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Higher oxidative stress in skeletal muscle of McArdle disease patients

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ABSTRACT

McArdle disease (MCD) is an autosomal recessive condition resulting from skeletal muscle glycogen phosphorylase deficiency. The resultant block in glycogenolysis leads to an increased flux through the xanthine oxidase pathway (myogenic hyperuricemia) and could lead to an increase in oxidative stress. We examined markers of oxidative stress (8-isoprostane and protein carbonyls), NAD(P)H-oxidase, xanthine oxidase and antioxidant enzyme (superoxide dismutase, catalase and glutathione peroxidase) activity in skeletal muscle of MCD patients (N = 12) and controls (N = 12). Eight-isoprostanes and protein carbonyls were higher in MCD patients as compared to controls ($p < 0.05$). There was a compensatory up-regulation of catalase protein content and activity ($p < 0.05$), mitochondrial superoxide dismutase (MnSOD) protein content ($p < 0.01$) and activity ($p < 0.05$) in MCD patients, yet this increase was not sufficient to protect the muscle against elevated oxidative damage. These results suggest that oxidative stress in McArdle patients occurs and future studies should evaluate a potential role for oxidative stress contributing to acute pathology (rhabdomyolysis) and possibly later onset fixed myopathy.

1. Introduction

McArdle disease (MCD) or Type V glycogen storage disease (OMIM #232600) is an autosomal recessive condition characterized by the absence of glycogen phosphorylase activity in skeletal muscle [1]. This enzyme is required for efficient glycogen breakdown during cellular energy need such as physical activity. Patients with this disorder often have exercise intolerance characterized by muscle pain and cramping during moderate- to high-intensity exercise as well as weakness and fatigability [1–5]. Additionally, more strenuous activities may lead to painful muscular contractures, rhabdomyolysis, and myoglobinuria [3,5]. The basal level of serum creatine kinase (CK) activity, an indicator of skeletal muscle damage due to loss of cell membrane integrity [6], is chronically elevated in MCD patients as compared to their age- and gender-matched sedentary controls [7]. Older patients with MCD often develop a slowly progressive proximal myopathy and fixed weakness, however active patients have a better clinical outcome and functional capacity [8].

A lack of physical activity in MCD patients attenuates mitochondrial biogenesis and enzyme activity [9,10]. Recently, it has been shown that moderate aerobic exercise training is well tolerated by MCD patients and leads to adaptations that increase oxidative capacity and health status [7,11–14]. It is well documented that physical activity induces physiological adaptations in healthy people including increased mitochondrial volume and content, and increased mitochondrial enzyme activities [15–17]. These adaptations may decrease oxidative stress in the following ways: (1) increased antioxidant enzyme content and/or activity, (2) reduced basal production of oxidants and (3) attenuation of reactive oxygen species (ROS) leakage during oxidative phosphorylation resulting in reduced oxidative damage to macromolecules [18–21].

There are several possible reasons why MCD patients would have higher levels of oxidative stress. Forearm exercise testing is associated with an exaggerated elevation of ammonia, hypoxanthine and uric acid in MCD patients versus controls [22–24]. This observation is consequent to increased flux through the uric acid pathway beginning with myoadenylate deaminase and ending with uric acid formation by xanthine oxidase (XO), which contributes to myogenic hyperuricemia. An increased flux through XO would lead to higher superoxide anion and/or H_2O_2 generation and result in a higher level of oxidative stress. An increase in inflammatory cells in muscle following an acute bout of rhabdomyolysis could also lead to a transient increase in oxidative stress from invading neutrophils [25,26]. Furthermore, rhabdomyolysis may perpetuate ROS generation by releasing myoglobin, which may act locally (muscle) and distally (kidney) to further induce macromolecular damage [27]. Finally, the repeated effects of varying degrees of rhabdomyolysis could contribute to the cumulative effect of normal aging

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associated oxidative stress [19], and produce a synergistic effect resulting in the later onset fixed myopathy seen in MCD patients. Recently, elevated levels of oxidative stress have been found in skeletal muscle of MCD patients [28]. We assume that oxidative stress can be a main cause of rhabdomyolysis in these patients (disruption of muscle fiber membrane and leakage of CK to the extracellular space). Therefore, higher oxidative damage in skeletal muscle of MCD patients may be one of explanatory mechanism because owing to their muscle metabolic deficiency.

The purpose of the present study was to characterize oxidative stress and a compensatory antioxidant enzyme responses in skeletal muscle of patients with MCD (glycogen phosphorylase deficiency) as compared to sedentary control patients. Based partially on the theory put forth by Russo and colleagues [29], and recent data [28], we hypothesized that higher oxidative stress occurs in skeletal muscle of sedentary MCD patients in association with: (1) higher levels of 8-isoprostanes and protein carbonyls, and (2) elevated antioxidant defenses as a compensatory response to chronic oxidative stress.

2. Materials and methods

2.1. Subjects

2.1.1. MCD patients

Twelve subjects (N = 4 women and N = 8 men) with MCD (all reported exercise induced myalgia) were included in this study. Myophosphorylase activity was absent (histochemistry) or < 1% of normal activity (biochemistry) in all MCD patients and electron microscopic examination of muscle tissue specimens revealed elevated glycogen accumulation [30]. In addition, all patients had known mutations in the *PYGM* gene in *trans* or a known mutation and a predicted pathogenic mutation or two alleles containing novel sequence variants predicted to be pathogenic [31]. None of the subjects had experienced a bout of clinically relevant rhabdomyolysis in the 12 months before the muscle biopsy and none were participating in a physical activity program at the time of the biopsy.

2.1.2. Control subjects

MCD patients (38.8 ± 10.8 y) were age- and sex- matched with control subjects (39.1 ± 10.8 y; N = 4 women and N = 8 men) who did not have MCD but were referred to the Neuromuscular and Neurometabolic Clinic for other reasons and were not clinically symptomatic for any other neuromuscular or neurometabolic disease. The control subjects had normal phosphorylase activity in muscle, normal electromyography and normal histology and ultrastructural assessment. A Likert-type scale was used to assess habitual exercise training status for each subject [32]. None of the patients were taking allopurinol. There was one smoker in each group (control and MCD), none had diabetes, and two MCD and two controls took 400 IU of vitamin E a day. None were taking anti-oxidant supplements (other than the subjects on vitamin E). All participants were working and independent in daily activity with no gait assistive devices. None of the subjects had fixed proximal weakness or myopathic/dystrophic changes in the skeletal muscle biopsy by histology (both controls and MCD). The training status of the two groups was similar indicating a sedentary activity level for both groups and no difference between them using a Student's 2-tailed unpaired *t*-test.

2.1.3. Study design

This study was completed using extra muscle following all diagnostic testing on each participant and was approved after muscle collection by the McMaster University Hamilton Health Sciences Human Research Ethics Board and conformed to the Declaration of Helsinki guidelines. Written informed consent was obtained from all study subjects for the muscle biopsy. The muscle biopsy was taken from *vastus lateralis* muscle under local anesthesia in the morning after an overnight fast as described [33]. The tissue was immediately frozen in liquid nitrogen and stored at − 80 °C until analysis.

2.1.4. RNA isolation

Frozen skeletal muscle tissues samples (25–40 mg) from all 24 participants were subjected to the Trizol method of total RNA extraction as per manufacturer's instructions (Invitrogen, Burlington, ON, Canada) and described by Mahoney et al. [34]. Selected samples were run on a denaturing agarose gel to verify RNA integrity. The ratio of the 28S to 18S rRNA was consistently > 1 for each sample selected indicating good quality RNA.

2.1.5. Real time RT-PCR

Real time RT-PCR was performed using TaqMan chemistry (TaqMan One Step RT-PCR Master Mix Reagents, Applied Biosystems, Streetsville, ON, Canada) according to the manufacturer's instructions and previously described [34]. Beta (2) microglobulin (β2M) was used as a housekeeping gene to which target gene threshold cycle values were normalized. The use of β2M as a housekeeping gene for this study was validated by ensuring that its mRNA expression was not significantly different between MCD and controls ($p = 0.2$; data not shown).

2.1.6. Muscle sample preparation

Frozen skeletal muscle tissue samples from subjects were homogenized in a 2 mL Tenbroeck homogenizer at 1:25 (wt/vol) in phosphate buffer (50 mM potassium phosphate, 5 mM EDTA, 0.5 mM DTT and 1.15% KCl at pH 7.4). Protease inhibitors (Sigma Chemical Co., St. Louis, MO) were added to the phosphate buffer immediately prior to use at a ratio of 1:1000 and 1 mM butylated hydroxytoluene was added to the samples that were designated for measuring 8-isoprostanes. Samples were centrifuged at 600*g* for 10 min at 4 °C and the supernatant was divided into serial aliquots for enzyme activity, western blot and markers of oxidative stress. Samples were frozen in liquid nitrogen and stored at − 80 °C. Protein content was determined using the method of Lowry and colleagues [35].

2.1.7. Immunoblot preparation and analysis

Western blots were performed as previously described [36]. Briefly, 5–20 μg of protein were loaded per sample and heat denatured for 10 min at 90–99 °C. Following electrophoresis, the proteins were transferred onto nitrocellulose membranes and electroblotted for 1 h at 100 V. Membranes were blocked overnight with 5% dry milk in TBS with 0.1% Tween-20. Membranes were probed with the primary antibody and anti-actin (BD Biosciences, Mississauga, ON, Canada) antibody, as a loading control, for 2 h. The antibodies were purchased from Abcam (Cambridge, MA) and Santa Cruz (Santa Cruz, CA) and used at the following dilutions: catalase (AB16731) 1:1000; SOD1 (AB16831) 1:5000; SOD2 (AB13534) 1:3000; GPx1 (AB16798) 1:1000; $p67^{phox}$ (SC15342) 1:100. A horseradish peroxidase-conjugated secondary antibody (Amersham, Piscataway, NJ) was used and visualized using enhanced chemiluminescence detection according to the manufacturer's instructions (ECL Plus, Amersham). Densitometry was performed on scanned images of X-ray film (Kodak XAR) using *ImageJ* v1.34s software.

2.2. Enzyme activity

2.2.1. Superoxide dismutase (SOD)

Total SOD activity was determined in muscle by measuring the kinetic consumption of O_2^- by superoxide dismutase in a competitive reaction with cytochrome

c as described by Flohe and Otting [37]. Briefly, 20 μL of supernatant were added to a cuvette containing 965 μL of medium (50 mM phosphate buffer, 0.1 mM EDTA, pH 7.8) with partially acetylated cytochrome *c* (25 mg/100 mL) and 0.5 μM xanthine. Fifteen microliters of