.* 2001).

The etiology of Duchenne muscular dystrophy is characterized by progressive damage, inflammation, fibrosis, and weakness of respiratory and limb skeletal muscles. Muscle damage and pathology with DMD are proposed to result from (a) high susceptibility to material fatigue injury and (b) chronic inflammation (Tidball & Wehling-Henricks, 2007). Material fatigue injuries occur in biological tissues, including skeletal muscle with repeated mechanical strain. Repeated lengthening or eccentric contractions of sufficient load and frequency induce a multifocal material fatigue injury in skeletal muscle (Warren *et al.* 1993). The load magnitude is often moderate, much lower than a maximal contraction, and material fatigue is believed to cause the initial damage phase associated with delayed-onset muscle soreness (Warren *et al.* 1993). Susceptibility to repetitive, eccentric-contraction damage and thus material fatigue injury is enhanced with DMD (Rousseau *et al.* 2010). Thus, a lower load magnitude in DMD muscles is required to induce damage with repeated eccentric contractions, possibly related to an impaired ability to transfer transverse or lateral forces (Ramaswamy *et al.* 2011).

Anti-inflammatory, corticosteroid drugs reduce oxidative stress, damage, apoptosis and disease progression with DMD (Lim *et al.* 2004), but have numerous side effects that limit their long-term use. While the link between inflammation with muscle damage and weakness in Duchenne muscular dystrophy is unresolved, elevated 'oxidative stress' has been proposed as a contributing mechanism (Tidball & Wehling-Henricks, 2007). Muscle damage, wasting and weakness have been noted with deficiencies in vitamin E and the Cu–Zn isoform of superoxide dismutase (Binder *et al.* 1965; Muller *et al.* 2006). Reduction in oxidative stress indeed correlates with slowed muscle wasting and relief of clinical symptoms with DMD, including respiratory muscle distress (Anderson *et al.* 2000; Bonafati *et al.* 2000; Carter & McDonald 2000). However, cause and effect were not established and the mechanisms by which glucocorticoids provide relief in DMD models remain uncertain. Perhaps oxidative stress may be upstream of nuclear factor-kappaB (NF-κB) and pro-inflammatory targets.

Oxidative stress and upregulation of the inflammatory transcription factor NF-κB are believed to contribute to myopathy during disuse, cachexia, chronic heart failure, chronic obstructive pulmonary disease, AIDS and cancer (Buck & Chojkier 1996; Adams *et al.* 1999; Dalla Libera *et al.* 2001; Lawler *et al.* 2003; Abrogast *et al.* 2007; Powers *et al.* 2007). While oxidative stress is elevated and integrated with inflammatory cell (macrophages, T-cells), cause and effect have remained uncertain with Duchenne muscular dystrophy (Tidball & Wehling-Henricks, 2007). Unfortunately, our understanding of the importance of redox signalling in DMD pathology has been partially obscured by the use of non-specific scavenger approaches and scientific designs that have yielded inconsistent results. For example, green tea extract (Buetler *et al.* 2002), purified epigallocatechin-3-gallate from green tea (Nakae, 2008), and low-iron diet (Bomman *et al.* 1998) reduced oxidative stress and muscle damage with DMD. In contrast, vitamin E, vitamin C and superoxide dismutase treatments have resulted in no clinical improvements (Walton & Nattrass, 1954; Roelofs *et al.* 1979; Fenichel *et al.* 1988; Stern *et al.* 1998). Inconsistency of non-specific antioxidant interventions in ameliorating Duchenne muscular dystrophy may lie within the nature of redox microenvironments of the sarcolemma (Fisher, 2009; Ushio-Fukai, 2009). For example, the reactive oxygen species (ROS)-generating NAD(P)H oxidase complex (Nox2) is elevated in the sarcolemma with both Duchenne muscular dystrophy and disuse (Williams & Allen, 2007).

Recent publications suggest that oxidative stress may play a more important role than previously understood. The antioxidant *N*-acetylcysteine was found to protect against extensor digitorum longus muscle (EDL) muscle damage, internal nuclei and weakness in locomotor muscles of *mdx* mice (Whitehead *et al.* 2008). NAD(P)H

oxidase is upregulated in the *mdx* mouse, and thus a potential contributor to oxidative stress and DMD pathology (Williams & Allen, 2007; Spurney *et al.* 2008; Whitehead *et al.* 2008). Inhibition of NAD(P)H oxidase protected against delayed muscle development and apoptosis (Whitehead *et al.* 2008). It is possible that Nox2 may hyper-respond to stretch in DMD muscles, possibly as a result of upregulation of stretch-activated Ca^{2+} channels (SACs), including transient receptor potential channel 1 (TRPC1) and caveolin-3 (Whitehead *et al.* 2006; Gervásio *et al.* 2008). Indeed, new evidence indicates that Nox2 is activated by stretch and contributes to muscle damage in the tibialis anterior of *mdx* mice (Whitehead *et al.* 2010). Downstream of oxidative stress, NF-κB may exacerbate DGC disruption and muscle damage through matrix metalloproteinase-9 (MMP-9) (Li *et al.* 2009). A model is proposed that connects ROS to exacerbation of pathology of DMD in diaphragm and limb muscles via NAD(P)H oxidase activation of caveolin-3 and NF-κB (Fig. 1).

The subsarcolemmal cytoskeleton

Skeletal muscle is a highly specialized and adaptive mesodermic tissue capable of rapid remodelling in response to changes in loading and stretch (i.e. mechanotransduction) (Smith *et al.* 2002). The subsarcolemmal cytoskeleton ensures mechanical integrity of skeletal muscle and initiates cell signalling in response to alterations in mechanical stress (i.e. mechanotransduction) (Rando, 2001). The subsarcolemmal cytoskeleton is anchored to the sarcolemma where it (a) transfers forces between the muscle cells and extracellular matrix (ECM) proteins, and (b) initiates cell signalling responses to mechanical strain, endocrine influences and the chemical environment (Disatnik & Rando, 1999). The cytoskeleton forms two aggregates in skeletal muscle secured to extracellular proteins such as laminin: (a) the dystroglycan complex and (b) the focal adhesion complex (Yang *et al.* 1995; Rando, 2001). The dystroglycan complex includes α-,ß-dystroglycan, α-,ß-,γ-,∂-sarcoglycan subunits, biglycan, the scaffolding proteins dystrophin and dystrobrevin, utrophin, syncoilin, Grb2, α-,ß-syntrophin and neuronal nitric oxide synthase (nNOS), and is associated with caveolin-3. The dystroglycan complex plays a major role in linking the actin cytoskeleton to the extracellular matrix, stabilizing the myocyte during contraction and relaxation, and transmitting force generated in the muscle sarcomeres to the ECM. DGC proteins regulate cell signalling involved in mechanical strain, protein turnover, growth, blood flow and adhesion proteins, including satellite cell activation and protein turnover (Rando, 2001; Kosek & Bamman, 2008).

The dystrophin protein is a large (427 kD), rod-like scaffolding protein co-localized with α-syntrophin, nNOS and calmodulin in the DGC (Jarrett & Foster 1999; Rando, 2001; Tidball & Wehling-Henricks, 2007; Kosek & Bamman, 2008). Dystrophin appears be important in cytoskeleton-dependent stiffness and the elastic properties of skeletal muscle (Puttini *et al.* 2009). The μ-splice variant of nNOS is a key signalling protein integral to the DGC (Percival *et al.* 2008). nNOS is anchored to the sarcolemmal cytoskeleton by binding of a PDX motif at its N-terminus to α- or ß-syntrophin (Rando 2001; Compton *et al.* 2005; Fanin *et al.* 2009). Both α- and ß-syntrophin bind nNOS, which in turn attachs to dystrophin and dystrobrevin (Kameya *et al.* 1999; Rando, 2001; Jones *et al.* 2003; Compton *et al.* 2005). Caveolin-3 is a small muscle-specific sarcolemmal protein localized in caveolae, invaginations central to growth and insulin signalling (Fecchi *et al.* 2006). Caveolin-3 also binds to dystroglycan complex proteins including ß-dystroglycan and nNOS, possibly regulating DGC assembly (Venema *et al.* 1997; Galbiati *et al.* 2001; Allen *et al.* 2010). Caveolin-3 inhibits nNOS via distinct scaffolding domains (Venema *et al.* 1997; Sunada *et al.* 2001; Whitehead *et al.* 2008), and also binds to transient receptor potential channel-1 (TRPC1), a stretch-activated Ca^{2+} channel (Gervásio *et al.* 2008). Either upregulation of caveolin-3 or suppression via genetic ablation exacerbates Duchenne muscular dystrophy or causes limb-girdle muscular dystrophy (LGMD-1C) (Galbiati *et al.* 2001).

Etiology of Duchenne muscular dystophy

Disturbances in the dystrglycan complex, including loss of dystrophin and attached proteins (e.g. ß-dystroglycan,

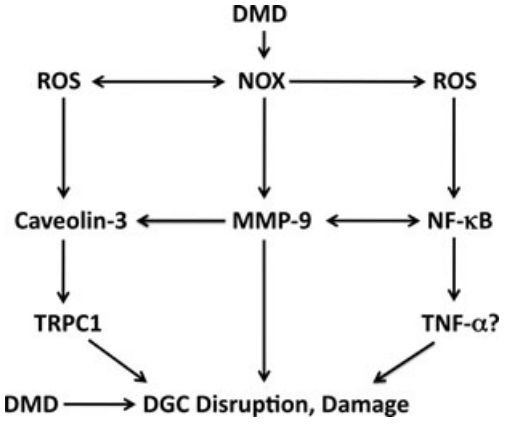

Figure 1. Redox signalling model for Duchenne muscular dystrophy (DMD), proposing oxidative stress as an amplifier of dystroglycan complex (DGC) disruption, muscle damage and pathology
In this model, NAD(P)H oxidase and NOX contribute to upregulation of caveolin-3 and transient receptor potential channel 1 (TRPC1), as well as activation of nuclear factor kappaB (NF-κB). NF-kB may contribute to damage via tumour-necrosis factor-alpha (TNF-α). ROS, reactive oxygen species.

α-syntrophin, nNOS), reduce the mechanical integrity of skeletal muscle eliciting a lower threshold to injuries with repeated stretch or eccentric contractions (Warren *et al.* 1993; Childers *et al.* 2002). DMD compromises the mechanical resilience of the cytoskeleton and cellular membrane to repeated mechanical strain resulting in material fatigue injury to muscles (Warren *et al.* 1993; Rando, 2001). Thus, muscles in DMD patients and animal models display a lower threshold for injury with repeated stretch or eccentric contractions (Childers *et al.* 2002; Rousseau *et al.* 2010). Importantly, Ramaswamy *et al.* (2011) recently published exciting new data that suggested that the dystroglycan complex is the weak link in transfer of lateral or transverse forces from the contractile apparatus with Duchenne muscular dystrophy, in a manner shared with the ageing process.

Loss of nNOS from the sarcolemma appears central to damage and myopathy with DMD as well (Tidball & Wehling-Henricks, 2004; Kim, 2009). Canonical thought is that damage leads to necrosis, apoptosis and weakness in Duchenne muscular dystrophy (Rando, 2001; Tidball & Wehling-Henricks, 2007). Apoptosis and necrosis may lead to loss of satellite cells, myonuclei and muscle fibres with a profound effect on muscle weakness, wasting, impaired muscle regeneration and etiology of the disease (Sandri *et al.* 2001; Mikhailov *et al.* 2002; Sandri & Carraro, 2002). An alternative hypothesis suggests that disruption of the dystroglycan complex elevates stretch-activated channels such as TRPC1, and elicits a Ca^{2+}-driven cascade (Allen *et al.* 2010). In addition, caveolin-3 may exacerbate loss of DGC proteins and disease pathogensis, possibly via TRPC1-regulated Ca^{2+} release (Gervásio *et al.* 2008).

Recent evidence suggests that matrix metalloproteinase-9 (MMP-9) cleaves ß-dystroglycan and exacerbates loss of DGC proteins from the sarcolemma, enhancing muscle damage with DMD (Li *et al.* 2009). Resultant muscle damage invites infiltration of inflammatory cells, primarily macrophages and T-cells, and initiates necrosis and apoptosis (Spencer & Tidball, 2001). Necrosis and apoptosis may remove satellite cells, myonuclei and muscle fibres, with a profound effect on muscle contractility and regeneration (Sandri *et al.* 2001; Mikhailov *et al.* 2002; Sandri & Carraro, 2002). Thus, alterations in the DCG also may impair regeneration and healing (Tidball & Wehling-Henricks, 2007). With repeated chronic cycling of damage and repair, fibrotic tissue fills in lost sarcomeres and muscle fibres over time (Gosselin *et al.* 1994; Gosselin & Martinez, 2004). As damage–repair cycling continues, a catenation of Wg (wingless), from wing development in Drosophila and Int, a homologous breast tumor gene (wnt) signalling and satellite cell function are impaired and satellite cells display shortened telomeres, and may differentiate to adipocytes, fibroblasts or cease to divide (Alexakis *et al.* 2007; Lund *et al.* 2007; Pescatori *et al.* 2007). Differentiation and reduction of satellite cell activation may exacerbate weakening and wasting of muscle fibres, further impairing function and thus respiratory muscle failure.

DMD and inflammation

Increasing evidence indicates inflammatory processes are highly integrated into muscle wasting with DMD (Spencer & Tidball, 2001). Autoreactive immune cells including T-cells and macrophages invade skeletal muscle in DMD (Spencer & Tidball, 2001). Indeed, $CD4^+$ and $CD8^+$ T-cell depletion decreases histopathology of *mdx* mouse muscle (Spencer & Tidball, 2000). Circulating inflammatory cytokines may be a 1000 times higher in DMD human patients than healthy controls (Watanabe, 2001). Stretch, damage, cytokines, inflammation and oxidative stress activate the transcription factor NF-κB through the phosphorylation and release of the inhibitor protein I-κB (inhibitory kappaB) (Gius *et al.* 1999). Indeed, the inflammatory cytokines tumour necrosis factor-α (TNF-α), transforming growth factor-beta (TGF-ß) and interleukin 1ß as well as the inflammatory transcription factor NF-κB are significantly elevated with DMD and in the *mdx* mouse (Kumar & Boriek, 2003; Monici *et al.* 2003). NF-κB translocates to the nucleus where it binds with DNA (i.e. activation), and then amplifies release of ROS and pro-inflammatory proteins and peptides (Crépieux *et al.* 1997; Kumar *et al.* 2004). Indeed, Monici *et al.* (2003) and Boriek and colleagues (Kumar & Boriek, 2003, Kumar *et al.* 2004) reported that NF-κB activity was higher in limb muscle and the diaphragm of *mdx* mice compared with wild-types. As a consequence, interventions that reduce NF-κB in DMD models significantly reduce damage and pathology (Nakae *et al.* 2001; Lim *et al.* 2004; Messina *et al.* 2006; Peterson *et al.* 2011). While the mechanisms remain unclear, NF-κB is linked with necrosis, activation of ubiquitin ligases and muscle wasting (Messina *et al.* 2006; Senf *et al.* 2008).

Cyclic damage and inflammation can also promote fibrosis or accumulation of connective tissue proteins such as collagen (Gosselin & Martinez, 2004). Collagen accumulation with repeated damage and repair is exacerbated by inflammatory cytokines (e.g. TNF-α, TGF-ß) and oxidative stress (Gosselin & Martinez, 2004). Collagen turnover is regulated by MMPs and by upstream tissue inhibitors of MMPs (TIMPs) (Kassiri & Khokha, 2005).

Anti-inflammatory and immunosuppressant drugs such as glucocorticoids (e.g. prednisone and deflazacort) consistently reduce skeletal muscle weakness, damage and progression of DMD in patients (Anderson *et al.* 2000; Bonafati *et al.* 2000; Carter & McDonald 2000; Sussman 2002). Immunosuppressants such as cyclosporine A

reduce muscle damage in *mdx* mice (De Luca *et al.* 2005). Corticosteroids also reduce oxidative stress (Tarnopolsky *et al.* 2004). Unfortunately, steroidal anti-inflammatories cause serious side-effects including impaired growth and maturation, weight gain, osteopaenia, immunosuppression and susceptibility to infection (Carter & McDonald 2000; Skrabek & Anderson, 2001).

Oxidative stress and DMD

Sources of oxidative stress in respiratory and locomotor muscles with DMD are thought to include infiltration of inflammatory cells (e.g. myeloperoxidase), NAD(P)H oxidase, mitochondria and decoupling of inducible nitric oxide synthase (iNOS) (Adams *et al.* 1999; Williams & Allen 2007); Spurney *et al.* 2008; Tidball & Wehling-Henricks, 2007; Whitehead *et al.* 2008. Furthermore, oxidative stress may also be exacerbated in muscle wasting disease by insufficient stress response including heat shock proteins and insulin-like growth factor (Bouchentouf *et al.* 2004; Lawler *et al.* 2006; Senf *et al.* 2008). Elevated oxidative stress may promote inflammatory cell invasion, exacerbate damage and interfere with cell signalling that can promote repair (Tidball & Wehling-Henricks, 2007). Markers of oxidative stress and lipid peroxidation are elevated with DMD (Grinio *et al.* 1984; Tidball & Wehling-Henricks, 2007). Muscles in Duchenne muscular dystrophy patients may also be more susceptible to oxidative stress. For example, Rando *et al.* (1998) showed that mdx myotubes are killed more easily by oxidants. He proposed a 'two-hit' hypothesis where the combination of oxidative stress plus disturbances in the dystroglycan complex lead to pathology with DMD.

Downregulation and dislocation of nNOS from the dystroglycan complex may also elevate oxidative stress and lead to muscle damage (Wehling *et al.* 2001; Nguyen & Tidball, 2003; Shiao *et al.* 2004; Tidball & Wehling-Henricks, 2004). nNOS in skeletal muscle appears to be attached to dystroglycan complex scaffolding proteins dystrophin and dystrobrevin via α- and ß-syntrophin (Rando, 2001). Dislocation of nNOS from the dystroglycan complex could (a) increase NAD(P)H oxidase activity, (b) increase inflammation, (c) increase protein degradation via activation of ubiquitin ligases, (d) impair satellite cell activation, and (e) increase the risk of material fatigue injury (Nguyen & Tidball, 2003; Tidball & Wehling-Henricks, 2004; Tidball & Wehling-Henricks, 2007; Suzuki *et al.* 2008). Further, impairment of mitochondrial function with DMD can also lead to oxidative stress, apoptosis and necrosis (Bernardi, 1999).

The Nox2 isoform of NAD(P)H oxidase contains membrane-bound gp91phox, p22phox and the cytosolic subunits p47phox and p67phox (Nguyen & Tidball, 2003; Whitehead *et al.* 2008). NAD(P)H oxidase is a source of oxidative stress, localized in cell membranes and inflammatory cells (Nguyen & Tidball, 2003). While NAD(P)H oxidase releases superoxide anions ($O_2^{\bullet -}$) into the interstitial space, $O_2^{\bullet -}$ may enter a cell easily via chloride channel-3 (ClC3) while H_2O_2, following dismutation, diffuses across cell membranes facilitated by aquaporin channels (Fisher 2009). Mounting data suggest that NAD(P)H oxidase is an important contributor of skeletal muscle pathology and muscle wasting including mechanical ventilation (McClung *et al.* 2007) and ageing (Vasilaki *et al.* 2006). Further, knockout of the NAD(P)H oxidase regulatory subunit $gp91^{phox}$ reduces membrane and fibre damage during reloading following hindlimb unloading (Nguyen & Tidball, 2003). Importantly, elevations of NAD(P)H oxidase have recently been identified in the heart and muscles of *mdx* mice that indeed may play a significant role in muscle damage, weakness and wasting (Williams & Allen, 2007; Spurney *et al.* 2008).

Recent evidence suggests that oxidative stress may sensitise stretch-activated Ca^{2+} channels, including TRPC1, thus upregulating caveolin-3 (Allen *et al.* 2010). Now evidence suggests NAD(P)H oxidase is involved in response to stretch and damage in *mdx* mice (Whitehead *et al.* 2010). Upregulation of caveolin-3 is believed to contribute to enhanced dislocation of DGC proteins with DMD, including nNOS dislocation from the DGC (Gervásio *et al.* 2008). MMP-9 cleaves ß-dystroglycan from α-dystroglycan, and is potentially under redox modulation via NF-κB (Li *et al.* 2009).

Data from Suzuki *et al.* (2007) implicated disruption in DGC integrity and nNOS dissociation with activation of muscle wasting. DGC disruption may lead to muscle wasting with other models. Ageing results in dissociation of dystrophin (Rice *et al.* 2006) from the sarcolemmal and reduces nNOS (Song *et al.* 2009), linked with sarcopenia and weakness (Rice *et al.* 2006; Marzetti *et al.* 2007, Kim 2009). Indeed, a very new publication reported that the dystroglycan is the mechanical weak link in the transfer of transverse forces linking the contractile apparatus and z-disc with ageing (Ramaswamy *et al.* 2011). Therefore, disruption of the DGC and subsarcolemmal cytoskeleton may be a shared signalling mechanism that elicits myopathy across a number of pathologies.

The diaphragm muscle in particular suffers from significant fibrosis, damage and weakness with DMD, and is also susceptible to oxidative stress (Tkatchenko *et al.* 2000; Van Gammeren *et al.* 2004). Oxidant production may be higher in the *mdx* diaphragm than limb muscles, which could contribute to more profound fibrosis, weakness and fatigue in that muscle (Stevens & Faulkner, 2000; Hartel *et al.* 2001). In addition, the diaphragm muscle in the *mdx* genetic mouse model experiences muscle damage, disease progression and gene expression profiles similar to human DMD pathology (Tkatchenko

et al. 2000; Tidball & Wehling-Henricks, 2007). Therefore, the study of the mechanisms by which oxidative stress causes pathology in the *mdx* diaphragm, and development and testing of targeted, antioxidant therapeutics is vital in translation to human health and pathology with DMD.

Targeted antioxidant therapeutic development and DMD

Oxidative stress is a potential upstream contributor to inflammatory signalling and DMD pathology, and thus a therapeutic target (Tidball & Wehling-Henricks, 2007; Whitehead *et al.* 2008). Reduction in oxidative stress indeed correlates with slowed muscle wasting and relief of clinical symptoms with DMD including respiratory muscle distress (Anderson *et al.* 2000; Bonafati *et al.* 2000; Carter & McDonald 2000). Sources of oxidative stress with DMD include inflammatory cells (e.g. myeloperoxidase), NAD(P)H oxidase, mitochondria and decoupling of nitric oxide synthases (Adams *et al.* 1999; Tidball & Wehling-Henricks, 2007; Williams & Allen, 2007; Spurney *et al.* 2008; Whitehead *et al.* 2008). Dislocation of nNOS from the dystroglycan complex could also (a) increase NAD(P)H oxidase activity, (b) increase inflammation, (c) increase protein degradation via activation of ubiquitin ligases, (d) impair satellite cell activation, and (e) increase the risk of material fatigue injury (Nguyen & Tidball, 2003; Tidball & Wehling-Henricks, 2004; Suzuki *et al.* 2007; Tidball & Wehling-Henricks, 2007).

Unfortunately, non-specific scavenger approaches have yielded mixed and confounding results in ameliorating DMD pathology. Green tea extracts including epigallocatechin-3-gallate (Buetler *et al.* 2002; Nakae *et al.* 2008), low-iron diet (Bomman *et al.* 1998) and *N*-acetylcysteine (Whitehead *et al.* 2008) protected against muscle damage, while tocopherols, ascorbate and penicillamine treatments (Walton & Nattrass 1954; Roelofs *et al.* 1979; Stern *et al.* 1982; Fenichel *et al.* 1988) elicited no improvements. Only more recently has the tie between oxidative stress and pathology in muscles in Duchenne muscular dystrophy moved beyond association.

Loss of nNOS from the sarcolemma may have a profound effect on oxidative stress, mechanical integrity and capability for satellite cell activation, growth and repair (Suzuki *et al.* 2007; Tidball & Wehling-Henricks, 2007). Tidball and colleagues (Wehling *et al.* 2001; Tidball & Wehling-Henricks, 2004) reported that overexpression of nNOS increased nNOS localization at the sarcolemma, and partially rescued ß-dystroglycan, α-sarcoglycan and ß-sarcoglycan, concomitant with reduced oxidative stress and muscle damage in *mdx* mice. Thus, interventions that stabilize sarcolemmal localization of nNOS and other DGC proteins may prove effective in ameliorating DMD.

Whitehead *et al.* (2008) demonstrated that 6 week treatment of the antioxidant *N*-acetylcysteine was found to protect against damage, incidence of internal nuclei and weakness in the EDL muscle of 8-week-old *mdx* mice. In addition, *N*-acetylcysteine also increased protein levels of the ß-dystroglycan and utrophin while caveolin-3 was reduced by *N*-acetylcysteine. *N*-acetylcysteine also reduced dihydroethidine oxidation (marker of oxidative stress) and protein expression of the p65 subunit of NF-κB (Whitehead *et al.* 2008), an inflammatory transcription factor that also stimulates proteolysis via the ubiquitin ligase (muscle ring finger-1: MuRF1, atrogin-1) activation. *N*-acetylcysteine treatment also reduced macrophage invasion and collagen accumulation in the hearts of *mdx* mice (Williams & Allen, 2007). *N*-acetylecysteine also attenuated the elevation of caveolin-3 protein expression noted in the EDL of *mdx* mice.

New evidence indicates that NAD(P)H oxidase is upregulated in the *mdx* mouse, and is thus a potential source of oxidative stress and pathology (Williams & Allen, 2007; Spurney *et al.* 2008; Whitehead *et al.* 2008). Inhibition of NAD(P)H oxidase appears to reduce markers of apoptosis in the diaphragm of *mdx* mice by elevating Bcl-2 (B-cell lymphoma 2) and BAG-4 (Bcl-2-associated athanogene-4) are mitochondrial-expressed stress proteins that are anti-apoptotic. in the diaphragm (Kwak *et al.* 2008). Nox may be an important regulator of DMD pathology; however, additional studies are required.

Inhibition of TRPC1 by streptomycin reduces muscle damage and depresses muscle contractility in *mdx* mice (Whitehead *et al.* 2006; Gervásio *et al.* 2008). TRPC1 appears to co-localize with caveolin-3, and both are upregulated in DMD (Gervásio *et al.* 2008). Indeed, TRPC1 upregulation was dependent on caveolin-3. H_2O_2 increased TRPC1 protein expression in myotubes, suggesting that upregulation of TPRC1 via caveolin-3 may be redox dependent (Gervásio *et al.* 2008).

Li *et al.* (2009) reported that matrix MMP-9 appears to be upstream of NF-κB activation. Inhibition or genetic ablation of MMP-9 reduced muscle membrane damage and necrosis. Furthermore, MMP-9 knockout rescued ß-dystroglycan protein expression and reduced caveolin-3 with a small increase in nNOS levels. Although uncertain, MMP-9 may be a nexus of control in the regulation of caveolin-3 and amplification of DMD-induced disruption of DGC integrity, inflammation and myopathy.

Green tea extract, which contains antioxidant compounds such as epigallocatechin-3-gallate (EGCG), reduced oxidative stress in a dose-dependent manner and attenuated the number of necrotic and regenerating fibres in the EDL muscle (Buetler *et al.* 2002). Injection of EGCG extract reduced oxidative stress, lipofuscin, membrane damage (creatine kinase leakage) and necrosis (Nakae *et al.* 2008). Long-term EGCG treatment also blunts NF-κB activity (Evans *et al.* 2010). EGCG is a powerful antioxidant and inhibitor of NF-κB (Aggarwal & Shishodia, 2004,

Ichiyanagi *et al.* 2004), and thus offers a more targeted therapeutic strategy than many nutraceuticals.

In conclusion, emerging evidence is suggesting that oxidative stress may be an important amplifier of pathology in respiratory and limb muscles with Duchenne muscular dystrophy. Based upon recent evidence, a cascade involving TRPC1, NAD(P)H oxidase, caveolin-3 and NF-κB may contribute to damage, inflammation and impaired contractile function in skeletal muscles of patients suffering from DMD. Pharmaceutical developments that target specific mechanisms elevating oxidative stress with DMD could attenuate myopathy and provide relief for patients.

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