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Age-related alterations in the sarcolemmal environment are attenuated by lifelong caloric restriction and voluntary exercise

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ABSTRACT

Age-related loss of skeletal muscle mass and function, referred to as sarcopenia, is mitigated by lifelong calorie restriction as well as exercise. In aged skeletal muscle fibers there is compromised integrity of the cell membrane that may contribute to sarcopenia. The purpose of this study was to determine if lifelong mild (8%) caloric restriction (CR) and lifelong CR + voluntary wheel running (WR) could ameliorate disruption of membrane scaffolding and signaling proteins during the aging process, thus maintaining a favorable, healthy membrane environment in plantaris muscle fibers. Fischer-344 rats were divided into four groups: 24-month old adults fed *ad libitum* (OAL); 24-month old on 8% caloric restriction (OCR); 24 month old 8% caloric restriction + wheel running (OCRWR); and 6-month old sedentary adults fed *ad libitum* (YAL) were used to determine age-related changes. Aging resulted in discontinuous membrane expression of dystrophin glycoprotein complex (DGC) proteins: dystrophin and α-syntrophin. Older muscle also displayed decreased content of neuronal nitric oxide synthase (nNOS), a key DGC signaling protein. In contrast, OCR and OCRWR provided significant protection against age-related DGC disruption. In conjunction with the age-related decline in membrane DGC patency, key membrane repair proteins (MG53, dysferlin, annexin A6, and annexin A2) were significantly increased in the OAL plantaris. However, lifelong CR and CRWR interventions were effective at maintaining membrane repair proteins near YAL levels of. OAL fibers also displayed reduced protein content of NADPH oxidase isoform 2 (Nox2) subunits (p67phox and p47phox), consistent with a perturbed sarcolemmal environment. Loss of Nox2 subunits was prevented by lifelong CR and CRWR. Our results are therefore consistent with the hypothesis that lifelong CR and WR are effective countermeasures against age-related alterations in the myofiber membrane environment.

1. Introduction

Skeletal muscle is crucial for stability, mobility and whole body metabolism. As with all other organs, aging has a detrimental impact on skeletal muscle that can be observed phenotypically as atrophy, susceptibility to damage, fibrosis, and weakness. This age-related loss of skeletal muscle mass and function is often referred to as "sarcopenia." Sarcopenia occurs in rodents and humans (Iannuzzi-Sucich et al., 2002; Kim et al., 2008; Lushaj et al., 2008; Rice et al., 2006). In the human population sarcopenia affects up to 25% of humans that are 65 years and older, and >40% of the population that is ≥80 years old (Iannuzzi-Sucich et al., 2002). The affect that sarcopenia has on muscle function is clinically important, exemplified by increased susceptibility to muscle fatigue and weakness that lead to a higher risk of fall-related injuries (Marzetti and Leeuwenburgh, 2006; Metter et al., 2002). With mean lifespan continuing to increase, finding treatments and interventions to slow the process or lessen the impact of sarcopenia on health and quality of life are of critical importance.

The plasma membrane of skeletal muscle cells, commonly referred to as the sarcolemma, relies on binding of the sarcolemmal scaffolding to the extracellular matrix (ECM) for proper membrane integrity. Sarcolemmal scaffolding is manifested as costameres, which are comprised of the dystrophin-glycoprotein complex (DGC) and integrin/focal adhesion complexes (Clark et al., 2002; Davies and Nowak, 2006; Grounds et al., 2005). Damage to the sarcolemmal environment can lead to a reduced ability to sense and respond to dynamic changes in mechanical stimuli. Indeed, impairment of sarcolemmal scaffolding and signaling promotes myofiber fragility and leads to overall muscle weakness and limited mobility (Grounds et al., 2005; Wu et al., 2011). The sarcolemma is increasingly prone to mechanically-induced damage in aged muscle (Faulkner et al., 1995). Intriguing observations indicate that compromised integrity of the sarcolemma in conjunction with diminished

Abbreviations: CR, calorie restriction; WR, wheel running; OAL, old *ad libitum*; OCR, old calorie restriction; OCRWR, old calorie restriction + wheel running; YAL, young *ad libitum*; DGC, dystrophin glycoprotein complex; nNOS, neuronal nitric oxide synthase; NADPH, nicotinomide adenine dinucleotide phosphate; Nox2, NADPH oxidase isoform 2.

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mechano-signaling contributes to sarcopenia (Ramaswamy et al., 2011; Rice et al., 2006). For instance, in skeletal muscle from very old mice, sarcolemmal dystrophin becomes discontinuous and elicits decreased membrane integrity and lateral force transmission through the Z-disc to the extracellular matrix (Ramaswamy et al., 2011; Rice et al., 2006). These findings indicate that muscular dystrophic-like DGC disruptions occur in aging muscle.

In various forms of muscular dystrophy, where there is a deficit in sarcolemmal integrity and force transmission, membrane repair proteins are found to be increased compared to muscles from controls (Cagliani et al., 2005; Ramaswamy et al., 2011). Mechanical and/or biochemical (e.g., lipid peroxidation) stressors can damage the sarcolemma and induce the membrane repair response. Shortly after the damage-inducing event, the membrane repair response occurs in a coordinated fashion, mitsugumin-53 (MG53) is recruited to the site of injury followed by dysferlin, annexin A6, and then annexins A1 and A2 (Cai et al., 2009; Roostalu and Strahle, 2012). While little is known about the relationship between sarcopenia and cell membrane repair, a similar repair response may be occurring in aged muscle as in the dystrophic muscles. Similar to the various forms of muscular dystrophy, aging is accompanied by increased levels of oxidative stress and susceptibility to damage – possibly triggering and contributing to the altered sarcolemmal environment.

Skeletal muscle wasting associated with aging is postulated to be due in part to increased levels of oxidative stress (Fulle et al., 2004; Kim et al., 2008; Ryan et al., 2008). In skeletal muscle, there are several oxidant producing sources (Bejma and Ji, 1999; Reid, 2001), and while many studies have examined the role of mitochondrial-derived reactive oxygen species (ROS) in aging skeletal muscle (Melov, 2000; Van Remmen and Jones, 2009), further examination of non-mitochondrial sources of superoxide is needed. NADPH oxidase (Nox) protein complexes have been found in skeletal muscle, with the Nox2 isoform serving as a major source of superoxide generation during contractile activity (Khairallah et al., 2012; Pearson et al., 2014; Sakellariou et al., 2013). In healthy muscle, contractile-induced activity of the membrane-localized Nox2 is crucial for redox signaling and upregulation of cytoprotective functions after cessation of contractions. However, in *mdx* mice, a model of Duchenne muscular dystrophy, Nox2 becomes hypersensitive to stretch while Nox2 abundance and activity are elevated, thus exacerbating disease pathology (Whitehead et al., 2010). Although recent Nox2 findings in aged skeletal muscle remain inconclusive (Barrientos et al., 2015; Nyberg et al., 2014; Ryan et al., 2010), it is an intriguing possibility that alterations in Nox2 may contribute to the age-associated reduction in membrane integrity.

Habitual or lifelong exercise training can limit the rate of sarcopenia (Kim et al., 2008; Lawler and Hindle, 2011). We have previously demonstrated that lifelong wheel running and mild caloric restriction attenuated age-associated muscle fiber atrophy (Kim et al., 2008). However, the effects of lifelong caloric restriction and voluntary exercise on the sarcolemmal environment (i.e., DGC, membrane repair proteins, Nox2) are unknown. Therefore, the purpose of our study was to investigate (i) the influence of aging on the sarcolemmal environment in the predominately fast-twitch plantaris muscle, and (ii) how lifelong mild (8%) CR and WR would affect the age-related affects. We hypothesized that aging would lead to a membrane environment that is indicative of reduced integrity and an exaggerated repair response that would be accompanied by increased Nox2 abundance.

2. Methods

2.1. Animals

Male Fischer-344 rats were purchased from Harlan (Indianapolis, IN) at 10–11 weeks of age. Rats were housed at the University of Florida's Animal Care Services facilities until time of sacrifice at 6 (young) or 24 (old) months of age. A week after arriving at the University of Florida's facilities, rats were randomly assigned to one of four groups: 6-month-old sedentary *ad libitium* (YAL; $n = 12$), 24-month-old sedentary *ad libitium* group (OAL; $n = 12$), 8% caloric restriction (CR) from 11 months–24 months (OCR; $n = 12$), and 8% CR plus voluntary wheel running (WR) from 11 to 24 months (OCRWR; $n = 12$). Studies have shown that rats fed an *ad libitum* diet have a tendency to run less, but mild CR (8–10%) is substantial enough to prevent the decline in running activity. Therefore, food intake for the two CR groups, OCR and OCRWR, was restricted by 8% below the *ad libitum* food intake of a separate group of sedentary, age-matched, male Fischer-344 rats that were housed in the same facilities. Although calories were reduced, nutritional balance was maintained for vitamins and trace minerals, as previously described (Kim et al., 2008). Throughout the length of the study, food intake in the OCR and OCRWR groups was adjusted weekly based on *ad libitum* food intake from the previous week. Animals were housed one per cage in a temperature-controlled (20° ± 2.5 °C) and light-controlled (12:12 h light-dark diurnal cycle) room. Sedentary rats were housed under standard conditions in rat cages purchased from Fisher Scientific. OCRWR rats were housed in standard cages equipped with Nalgene Activity Wheels purchased from Fisher Scientific. Activity wheels had a circumference of 1.08 m and were equipped with a magnetic switch and an LCD counter for recording the number of wheel revolutions. The number of revolutions was recorded for each animal on a daily basis. Body weights of all rats were recorded weekly. Experimental procedures were approved by the University of Florida's Institute on Animal Care and Use Committee.

2.2. Tissue preparation

Anesthetization of the Fischer-344 rats was accomplished by way of isoflurane inhalation and sacrificed by cardiac puncture. Upon sacrifice, the plantaris muscle was removed, trimmed of excess tissue (tendons and/or fat), rinsed in PBS, blotted dry, weighed and then frozen in liquid nitrogen. Muscle samples for histochemical analysis were washed with PBS, blotted dry, weighed, snap frozen in liquid nitrogen at optimal length in optimal cutting temperature (O.C.T.) solution and stored at −80 °C until analysis (Kim et al., 2008). Plantaris muscles were a kind gift to our lab from Dr. Christiaan Leeuwenburgh at the University of Florida.

2.3. Histochemical staining

Plantaris muscle samples were mounted in tissue freezing medium before being cut at a temperature of −15 °C and allowed to air-dry for 30 min. Hematoxylin and eosin (H&E) staining involved incubating the samples with hematoxylin (VWR, cat # 95,057–844) for 4 min followed by rinsing with tap water. Differentiation was performed using a 75% ethanol and 0.3% glacial acetic acid solution for 15 min. Muscle sections were then incubated in eosin (VWR, cat # 95057-846) for 4 min. Dehydration of the samples involved two incubations in 95% ethanol, followed by two subsequent incubations in 100% ethanol. Samples and slides were cleared with xylene prior to mounting the cover slips (Vectamount permanent mounting medium, Vector Laboratories, cat # H-5000). H&E stains were visualized and images captured on an Olympus IX-51 (Olympus Imaging America, Inc.) inverted microscope. Muscle cross-sectional area (CSA) was measured, recorded, and calibrated against a stage micrometer. Plantaris muscles ($n = 6$ per group) were analyzed with each fiber CSA in square micrometer units calculated using NIH ImageJ software against the stage micrometer standard.

2.4. Immunohistochemistry

To examine protein localization, plantaris muscle sections ($n = 6$ per group) obtained from the midbelly were serially sectioned at 10 μm thick in a cryostat (Thermo Scientific, Shandon Cryotome FSE) at