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Levels of inflammation and oxidative stress, and a role for taurine in dystropathology of the Golden Retriever Muscular Dystrophy dog model for Duchenne Muscular Dystrophy

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ABSTRACT

Duchenne Muscular Dystrophy (DMD) is a fatal skeletal muscle wasting disease presenting with excessive myofibre necrosis and increased inflammation and oxidative stress. In the *mdx* mouse model of DMD, homeostasis of the amino acid taurine is altered, and taurine administration drastically decreases muscle necrosis, dystropathology, inflammation and protein thiol oxidation. Since the severe pathology of the Golden Retriever Muscular Dystrophy (GRMD) dog model more closely resembles the human DMD condition, we aimed to assess the generation of oxidants by inflammatory cells and taurine metabolism in this species. In muscles of 8 month GRMD dogs there was an increase in the content of neutrophils and macrophages, and an associated increase in elevated myeloperoxidase, a protein secreted by neutrophils that catalyses production of the highly reactive hypochlorous acid (HOCl). There was also increased chlorination of tyrosines, a marker of HOCl generation, increased thiol oxidation of many proteins and irreversible oxidative protein damage. Taurine, which functions as an antioxidant by trapping HOCl, was reduced in GRMD plasma; however taurine was increased in GRMD muscle tissue, potentially due to increased muscle taurine transport and synthesis. These data indicate a role for HOCl generated by neutrophils in the severe dystropathology of GRMD dogs, which may be exacerbated by decreased availability of taurine in the blood. These novel data support continued research into the precise roles of oxidative stress and taurine in DMD and emphasise the value of the GRMD dogs as a suitable pre-clinical model for testing taurine as a therapeutic intervention for DMD boys.

1. Introduction

Duchenne Muscular Dystrophy (DMD) is a lethal, X-chromosome linked muscle disease affecting about 1 in 3500–6000 boys worldwide (Reviewed in [1,2]). DMD is characterised by severe muscle weakness caused by mutations in the dystrophin gene, which result in the loss of functional dystrophin protein. In skeletal muscles this defect increases susceptibility to sarcolemma damage after muscle contraction leading to myofibre necrosis, with inflammation and excessive fibrosis initially associated with muscle regeneration [3–5]. Repeated cycles of widespread myofibre necrosis and progressive failure of regeneration over time in DMD boys (with replacement of myofibres by fatty and fibrous connective tissue) lead to the loss of muscle mass and function with premature death of DMD boys, often due to respiratory or cardiac failure (Reviewed in [1,5,6]).

Abbreviations: (2ME;, 2-mercaptoethanol; ACN, acetonitrile; BSA, bovine serum albumin; CD, cysteine deoxygenase; CSD, cysteine sulfinate decarboxylase; DC, detergent-compatible; DMD, Duchenne Muscular Dystrophy; DNPH, 2,4-dinitrophenylhydrazine; EDTA, ethylene diamine tetra acetic acid; FA, formic acid; FLM, BODIPY FL-N-(2-aminoethyl) maleimide; GAP, glyceraldehyde 3-phosphate dehydrogenase; GRMD, Golden Retriever Muscular Dystrophy; HOCl, hypochlorous acid; HPLC, high performance liquid chromatography; IκB-α, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha; MPO, myeloperoxidase; MS, mass spectrometry; MS/MS, tandem mass spectrometry; NAC, N-acetylcysteine; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; NO, nitric oxide; NOS, nitric oxide synthase; nNOS, neuronal nitric oxide synthase; OPA, o-phthalaldehyde; OTC, L-2-Oxothiazolidine-4-Carboxylate; PBS, phosphate buffered saline; SDS, sodium dodecyl sulphate; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; SERCA, sarcoplasmic/endoplasmic reticulum calcium ATPase; Tau-Cl, taurine chloramine; TauT, taurine transporter protein; TCA, trichloroacetic acid; TCEP, tris(2-carboxyethyl)phosphine; Texas red, Texas Red C2-maleimide; TNF, tumour necrosis factor

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While the mechanisms for loss of muscle function in DMD (and animal models of DMD) are not fully understood, disturbed intracellular calcium homeostasis, inflammation and oxidative stress are implicated [7,8]. Proposed sources of various oxidants in dystrophic muscle include mitochondria, inflammatory cells, NAD(P)H oxidase, xanthine oxidase, and decoupling of NOS (via dislocation or translocation of nNOS from the dystroglycan complex of the sarcolemma) (reviewed in [9]). Oxidative damage to muscle proteins has been observed in both DMD boys and the widely studied *mdx* mouse model of DMD [10–14]. Another major cellular consequence of oxidant exposure is the reversible oxidation of protein thiol side chains (-SH, in the cysteine residue). Protein thiols can undergo numerous reactions, which are dependent on the species and concentration of oxidants they encounter [15]. For example, oxidants such as hydrogen peroxide, can cause reversible oxidation (disulphide formation) of thiols, for which the reduction/oxidation (redox) state is an important regulator of protein function [16]. We have previously shown that reversible protein thiol oxidation is increased in *mdx* muscle, and is especially pronounced around areas of necrosis, occurring on muscle proteins such as myosin heavy chain, myosin light chain and tropomyosin, as well as on glycolytic proteins phosphoglycerate mutase and triosephosphate isomerase [14,17–22].

Antioxidants that target protein thiol oxidation, such as the cysteine/glutathione precursors *n*-acetylcysteine (NAC) and L-2-Oxothiazolidine-4-Carboxylate (OTC) have been investigated in *mdx* mice as therapeutic interventions for DMD [19,20,23–26]. Treatment of *mdx* mice with NAC or OTC reduces muscle pathology, as shown by decreased myofibre necrosis, inflammatory cells and TNF levels, and improved grip strength [19,20,23–25]. NAC and OTC are derivatives of the amino acid cysteine, and can increase tissue content of both cysteine and GSH, two major cellular thiol antioxidants [27,28]. However we established that the mechanism of action of NAC and OTC in *mdx* mice was not via an increase in either cysteine or glutathione in muscle, liver and plasma [19,20,23]. Instead, we showed that OTC treatment of *mdx* mice leads to an increase in the content of the semi-essential amino acid taurine (2-aminoethanesulfonic acid) in muscle, liver and plasma [19,23].

Taurine is synthesised from cysteine, as a mechanism for removing excess cysteine, which is toxic in mammals [29,30]. Taurine is found in many tissues and is considered important for the function of skeletal muscle; the concentration of taurine in tissues is regulated by interactions between dietary intake, biosynthetic rate (mainly in the liver) tissue uptake and elimination via the kidney [31]. We recently showed in *mdx* mice that systemic taurine homeostasis is perturbed and may correlate with the onset of pathology [31], and treatment of adult *mdx* mice with taurine improves both in vivo and *ex vivo* muscle strength [23,32,33]. Importantly, in young *mdx* mice aged 22 days, taurine treatment (from day 14) prevents the acute onset of myofibre necrosis [34]. Taurine is hypothesised to modulate ion channel function, membrane stability and calcium homeostasis [35–40]. However we showed that taurine is also a potent thiol antioxidant in *mdx* muscle and can dramatically decrease muscle tissue content of neutrophils and myeloperoxidase (MPO) [23,34]. MPO is a heme protein secreted primarily by neutrophils, the key cells involved in acute inflammation that are phagocytes responsible for microbial killing and generation of various pro-inflammatory mediators that attract macrophages to the site of tissue damage [41]. MPO is also secreted (to a lesser extent) by monocytes and can be secreted by some macrophages [42]. MPO oxidises chloride in the presence of hydrogen peroxide to form the potent oxidant hypochlorous acid (HOCl) that targets proteins by reacting with thiols and by causing oxidative damage [43]. Amino acids such as taurine can function as antioxidants by forming chloramines which can trap HOCl [41]. We propose that a possible mechanism for elevated oxidative stress in dystrophic muscles is the excessive generation of HOCl (by inflammatory cells), combined with a disruption in taurine metabolism, that leaves tissues susceptible to oxidative damage by HOCl.

The golden retriever muscular dystrophy (GRMD) dog model manifests a more severe dystropathology with a rapidly progressing and fatal disease similar to DMD boys, in marked contrast with the *mdx* mouse model [43]. The disruption in taurine metabolism documented for *mdx* mice has the potential to be species specific, since the homeostasis of taurine differs greatly between carnivores such as dogs and humans, and mice [44]. Furthermore, compared with GRMD dogs and DMD boys, the *mdx* mice exhibit a very mild pathology, possibly due to the very short growth phase and lifespan as well as the small size of *mdx* mice [3,45]. Like the human DMD condition, persistent muscle necrosis in GRMD dogs results in incomplete muscle repair leading to loss of myofibres and increased fibrosis, with progressive weakness and gait abnormalities around 6–9 weeks of age and contractures by 6 months [46]. Death of GRMD dogs usually occurs around 1 year of age as a result of failure of respiratory muscles as well as feeding difficulties (severe dysphagia) [43,47,48]. Whilst dystrophin deficiency has now been identified in many breeds of dogs [49], GRMD dogs are the favoured model for pre-clinical trials in DMD research [46,51,52], although the colonies are expensive and hard to maintain and show high variation between individual dogs [50]. There is little information on the role of oxidative stress in GRMD muscles and the role of taurine in GRMD dystropathology has yet to be investigated; however it is known that diet induced taurine deficiency predisposes healthy dogs to cardiomyopathy [51].

The present study investigated inflammation, oxidative stress and taurine homeostasis in muscle and blood from GRMD and healthy wild-type dogs, to determine whether taurine deficiency in GRMD dogs may render the dystrophic muscles susceptible to oxidative damage and protein thiol modifications, caused by inflammation. Inflammation was assessed in GRMD and healthy dog muscles by quantifying the presence of neutrophils and macrophages; the contribution of these cells to oxidative stress was assessed by measuring MPO and chlorotyrosines, which are biomarkers of HOCl generation [52]. Oxidative stress was quantified by measuring levels of protein thiol oxidation (including the thiol oxidative status of specific abundant proteins) and protein carbonylation. Taurine can accumulate in cells through two mechanisms; by uptake from the extracellular space by the sodium dependent transporter TauT, and by endogenous local synthesis from cysteine by two enzymes, cysteine deoxygenase and cysteine sulfinate decarboxylase [29,53]. Since we have previously shown that both mechanisms are perturbed in *mdx* muscle [31], we measured TauT, cysteine deoxygenase and cysteine sulfinate decarboxylase in GRMD muscle, as well as taurine content of the muscle and plasma (to establish extracellular taurine concentrations). We show that GRMD muscle has an increased content of neutrophils and macrophages with resultant high levels of MPO and HOCl, associated with high levels of protein thiol oxidation and irreversible oxidative damage of both intracellular and extracellular proteins; we also show perturbations of taurine homeostasis in GRMD dogs. These data emphasise the value of using GRMD dogs to evaluate the role of immune cell generated HOCl in dystropathology, and support the use of this dystrophic dog model for pre-clinical trials of interventions that target this pathway.