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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); 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.

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Sarcopenia; Aging Muscle; Calorie Restriction; Exercise; Wheel Running; Sarcolemma

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 and others 2002; Kim and others 2008; Lushaj and others 2008; Rice and others 2006). In the human population sarcopenia affects up to 25% of humans that are 65 years and older, and greater than 40% of the population that is ≥ 80 years old (Iannuzzi-Sucich and others 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 and others 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 and others 2002; Davies and Nowak 2006; Grounds and others 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 and others 2005; Wu and others 2011). The sarcolemma is increasingly prone to mechanically-induced damage in aged muscle (Faulkner and others 1995). Intriguing observations indicate that compromised integrity of the sarcolemma in conjunction with diminished mechano-signaling contributes to sarcopenia (Ramaswamy and others 2011; Rice and others 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 and others 2011; Rice and others 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 and others 2005; Ramaswamy and others 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 and others

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 and others 2004; Kim and others 2008; Ryan and others 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 and others 2012; Pearson and others 2014; Sakellariou and others 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 and others 2010). Although recent Nox2 findings in aged skeletal muscle remain inconclusive (Barrientos and others 2015; Nyberg and others 2014; Ryan and others 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 and others 2008; Lawler and Hindle 2011). We have previously demonstrated that lifelong wheel running and mild caloric restriction attenuated age-associated muscle fiber atrophy (Kim and others 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 11–24 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 and others 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 hour 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 and others 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 minutes. Hematoxylin and eosin (H&E) staining involved incubating the samples with hematoxylin (VWR, cat # 95057-844) for 4 minutes followed by rinsing with tap water. Differentiation was performed using a 75% ethanol and 0.3% glacial acetic acid solution for 15 minutes. Muscle sections were then incubated in eosin (VWR, cat # 95057-846) for 4 minutes. 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 −15° C and placed onto microscope slides. After acetone fixation, slides were washed in PBS with 0.1% Tween20. Sections were blocked in TBS with 0.05% Tween20 and 10% goat serum for 15 minutes. After blocking, sections incubated with specific primary antibodies: dystrophin (1:100, Santa Cruz Biotechnology cat # sc-15376), α-1-syntrophin (1:100, Abcam, cat # ab11187), or nNOS (1:100, Cayman Chemical, cat #160870) for 1 hour in an enclosed chamber at room temperature. After plantaris sections were washed, a 30 minute incubation with the biotinylated goat anti-rabbit IgG (H+L) (1:200, Vector Laboratories, cat # BA-1000) secondary antibody in PBS commenced. Cross-sections were washed in PBS before incubating with Vectastain Elite ABC Reagent (Vector Laboratories, cat # PK-61000) for 30 minutes, followed by a 5 min incubation in NovaRed Peroxidase Substrate Solution (Vector Laboratories, cat # SK-4800). Slides were washed and subsequently mounted with Vectashield Mounting Medium Hard Set (Vector Laboratories, cat # H-1000). Membrane protein localization for dystrophin, α-syntrophin, and nNOS was quantified using an edge filter macro developed for the NIH ImageJ software program. Samples in each group were analyzed under identical conditions.

2.5. Western Blot Analysis

Upon sacrifice, plantaris muscles were harvested and frozen in liquid nitrogen prior to being stored at −80°C until subsequent analysis. Plantaris muscles were pooled together (n = 5 per group), minced, weighed and suspended (15:1 w/v) in ice-cold lysis buffer composed of 25 mM Tris-HCl [pH 7.4], 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, and protease inhibitor cocktail (Pierce protease inhibitor, Thermo Scientific, cat # 88666). Muscles were homogenized in a ground glass-on-ground glass mortar and pestle at 4° C. Homogenates were then centrifuged for 10 min at $1000 \times g$ (4°C). The resulting supernatant was used for protein quantification. Protein concentration of the supernatant fraction was measured using the Bradford protein assay, following the manufacturer's instructions (Bio-Rad, cat # 500-0006). Tissue extracts were subsequently aliquoted and stored at −80°C until western blot analysis.

Plantaris tissue extracts (20 μg or 40 μg) along with sample buffer were loaded into wells of 8% or 10% SDS-PAGE gels. Electrophoresis was conducted at 120 V for ~70 min. Gels were transferred at 100 V for 60 min onto a nitrocellulose membrane (BioRad, cat # 162-0112). Membranes were blocked in 5% nonfat milk in TBS for 1 h. After blocking, membranes incubated overnight at 4°C in blocking buffer with the appropriate primary antibody: anti-syntrophin alpha 1 (1:2500, Abcam, cat # ab11187), anti-nNOS (1:1000, BD Biosciences, cat # 610309), anti-MG53/TRIM72 (1:2500, ProSci, cat # 25-812), anti-dysferlin (1:2500, Novacastra, NCL-Hamlet), anti-annexin II (A2) (1:1000, BD Biosciences, cat # 610068), anti-annexin VI (A6) (1:1000, Abcam, cat # ab31206), anti-gp91phox (1:1000, BD Biosciences, cat # 611415), anti-p67phox (1:2500, BD Biosciences, cat # 610913), anti-p47phox (1:750, BD Biosciences, cat # 610355), and anti-Rac1 (1:500, EMD Millipore, cat # 07-1464). Following TBS with 0.1% Tween-20 washes, membranes were incubated with the appropriate HRP-conjugated secondary antibodies (Santa Cruz Biotechnology, cat # sc-2004 or sc-2005) in blocking buffer at room temperature for 1 h. After TBS-T washes, proteins were visualized by Super Signal West Dura Extended Duration Substrate (Thermo Scientific, cat # 34076) enhanced chemiluminescence detection

and developed with the Fuji LAS-3000 Luminescent Image Analyzer (FujiFilm Medical Systems). Quantification was performed using NIH ImageJ software. Ponceau-S staining (band ~38 kDa mark) was used as a loading control.

2.6. Statistical Analysis

All data reported here are as mean ± SEM. Statistical analysis was performed using GraphPad 6 software (GraphPad Software, Inc., San Diego, CA). One-way ANOVA was used to identify differences among YA, OA, OCR, and OCRWR. Tukey's post-hoc analysis was utilized when necessary. Significance level for all tests was set at $p < 0.05$.

3. Results

3.1. Age-related decrease in plantaris fiber CSA is mitigated by lifelong CR and WR

As published by Kim *et al.*, average body mass of OAL rats (389.7 g) was not significantly different than YAL rats (374.3 g) (Kim and others 2008). However, mean body mass of the OCRWR rats (341.3 g) was significantly lower than the other three groups – YAL, OAL, and OCR. Relative muscle mass to body mass ratio was not significantly different between OAL and YAL rats (1.67 g/kg v. 1.74 g/kg). In contrast, muscle to body mass ratio was significantly increased in OCRWR rats (1.80 g/kg) when compared to OAL ($p<0.01$) and OCR ($p<0.001$) groups (Kim and others 2008).

Fiber cross-sectional area (CSA) in the plantaris muscles was quantified following Hematoxylin and Eosin (H&E) staining (Fig. 1 A–C). CSA of muscle fibers is an indicator of muscle atrophy and thus sarcopenia. Compared to YAL fibers (3148 μm^2 ± 82.17), each of the old groups were found to have significantly smaller fiber CSA ($p<0.0001$ OAL; $p=0.0025$ OCR; $p=0.0005$ OCRWR). However, both lifelong interventions (CR and CRWR) mitigated the age-related decline in mean muscle fiber CSA, with fiber CSA in OCR (2786 μm^2 ± 77.01) and OCRWR (2778 μm^2 ± 64.43) groups significantly larger than those found in the OAL group (2264 μm^2 ± 49.21; $p<0.0001$ in each instance) (Fig. 1B). Distribution of plantaris fibers from OAL were mostly under 2500 μm^2 (~74% of all fibers), whereas OCR muscles contained approximately 58% and OCRWR muscles only 55% of fibers that were smaller than 2500 μm^2 (Fig. 1C). Altogether, these findings indicate that lifelong CR and CRWR limit the age-related loss of skeletal muscle mass and fiber CSA, thus limiting the decline in muscle function.

3.2. Lifelong CR and WR attenuated disruption of sarcolemmal localized DGC proteins

We examined the localization of three key DGC proteins: dystrophin, α-syntrophin, and nNOS. These DGC proteins are normally localized near the sarcolemma. Dystrophin, which normally binds to the transmembrane protein β-dystroglycan, as well as α-syntrophin and dystrobrevin, is tightly associated with the sarcolemma. nNOSμ is bound to α- or β-syntrophin in healthy, skeletal muscle, but may detach during Duchenne and other muscular dystrophies and disuse (Brenman and others 1995; Finanger Hedderick and others 2011; Suzuki and others 2007). Aging did not have an effect on the sarcolemmal content of dystrophin in the plantaris muscle fibers (Fig. 2A and B). In contrast, CR and WR interventions contained significantly greater levels of sarcolemmal-localized dystrophin

compared to the OAL fibers (+18.4%, p=0.0002 OCR and +24.4%, p<0.0001 OCRWR compared to OAL; Fig. 2A and B). In addition, OCR and OCRWR fibers had significantly greater sarcolemmal dystrophin than YAL (+17% OCR, p=0.0012 and +22.9%, p<0.0001 OCRWR compared to YAL; Fig. 2A and B). Although the overall sarcolemmal content of dystrophin between YAL and OAL was not deemed significantly different, it was apparent that a discontinuous sarcolemmal pattern existed for the OAL fibers (Fig. 2A). This was an effect that was ameliorated by the lifelong interventions of CR and CRWR, with OCRWR dystrophin immunostaining appearing very similar in consistency to that of the YAL plantaris fibers (Fig. 2A).

As mentioned above, α-syntrophin binds to dystrophin and is commonly found near the sarcolemma. In aged plantaris muscle fibers, α-syntrophin was largely disassociated from the sarcolemma (Fig 2A). However, CR and CRWR led to significant increases in sarcolemmal localized α-syntrophin compared to the ad-libitum fed groups, with OCR having 25.8% more than OAL and 17% more than YAL (p<0.0001 for OAL v. OCR and OCRWR; Fig. 2A and B), while OCRWR contained 31.2% more than OAL and 22.7% more than YAL (p<0.0001 for YAL v. OCR and OCRWR; Fig. 2A and B). As can be seen in Fig. 2A, continuity of α-syntrophin near the membrane is best preserved with the combination of WR with CR. Overall protein content of α-syntrophin was found to be significantly higher in the OAL plantaris fibers compared to YAL (p = 0.01), whereas the OCR and OCRWR content was not deemed significantly higher than YAL or significantly lower than OAL (Fig. 2C).

The mu splice variant of neuronal nitric oxide synthase (nNOSμ) is normally found bound to α-syntrophin and dystrobrevin and indirectly to dystrophin. In healthy muscle nNOS is involved in several signaling events related to regulation of muscle mass, force generation and susceptibility to fatigue (Brenman and others 1995; Kobayashi and others 2008; Percival and others 2008). Protein concentration of nNOS (relative to ponceau red staining) was not found to be significantly different between YAL and OAL (Fig. 2C and D). Although CR and CRWR interventions did not affect nNOS protein abundance significantly, compared to OAL, OCRWR trended toward a significant increase with a p-value of 0.08 (+26% compared to OAL, Fig. 2D). Even though nNOS protein content was not found to be significantly different across groups, nNOS localization was altered with aging (Fig. 2A and B). Aging resulted in a significant decrease of sarcolemmal localized nNOS when comparing YAL to OAL plantaris fibers (−59%, p<0.0001 OAL compared to YAL, Fig. 2B). Lifelong CR and the addition of WR mitigated the mislocalization of nNOS, with 94.4% more in the OCR (p <0.0001) and 100.7% more sarcolemmal nNOS in the OCRWR (p <0.0001) compared to OAL (Fig. 2B). However, the interventions were not able to completely maintain the YAL sarcolemmal phenotype.

3.3. Sedentary, ad-libitum aging is associated with dramatic increases in membrane repair proteins

As indicated by previous studies (Leiter and others 2012; Rice and others 2006) and suggested by our DGC findings, sarcolemmal damage occurs with sedentary, *ad libitum* aging. To examine this further, we investigated how aging as well as lifelong diet and

exercise interventions, on membrane-repair machinery in aged muscle fibers. We chose to measure the protein content of four proteins that have been strongly tied to membrane repair processes: MG53, dysferlin, annexin A6, and annexin A2. Membrane damage is normally followed by a repair response to restore integrity to the membrane and maintain homeostasis. Our observations indicate that there is significant increase in MG53 ($p < 0.0001$), dysferlin ($p < 0.0001$), annexin A6 ($p < 0.0001$) and annexin A2 ($p = 0.004$) abundance in the OAL rats compared to YAL (Fig. 3). The lifelong intervention of CR+WR was capable of maintaining at or near control YAL levels of MG53, dysferlin, annexin A6, and annexin A2 (Fig. 3A and B). Plantaris muscles from OCR rats were found to contain significantly more dysferlin ($p = 0.011$) than OCRWR, but significantly less than OAL ($p = 0.0005$). Old CR muscles also contained significantly less annexin A6 ($p < 0.0001$) than OAL, but significantly more than YAL ($p = 0.026$) and OCRWR ($p = 0.0003$). Interestingly, the exercised group (OCRWR) contained the lowest levels of repair proteins out of the four groups (Fig. 3B).

3.4. Lifelong CR and WR ameliorate the age-related reduction in Nox2 abundance

Presently, the level of involvement that Nox2 plays during the sarcopenic process remains unclear, and thus investigation of Nox2 isoforms in the aging plantaris and the effects of lifelong mild caloric restriction and wheel running were investigated. In the present study, we found that aging was associated with a reduced abundance of Nox2 subunits (Fig. 4A and B). There was a trend for an age-related decrease (−45%) in the transmembrane catalytic subunit, gp91phox, comparing YAL to OAL ($p=0.096$; Fig. 4B). Regulatory cytosolic subunits, p47phox and p67phox, displayed a significant age-related reduction in protein content ($p=0.01$ and $p=0.03$, respectively; Fig. 4B). No significant difference was observed between YAL and OAL in the levels of the microtubule-associated Nox2 subunit Rac-1 (Fig. 4B). Interestingly, gp91phox, p67phox, and p47phox were expressed at significantly higher protein levels in the OCR compared to OAL plantaris (Fig. 4B). Lifelong CR+WR muscles trended toward a significant increase in levels of gp91phox (+81.1%, $p=0.096$) and reached significant increases in protein abundance of p67phox (+70.9%, $p = 0.0009$) and p47phox (+128.4%, $p = 0.0002$) compared to OAL (Fig. 4B). Overall, each Nox2 subunit exhibited a similar trend of an age-related decrease. With the exception of p67phox, which was significantly higher in the OCR than YAL group ($p=0.03$), Nox2 subunits in OCR and OCRWR muscle were not significantly different than YAL.

4. Discussion

In the current study, we report that lifelong mild (8%) caloric restriction (CR) and CR plus lifelong voluntary wheel running (WR) are interventions capable of partially preventing the age-associated decrease in plantaris muscle fiber cross-sectional area (CSA). Additionally, lifelong CR and CRWR offered protection against age-related alterations in the sarcolemmal environment, which may aid in maintenance of skeletal muscle functionality and overall health. We observed that sedentary aging led to discontinuous sarcolemmal patterning of key DGC proteins, such as dystrophin, α-syntrophin, and nNOS, an effect that was prevented by the addition of lifelong CR and CRWR. Inconsistent sarcolemmal patterning of DGC proteins is indicative of reduced membrane integrity; therefore, we examined proteins

involved in membrane repair. Sedentary, *ad libitum* aging (OAL) had increased abundance of membrane repair proteins: MG53, dysferlin, annexin A6 and annexin A2. In order to elucidate the potential role of membrane-generated oxidative stress in the age-related reduction in membrane integrity, we sought to determine the role of the membrane-bound superoxide producing NADPH oxidase isoform 2 (Nox2) complex. We identified several crucial Nox2 protein subunits that were significantly reduced with sedentary aging (OAL), which is prevented by lifelong CR and CRWR. Thus, our data indicates that sedentary, ad-libitum aging is associated with reduced sarcolemmal integrity, increased sarcolemmal damage, altered membrane redox environment, all of which are likely associated with the progression of sarcopenia. Lifelong CR and CRWR interventions were capable of mitigating the negative age-related alterations, consequently providing a myofiber environment conducive for better overall muscle health and function. A detailed discussion of our major findings follows.

4.1. Effect of CR and WR on muscle fiber atrophy

Previous studies showed that sarcopenia can be attenuated by dietary restriction (Hepple and others 2008; McKiernan and others 2004) and/or exercise (Kim and others 2008; Pasini and others 2012). While Wohlgemuth and colleagues did not find significantly increased muscle to body mass ratios in the old CR or old CR + exercise rats compared to OAL (Wohlgemuth and others 2010), our lab previously showed that lifelong dietary restriction + exercise resulted in a muscle : body mass that was significantly increased compared to sedentary, ad-libitum fed, aged rats (Kim and others 2008). Our previous findings exhibited an age-related decrease in plantaris fiber CSA, an effect prevented by lifelong CR and CRWR (Kim and others 2008).

Here, we have observed the partial protection of muscle fiber cross-sectional area (CSA) by lifelong mild (8%) CR and CR plus WR (Fig 1). The present study demonstrated a protective effect of lifelong CR and WR + CR on plantaris fiber cross-sectional area compared with lifelong, sedentary rats fed *ad libitum*. Our lab noted that lifelong CR and CRWR prevented the increase in extramyocyte space and connective tissue exhibited in sedentary aged rats (Kim and others 2008). However, unlike Kim *et al.* (Kim and others 2008), in the present study the plantaris fiber CSA in OCR and OCRWR rats remained significantly smaller than YAL. If muscle fiber cross-sectional area is decreased and fiber number is reduced with aging due to apoptosis (Marzetti and others 2008), then extracellular matrix of connective tissue and fat must compromise a larger proportion of aging muscle mass (Kim and others 2008). This is consistent with a reduction in muscle quality (force per cross-sectional area) with progressive age (Brooks and Faulkner 1994; Thompson 2009).

4.2. Protection by CR and CRWR against DGC alterations in aged plantaris muscle

The costameric DGC acts as a mechanosensitive scaffold of proteins that are responsible for force transmission, structural integrity of the cell, and transmission of cellular signaling processes from the extracellular environment (Grounds and others 2005; Gumerson and Michele 2011). Loss of, or decreased expression of, DGC proteins signifies a decrease in the structural integrity of the sarcolemma and leads to myofiber loss (Davies and Nowak 2006; Rice and others 2006). Here, we report a discontinuity of membrane-associated dystrophin

with aging, but not overall expression, and a dissociation of α-syntrophin was noted (Fig. 2). This discontinuity was mitigated by the long-term interventions of CR and CRWR. Moreover, the lifelong CR and CRWR interventions not only proved to be effective at maintaining the sarcolemmal localization of dystrophin and α-syntrophin, but also resulted in increased sarcolemmal content of the DGC proteins, concomitant with larger muscle fiber size than OAL.

Recently, Ramaswamy *et al.* identified the mechanical consequences of age-related disruption in the DGC, as manifested as a roughly 50% loss of lateral force transmission, and thus the integrity of the parallel elastic element in both rats and mice (Ramaswamy and others 2011). Very old rats (34–35 months of age) displayed impaired lateral transmission of force, there was a concomitant discontinuity in dystrophin (Ramaswamy and others 2011). When coupled with the current data that illustrate a substantial disruption of DGC proteins (dystrophin, α-syntrophin, nNOSμ), perturbations in DGC-ECM interface with aging could compromise myofiber structural integrity. For example, Rice *et al.* showed that aging led discontinuous positive staining on the sarcolemma for dystrophin, while membrane content increased in the EDL of 36 month old rats (Rice and others 2006). Indeed, dystrophin was absent from discrete sections of sarcolemma (Rice and others 2006) similar to the plantaris muscles from the sedentary, ad-libitum aged (OAL) group. When a discontinuous and patchy membrane accumulation of dystrophin exists, there is a reduction in membrane and muscle fiber integrity (Faulkner and others 1995). Bound to dystrophin, α-Syntrophin is a DGC protein that binds to and attaches nNOSμ to the DGC. We found a significant increase in total α-syntrophin in the OAL compared to YAL plantaris muscles (Fig. 2). Similar to the dystrophin data from Rice *et al.* (Rice and others 2006), an increase in α-syntrophin protein content was accompanied by discontinuous disruption of sarcolemmal α-syntrophin in the OAL group (Fig 2). Furthermore, mice lacking dystrophin are unable to localize α-syntrophin to the sarcolemma (Brenman and others 1995; Grounds and others 2005), consistent with shared poor linkages by costameres and lateral force generation (Ramaswamy and others 2011). Membrane-associated α-syntrophin may also regulate protein turnover and remodeling, where the laminin – β-dystroglycan interaction permits sarcolemmal-localized α-syntrophin mediated phosphorylation of Akt (Zhou and others 2007).

The mu splice variant of nNOS (nNOSμ) is normally bound to α-syntrophin and thus anchored near the sarcolemma. nNOSμ has been identified as a key signaling protein that regulates muscle mass in response to changes in loading, augments force production, and reduces muscular fatigue (Kobayashi and others 2008; Percival and others 2008; Percival and others 2010). Here lifelong CRWR resulted in a non-significant ($p = 0.08$) increase of total nNOS compared to sedentary, ad-libitum aging, and was not impacted by aging. Sarcolemmal nNOS was drastically mislocalized in aged plantaris muscle fibers, where it was observed predominately in the sarcoplasm. Previously, our group (Lawler and others 2014) and Suzuki *et al.* (Suzuki and others 2007) found that mechanical unloading and denervation cause translocation of nNOSμ away from the sarcolemma. Mislocalization of nNOS is also noted in Duchenne muscular dystrophy (Brenman and others 1995) and other types of neuromuscular disorders (Finanger Hedderick and others 2011), further emphasizing the physiological importance of the protein for proper muscle function to occur.

The current study identified a loss of sarcolemmal, but not overall nNOS. Recent aging literature indicates that nNOS location, muscle, and physical activity have impacted age-related changes. We previously observed that constitutive NOS activity is reduced by aging (Song and others 2009). Overall nNOS protein levels were reduced in the soleus, but not the rat gastrocnemius. Pearson *et al.* (Pearson and others 2015) did not find a reduction in total nNOS protein abundance from old C57Bl/6 mice. In contrast, Samengo *et al.*, showed a 41% decline of nNOSμ expression levels in the quadricep muscles of 2 year old mice along with a concomitant decrease in protein content (Samengo and others 2012). In our study, CR and CRWR, proved to be effective at maintaining sarcolemmal nNOS compared to OAL fibers (Fig. 2). Previously, we found that exercise training increased nNOS protein expression in both muscles (Song and others 2009). Lieter *et al.* (2012) found that cytoplasmic localization of nNOS is greater in quadriceps of 19.5 month old mice vs. young mice, which could exacerbate proteolysis and atrophy (Suzuki and others 2007). However, 6 weeks of administration of an NO-donor drug and 3 weeks of exercise in 18 month old mice prevented mislocalization of nNOS, concomitant with muscle hypertrophy (Leiter and others 2012). Consistently, loss of sarcolemmal nNOS was causal in skeletal muscle fiber cross-sectional area during disuse (Lawler and others 2014; Suzuki and others 2007). The physiological importance of DGC localization of nNOSμ for healthy regulation of skeletal muscle size, damage, and repair is substantial. Therefore, lifelong CR and CRWR preserves a more continuous membrane patterning of key DGC proteins (dystrophin, α-syntrophin, and nNOS), which could improve membrane and mechano-signaling, and protect against sarcopenia.

4.3. Effect of aging on sarcolemmal repair

Given that aging was associated with a discontinuous loss of DGC proteins (dystrophin, α-syntrophin, nNOS) from the sarcolemma, we postulate that aging may be conferring a “mild dystrophic phenotype” on skeletal muscle. Since the DGC offers stability to the sarcolemma (Blake and others 2002), a weakened DGC could compromise sarcolemmal integrity and increase the risk of membrane leakage and fiber damage (Petrof and others 1993). Indeed, loss of sarcolemmal dystrophin results in muscle fibers that are more prone to contraction-induced injury, due to impaired ability of lateral force transference from the Z-disc through the costameres into the ECM (Lovering and De Deyne 2004). When dystrophin is lost, shear forces are elevated which can trigger ROS and Ca2+ mediated damage to the muscle fiber with repeated eccentric contractions (Lovering and De Deyne 2004). Compromised DGC leads to uncontrolled damage-repair cycling, fiber death, and eventual replacement with fibrotic tissue (Mann and others 2011; Serrano and others 2011). Therefore, an intact plasma membrane environment and efficient repair of the cell membrane are crucial to maintenance of cellular homeostasis.

When stressed by mechanical and biochemical (e.g., lipid peroxidation) perturbations, a cadre of membrane repair proteins guide vacuole sequestration, repair, and patching of damaged phospholipid membranes. Specifically, MG53, dysferlin, and annexin A6 are sequestered to membrane lesions to repair and reseal damaged sarcolemma, followed by recruitment of annexins A2 and A1 to complete scaffolding of the membrane patch. In this coordinated membrane repair, MG53 is recruited to the site of injury followed by dysferlin,

annexin A6, and then annexins A1 and A2 (Cai and others 2009; Roostalu and Strahle 2012).

Given that old sedentary, fed *ad libitum* expressed high levels of membrane repair proteins (MG53, dysferlin, annexin A6, annexin A2) (Fig. 3), we postulate that there is an enhanced requirement for phospholipid membrane repair in aging skeletal muscle. Lifelong exercise + mild caloric restriction (diet) maintained membrane repair proteins near control (YAL) levels, suggesting protection against cell membrane damage and thus the need for heightened repair (Fig. 3). Interestingly, CR alone was unable to maintain membrane repair proteins at YAL levels, particularly for annexin A6 (Fig. 3). Thus chronic exercise is required to better protect the environment of the aging sarcolemma. Indeed, we previously noted (Kim and others 2008) that lifelong wheel running plus mild CR abrogated age-related elevation of total hydroperoxides; while caloric restriction alone did not prevent age-related elevation of lipid hydroperoxides. Cagliani *et al.* observed annexin A1 and A2 levels were overexpressed in parallel in *mdx* mice, a model of Duchenne muscular dystrophy characterized by high levels of membrane damage and lipid peroxidation (Cagliani and others 2005). Thus lipid peroxidation and upregulation of membrane repair proteins are characteristics shared by aging and dystrophic muscle.

In contrast, lifelong wheel running mitigated age-associated upregulation of MG53, dysferlin, annexin A6, and annexin A2 (Fig. 3). Given that Alloush *et al.* reported 8 weeks of treadmill exercise training in C57Bl/6 mice had no effect upon three repair proteins (MG53, dysferlin, caveolin-3) in the soleus muscle, long-term physical activity would then logically be a preservation effect. Thus we would argue that habitual exercise maintains long-term homeostasis of the lipid membrane environment, linked to preservation of the DGC, and reduction of sarcopenia.

4.4. Nox2 and aging muscle

Oxidative stress increases during the aging process in skeletal muscle, as with most tissues. (Ji and others 1998; Kim and others 2008). High levels of oxidative stress have been linked to sarcopenia: muscle fiber CSA, muscular strength, and performance (Bejma and Ji 1999; Kim and others 2008). Sources of ROS in skeletal muscle include the mitochondrial electron transport chain, prostaglandin metabolism, xanthine oxidase, and NADPH oxidases (Nox).

Nox2 is a multi-protein enzyme complex that consists of membrane-bound catalytic gp91phox and regulatory p22phox, and regulatory cytosolic subunits: p47phox, p67phox, and the small-GTPase Rac1. Nox2 has been found in the sarcoplasmic reticulum (Xia and others 2003), t-tubules (Espinosa and others 2006), and the sarcolemma (Javeshghani and others 2002). Nox2 is the most likely source of $O_2^{\bullet -}$ in skeletal muscle during contraction and stretch of healthy skeletal muscles, and not mitochondria (Sakellariou and others 2013). However, Nox2 becomes hyper-responsive to stretch in dystrophic mice, contributing to excessive Ca(2+) entry into the fibers and fueling debilitating myopathy (Whitehead and others 2010). Furthermore, Nox2 is elevated during mechanical unloading and may contribute to disuse atrophy (Lawler and others 2014).

Surprisingly, we found that *ad libitum*, sedentary aged (OAL) rats contained significantly lower protein levels of cytosolic Nox2 subunits: p67phox and p47phox. In addition, the microtubule-associated subunit Rac1 exhibited a similar trend downward with age. Recently, Barrientos *et al.* found that p47phox displayed a non-significant downward trend with age (p=0.08) (Barrientos and others 2015), while Ryan and colleagues reported that total NADPH oxidase activity in the gastrocnemius was unaltered by age, following protein trends for the Nox4 isoform (Ryan and others 2010). Therefore, it is possible age-related changes in Nox2 expression may vary with subcellular localization, species, and independent of Nox4 protein expression. We postulate that the aging membrane environment may be unsuitable for normal response of Nox2 to contractions. Whereas lifelong physical activity may act to preserve normal Nox2 complex structure via maintaining a healthy membrane environmental phenotype. Here we found that lifelong wheel running maintained high abundance of gp91phox and p47phox (Fig. 4). Thus we argue that inducibility of the Nox2 complex by exercise is maintained in healthy aging when exercise is chronic or habitual.

Given that cellular oxidative stress is increased in aging skeletal muscle, (Lawler and others 2014; Sakellariou and others 2014; Whitehead and others 2010), it can be inferred that the sources independent of Nox2 may drive age-related oxidative stress. In young healthy muscle, Nox2 participates in contractile – induced cell signaling (Khairallah and others 2012; Sakellariou and others 2013) and insulin signaling (Contreras-Ferrat and others 2014; Espinosa and others 2009). However, overproduction of Nox2 contributes to pathology with Duchenne muscular dystrophy (Khairallah and others 2012; Whitehead and others 2010), hypertension (Frey and others 2009), and possible disuse (Lawler and others 2014). In contrast, baseline ROS released from mitochondria are elevated with Type II diabetes and aging (Anderson and others 2009; Janssen-Heininger and others 2008). Therefore, the yin-yang relationship between Nox2 and mitochondria may be substantially disrupted with aging, yet protected by lifelong diet and exercise interventions. Clearly, additional investigations are necessary to unravel this intriguing mystery.

4.5. Limitations

Experimental design and tissue limitation did not allow sarcolemmal localization of Nox2 and repair proteins or membrane damage, other than previous morphological histology (Kim and others 2008). Unfortunately, we were unable to inject the rats with Evans blue dye to determine damage and necrosis since that could have interfered with other experimental objectives. Due to a limited amount of tissue reserved for histochemical analysis, we were also unable to stain the plantaris muscles with IgG in an effort to examine tissue damage. These are unfortunate short-comings that should definitely be addressed in future studies in order to provide more details and connections as to the sarcolemmal integrity issues that appear to be occurring in aged animals. However, we were able to show that proteins that are normally associated with membrane repair were elevated in OAL muscle. Another drawback is that we only used male rats, leaving the question of potential sex-related differences unanswered. In addition, tissue mass and assay demands limited our ability to isolate and analyze mitochondrial ROS. Since the experimental design did not call for a lifelong WR only exercise group because 8% CR is needed to encourage voluntary lifelong wheel

running activity, we were unable to differentiate the effects of WR from the effects of CR. We have previously shown that wheel running may have additive or synergistic protection against age-related changes in morphology and cell signaling (Kim and others 2008).

5. Conclusions

In summary, aging was associated with a disruption of the membrane environment, affecting sarcolemmal expression of dystrophin-glycoprotein complex proteins, membrane repair proteins, and Nox2 subunits. Furthermore, age-related perturbations in DGC, membrane repair proteins, and Nox2 subunits were consistently mitigated by lifelong mild caloric restriction and voluntary wheel running. These highly novel findings in protective cell signaling conferred lifelong mild caloric restriction and voluntary exercise were also linked to significant abrogation of aging-associated reduction in relative muscle weight and CSA. Therefore, lifelong CR and WR may prevent age-related impairment of overall muscle quality and sarcopenia, which could yield better quality of life and resistance to chronic disease and frailty.

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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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Highlights

- Lifelong CR and WR maintains continuous sarcolemmal DGC proteins compared to OAL.
- Age-related increase in membrane-repair proteins is mitigated by OCR and OCRWR.
- Age-induced reduction of Nox2 subunits was prevented by OCR and OCRWR.

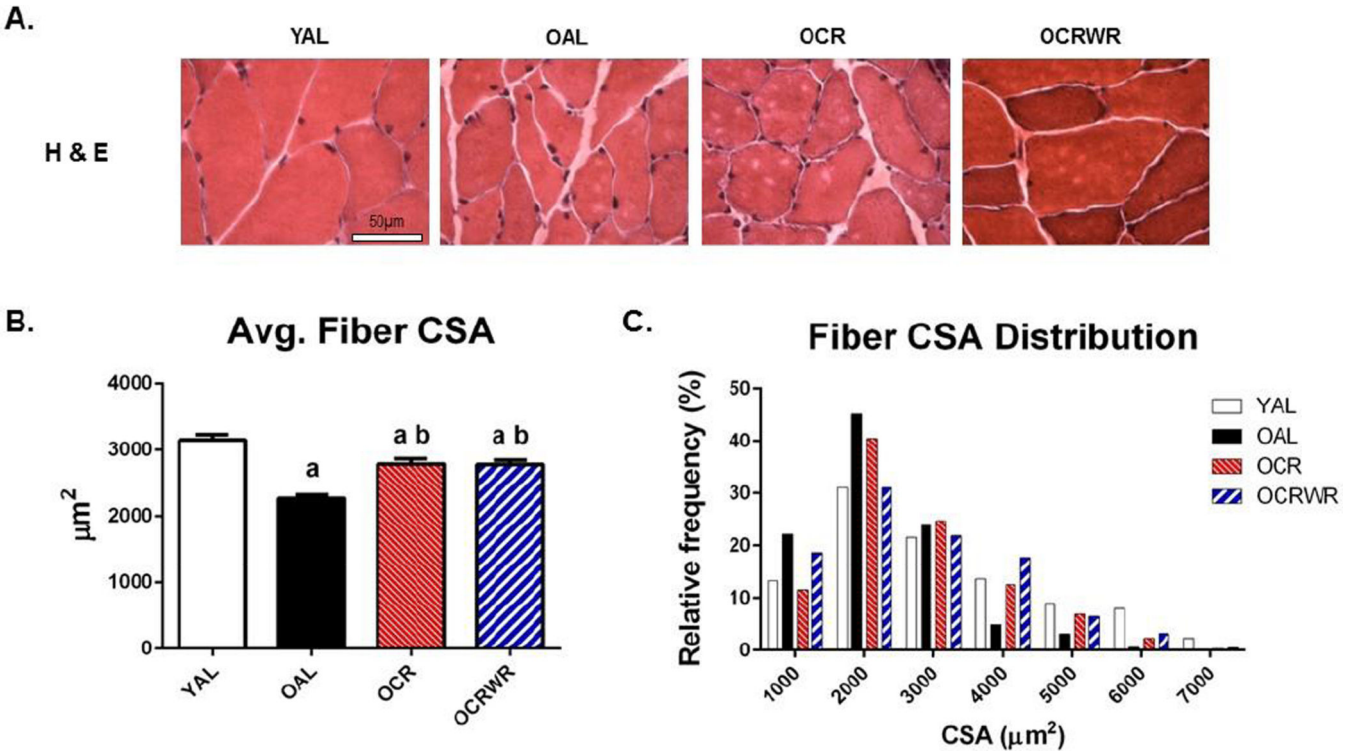

Fig. 1.

Cross-sectional area (CSA) of plantaris muscle fibers from young (6 months), old *ad libitum* fed rats (24 months), old, mild calorie restricted rats (8% CR, 24 months) and old, mild calorie restricted plus exercise rats (8% CR + WR, 24 months). (A) Hematoxylin and Eosin (H&E) stains of plantaris muscle fibers; (B) Average muscle fiber CSA; (C) Muscle fiber CSA frequency expressed as percentage. Data are presented as mean ± standard error of the mean (SEM). Significance level was set a $p < 0.05$. (a) Indicates significant difference from YAL group; (b) Indicates significant difference from OAL group. Representative images in (A) were taken at 40× magnification. Scale bar set at 50 μm.

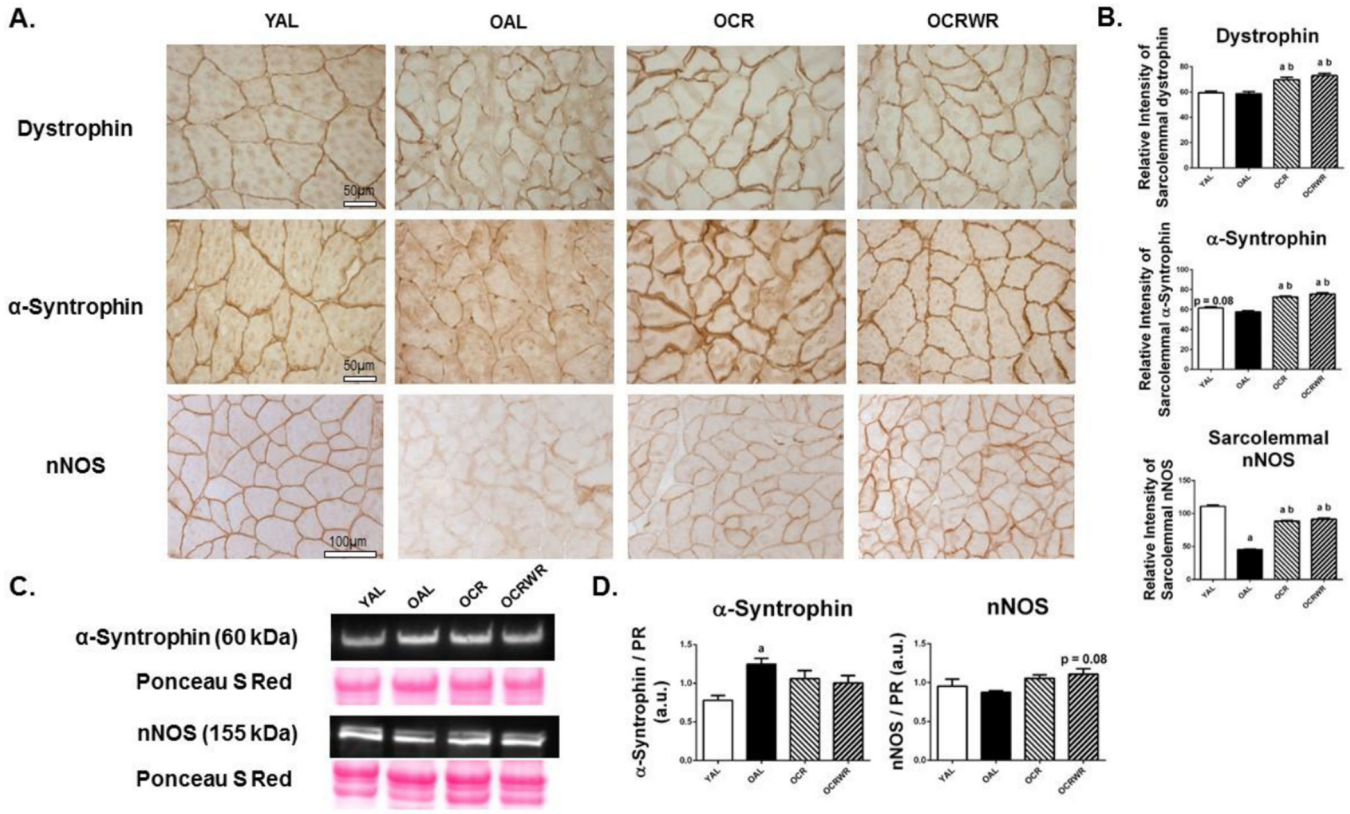

Fig. 2.

Immunostaining of dystrophin glycoprotein complex (DGC) proteins in plantaris muscle fibers from young *ad libitum* (YAL), old *ad libitum* (OAL), old - mild calorie restricted rats (OCR) and old - mild calorie restricted plus exercise rats (OCRWR). (A) Dystrophin immunostaining (top row), α-syntrophin immunostaining (middle row), and nNOS immunostaining (bottom row); (B) Quantification of sarcolemmal immunoreactivity of dystrophin (top graph), α-syntrophin (middle graph), and nNOS (bottom graph); (C) Representative western blots of α-syntrophin and nNOS proteins; (D) Western blot quantification. Data are presented as mean ± standard error of the mean (SEM). Significance level was set a $p < 0.05$. (a) Indicates significant difference from YAL group; (b) Indicates significant difference from OAL group. Dystrophin and α-syntrophin images were taken at 20× magnification (scale bar set at 50 μm) and nNOS images were taken at 16× magnification (scale bar set at 100 μm). Ponceau S Red stain (GAPDH – 38 kDa mark) used as a loading control for western blots.

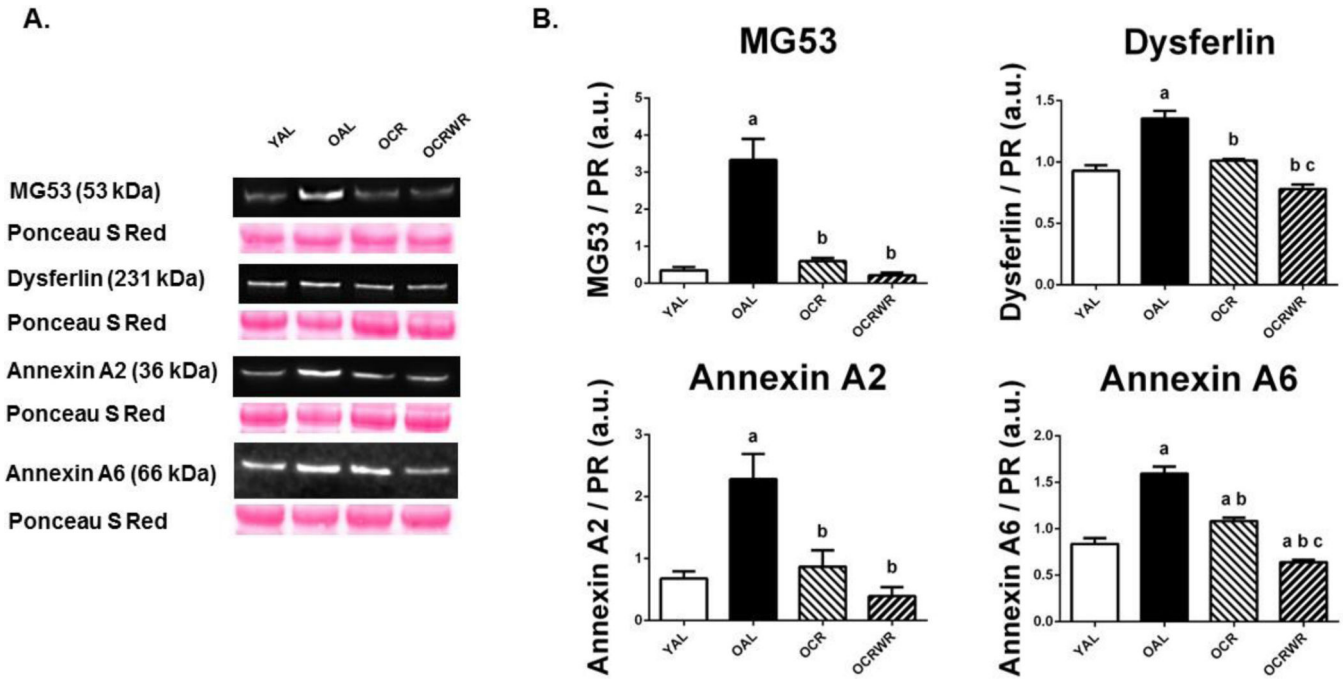

Fig. 3.

Protein content of select membrane repair-associated proteins from plantaris muscles from YAL, OAL, OCR, and OCRWR rats. (A) Representative western blot with corresponding Ponceau S Red stains of select membrane repair proteins (top to bottom: MG53, dysferlin, annexin A2, annexin A6); (B) Graphical output of the western blots. Data are presented as mean ± standard error of the mean (SEM). Significance level was set a $p < 0.05$. (a) Indicates significant difference from YAL group; (b) Indicates significant difference from OAL group. (c) Indicates significant difference from OCR group. Ponceau S Red stain (GAPDH – 38 kDa mark) was used as a loading control for western blots.

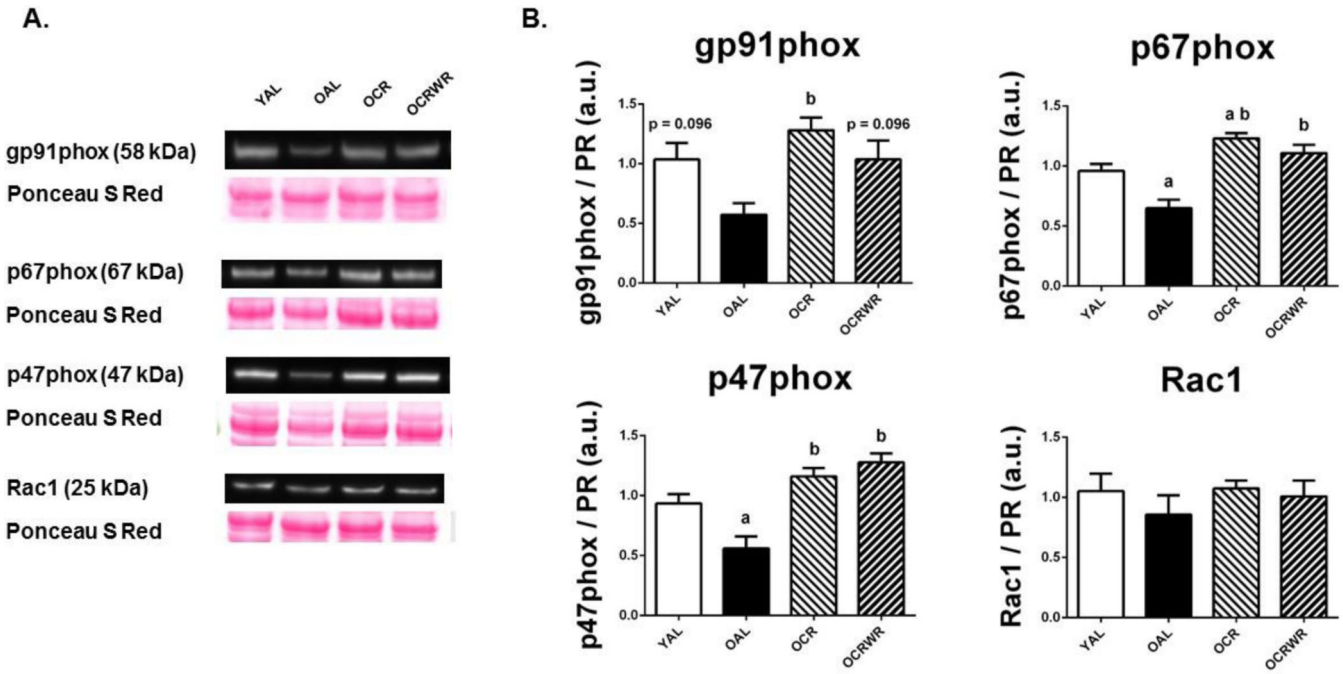

Fig. 4.

Protein content of select NAD(P)H Oxidase isoform 2 (Nox2) subunits from plantaris muscles from YAL, OAL, OCR, and OCRWR rats. (A) Representative western blots with corresponding Ponceau S Red stains of select Nox2 subunits (top to bottom: gp91phox, p67phox, p47phox, Rac1); (B) Graphical output of the western blots. Data are presented as mean ± standard error of the mean (SEM). Significance level was set a $p < 0.05$. (a) Indicates significant difference from YAL group; (b) Indicates significant difference from OAL group. Nonsignificant trend (p = 0.096) present comparing YAL to OAL and OCRWR to OAL. Ponceau S Red stain (GAPDH – 38 kDa mark) was used as a loading control for western blots.