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Involvement of oxidative stress, Nuclear Factor kappa B and the Ubiquitin proteasomal pathway in dysferlinopathy

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ABSTRACT

Aims: Dysferlinopathies are autosomal recessive neuromuscular disorders arising from mutations of the protein dysferlin that preferentially affect the limbs which waste and weaken. The pathomechanisms of the diseases are not known and effective treatment is not available. Although free radicals and upstream signaling by the redox sensitive transcription factor, NF-κB, in activation of the ubiquitin pathway are shown to occur in several muscle wasting disorders, their involvement in dysferlinopathy is not known. This study analyzed the role of oxidative stress, NF-κB and the ubiquitin pathway in dysferlinopathic muscle and in dysferlin knockdown human myoblasts and myotubes.

Main methods: Fourteen dysferlinopathic muscle biopsies and 8 healthy control muscle biopsies were analyzed for oxidative stress, NF-κB activation and protein ubiquitinylation and human primary myoblasts and myotubes knocked down for dysferlin were studied for their state of oxidative stress.

Key findings: Dysferlinopathic muscle biopsies showed NF-κB p65 signaling induced protein ubiquitinylation in response to oxidative stress. Dysferlin knock down primary muscle cell cultures confirmed that oxidative stress is induced in the absence of dysferlin in muscle.

Significance: Anti-oxidants that also inhibit nitrosative stress and NF-κB activation, might prove to be of therapeutic benefit in slowing the progression of muscle wasting that occurs with dysferlinopathy.

Introduction

Dysferlin is a 237 kDa transmembrane protein, expressed predominantly in muscle and involved in membrane repair (Bushby, 2000). Hereditary or *de novo* mutations of dysferlin cause the neuromuscular disorder, dysferlinopathy. Dysferlinopathies include Limb girdle muscular dystrophy 2B, (LGMD 2B) that affects proximal and distal limb muscles, Miyoshi myopathy (OMIM # 254130) that causes a preferential loss of gastrocnemius muscle, and DMAT (distal myopathy with anterior tibial involvement) (OMIM # 606678) (Amato and Brown, 2011; Bushby, 2000; Nguyen et al., 2005). The pathomechanisms that lead to progressive muscle wasting and weakness of dysferlinopathy remain elusive.

Muscle atrophy or wasting is common to various conditions. Sarcopenia is wasting associated with ageing, cachexia is a muscle wasting syndrome associated with cancer, sepsis, HIV, congestive heart failure, renal disorders, muscular dystrophies. Muscle loss also occurs during disuse of muscle, e.g., prolonged bed rest, space travel (Franch and Price, 2005; Hasselgren and Fischer, 2001; Lexell, 1995; Tisdale, 2007a). As reviewed by Tisdale (2007), muscle wasting of various cachectic conditions is associated with the activation of the ubiquitin proteasomal pathway while inhibition of the pathway reduces skeletal muscle wasting in animal models of muscle wasting. The transcription factor NF-κB is a primary activator of the ubiquitin pathway and of muscle specific E3 ligases of the ubiquitin pathway (Eddins et al., 2011; Foletta et al., 2011; Murton et al., 2008; Wyke and Tisdale, 2005). In mice activation of the IKKβ/NF-κB pathway induces skeletal muscle atrophy through activation of the ubiquitin pathway. Inhibition of NF-κB reduces muscle degeneration and improves muscle function in *mdx* mice, a model of Duchenne muscular dystrophy (DMD) (Acharyya et al., 2007; Cai et al., 2004; Karin, 2006; Mourkioti et al., 2006; Tang et al., 2010). Thaloor et al. (1999), demonstrated that systemic administration of the NF-κB inhibitor, curcumin, stimulates muscle regeneration after traumatic injury in mice. NF-κB p65 is shown to reduce MyoD levels and interfere in muscle regeneration in myoblasts. In muscle damage, IKKβ depletion facilitates skeletal muscle regeneration by enhancing satellite cell activation and reducing fibrosis in mice (Mourkioti et al., 2006).

Abbreviations: DMD, Duchenne muscular dystrophy; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); GSH, Reduced glutathione; IKK β, Inhibitory kappa B Kinase Beta; LGMD, Limb girdle muscular dystrophy; MAFbx/Atrogin 1, Muscle atrophy F-box protein; MuRF 1, Muscle specific RING finger protein; NF-κB, Nuclear factor kappa-light-chain-enhancer of activated B cell; ROS, Reactive oxygen species.

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Free radicals have been implicated as primary and secondary causes of several muscle wasting diseases (Buck and Chojkier, 1996; Rando, 2002; Sukhanov et al., 2011; Tidball and Wehling-Henricks, 2007; Tisdale, 2007). The role of reactive oxygen and nitrogen species is well documented in wasting of Duchenne muscular dystrophy, Fascioscapular muscular dystrophy, cancer, ageing, disuse induced atrophy and sepsis (Buck and Chojkier, 1996; Cui et al., 2012; Lawler, 2011; Li et al., 2011; Macaione et al., 2007; Menazza et al., 2010; Messina et al., 2006, 2011; Min et al., 2011; Ragusa et al., 1997; Rando, 2001, 2002; Sukhanov et al., 2011; Terrill et al., 2013; Whitehead et al., 2010; Yucel et al., 1998). The transcription factor NF-κB being redox sensitive could be activated by oxidative stress. Messina et al. (2006), showed that inhibition of lipid peroxidation prevented muscle wasting and improved muscle function in *mdx* mice. They also showed activation of NF-κB in response to oxidative stress in *mdx* mice. Based on existing literature on muscle wasting of various conditions including several forms of muscular dystrophies, we hypothesized that absence of dysferlin might induce oxidative stress. In response to oxidant stress, NF-κB might mediate protein degradation through the ubiquitin proteasomal system. To verify the hypothesis 14 dysferlinopathic muscle biopsies and 8 healthy control muscle biopsies were analyzed for oxidative stress, NF-κB activation and protein ubiquitinylation and human primary myoblasts and myotubes knocked down for dysferlin were studied for their state of oxidative stress. The results validated the hypothesis that the absence of dysferlin leads to oxidative stress.

Methods

Muscle biopsies

Fourteen muscle biopsies from patients aged between 9 and 35 years, confirmed for LGMD 2B and Miyoshi myopathy by clinical findings (including asymmetric calf atrophy, atrophy of hamstrings, difficulties in making a fist due to finger flexors atrophy and calf head sign, creatine kinase increase of 50–100 fold) and Western blots negative or reduced for dysferlin by more than 20% compared to healthy controls were included in the study (Cacciottolo et al., 2011; Rajakumar et al., 2011; Rajakumar et al., 2013; Renjini et al., 2012). Average normalized intensity of dysferlin band (237 kDa) of 8 healthy control biopsies was considered as 100%. Control muscles were obtained from patients undergoing orthopedic corrections of lower limb. All other muscle biopsies were obtained for routine diagnostics in the laboratory and stored at −70 °C until analysis. The study was approved by the Institutional Review Board and Human Ethics Committee of Christian Medical College, Vellore (EC Min No IRB (EC) 4/9/2007) and all muscle samples were obtained with informed consent.

Lipid peroxidation

Lipid peroxidation was assayed by estimation of malondialdehyde (MDA), formed by the thiobarbituric acid reaction (TBARS reaction), in muscle homogenized in 10 vol of 1.15% KCl (Ohkawa et al., 1979). A reaction mixture of 100 μl muscle homogenate, 1.5 ml acetic acid (20% pH 3.5), 200 μl SDS (8%), 1.5 ml thiobarbituric acid (0.8%) and 700 μl of water, was heated for 1 h at 95 °C. Samples were cooled to 25 °C, spun at 12,000 g for 10 min and MDA levels in the supernatant read at 535 nm. Lipid peroxidation was expressed as nmol MDA/gm tissue using tetra methoxy propane as standard.

Reduced glutathione (GSH)

GSH was estimated in the protein free supernatant of muscle homogenized in 10 vol of 5% trichloroacetic acid (TCA) containing 5 mM EDTA and spun at 13,000 g for 20 min at 4 °C. A reaction mixture of 200 μl protein free supernatant, 800 μl 0.4 M Tris HCl pH 8.9 and 40 μl 10 mM 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) in 100% methanol was incubated in the dark for 5 min and colour developed read at 412 nm. GSH was expressed as nmol/gm tissue calculated from the molar extinction coefficient of 5-thio-2-nitrobenzoic acid of 13,600 /M/cm at 412 nm (Messina et al., 2006).

Protein thiols

Protein thiols were estimated in muscles homogenized in 10 vol of phosphate buffered saline (PBS) and spun at 1000 g for 10 min at 4 °C. Fifty microlitres of the supernatant was precipitated with 1 ml of 10% TCA and spun at 5000 g for 10 min. To the pellet 1 ml of 0.1 M Tris HCl pH 7.4, 2 ml of 0.2 M Tris HCl pH 8.6 and 30 μl 10 mM DTNB were added and the reaction incubated for 15 min in the dark at 25 °C. Color development was read at 412 nm. Protein thiols were expressed as nmol 5-thio-2-nitrobenzoic acid/mg non-collagen protein calculated from the molar extinction coefficient of 5-thio-2-nitrobenzoic acid of 13,600 /M/cm at 412 nm (Yucel et al., 1998).

Muscle nitrite

Nitric oxide (NO) was estimated in muscle from levels of nitrate, the stable end product of NO, reduced to nitrite and assayed by the Greiss reaction. Muscles were homogenized in 10 vol of PBS and spun at 5000 g for 10 min. To 50 μl supernatant, 150 mg of copper/cadmium alloy was added and incubated at 25 °C for 1 h with thorough shaking. The reaction was stopped by addition of 100 μl 0.35 N sodium hydroxide and 400 μl 120 mM zinc sulphate and spun at 2000 g for 10 min. To 500 μl supernatant 250 μl 1% sulfanilamide in 3 N HCl and 250 μl of 0.1% napthylethylene diamine were added and color development read at 545 nm (Sastry et al., 2002). Units were expressed as nmoles nitrite/mg non-collagen protein read against a $NaNO_2$ standard.

Non-Collagen protein estimation

Protein was estimated as non-collagen protein, subsequent to alkaline hydrolysis of muscle, to exclude misinterpretation of fibrous proteins as muscle proteins. A known wet weight of muscle was hydrolyzed with 19 vol 0.1 N NaOH for 16 h at 25 °C, centrifuged at 5000 g for 15 min and the supernatant neutralized with 0.1 N HCl. NCP in the supernatant was estimated by the method of Lowry et al. (1951), using crystalline bovine serum albumin fraction V as standard (Rajakumar et al., 2011).

Western blots for dysferlin, NF-κB/IKK β and Ubiquitin/E3 ligases

Muscles were homogenized in 19 vol 0.125 M Tris/Hcl pH 7.6 containing 10% glycerol, 10% SDS, 4 M urea, 0.1 M EDTA, 10% β-mercaptoethanol and 0.05% bromophenol blue and 20 μg NCP protein subject to reducing SDS-PAGE (3.5%–12% gels). Non-collagen proteins (100 μg) were electrotransferred to polyvinylidene fluoride membranes for 1 h (80 V/4 °C) following SDS-PAGE (3.5%–12%) and separate blots probed with antibodies to dysferlin (1:100 dilution), antibodies to NF-κB p65, MuRF 1 and MAFbx, ubiquitin, IKK β, nitrotyrosine, nitrocysteine (1:2000 dilution) and loading control glyceraldehyde phosphate dehydrogenase (GAPDH) (1:20,000 dilution), secondary antibody conjugated with Biotin and developed with streptavidin peroxidase, H_2O_2 and diaminobenzidine hydrochloride. Densitometric image analysis of blots was performed with software developed in-house that correlated with Bio-Rad Quantity One Quantitation software version 4 ($r = 0.8$).

Antibodies

Goat anti-dysferlin, (Santcruz, Biotechnologies, USA), rabbit anti-NF-κB p65, MURF 1 and MAFbx were from Abcam, USA, mouse anti-ubiquitin, rabbit anti-IKK β, nitrotyrosine, nitrocysteine and mouse