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Lifelong Exercise and Mild (8%) Caloric Restriction Attenuate Age-induced Alterations in Plantaris Muscle Morphology, Oxidative Stress and IGF-1 in the Fischer-344 Rat

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Abstract

Muscle atrophy is a highly prevalent condition among older adults, and results from reduced muscle mass and fiber cross-sectional area. Resistive exercise training and moderate (30–40%) caloric restriction may reduce the rate of sarcopenia in animal models. We tested the hypothesis that lifelong, voluntary exercise combined with mild (8%) caloric restriction would attenuate the reduction of muscle fiber cross-sectional area in the rat plantaris. Fischer-344 rats were divided into: young adults (6 mo.) fed *ad libitum* (YAL); 24 mo. old fed *ad libitum* (OAL); 24 mo. old on 8% caloric restriction (OCR); lifelong wheel running with 8% CR (OExCR). Plantaris fiber cross-sectional area was significantly lower in OAL than YAL (–27%), but protected in OCR and OExCR, while mass/body mass ratio was preserved in OExCR only. Furthermore, 8% CR and lifelong wheel running attenuated the age-induced increases in extramyocyte space and connective tissue. Citrate synthase activity decreased with age, but was not significantly protected in OCR and OExCR. Total hydroperoxides were higher in OAL than YAL, but were not elevated in OExCR, with no change in MnSOD. IGF-1 levels were lower in OAL (–57%) than YAL, but partially protected in the OExCR group (+51%).

Keywords

skeletal muscle; aging; exercise; caloric restriction; plantaris

INTRODUCTION

Sarcopenia, a reduction in muscle mass and function, is one of the most apparent and consistent phenomenon associated with aging, and has been documented even in healthy elderly (Doherty, 2003; Rosenberg, 1997). Age-induced sarcopenia is associated with weakness and frailty, and is a product of a decrease in muscle fiber cross-sectional area and a reduction in the number of fibers (Brooks and Faulkner, 1994; Bua et al., 2002; Chung and Ng, 2006). In addition to a

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loss of muscle mass, a deterioration of muscle quality, or force generation per cross-sectional area also occurs with advancing age. Moreover, the amount of connective tissue also increases with aging, resulting in increased internal work, which in turn leads to increased energy requirement and thus reduced mechanical efficiency. Thus morphological changes in skeletal muscle with aging include a reduction of fiber cross-sectional area, loss of fiber number, and increases in extramyocyte space and connective tissue (Caccia et al., 1979; Chung and Ng, 2006; Grimby and Saltin, 1983; McCarter and McGee, 1987; Payne et al., 2003). The etiology of age-associated changes in muscle morphology is believed to be multifactorial, and includes reduced physical activity, increased insulin resistance, and impaired endocrine function (e.g., low testosterone, growth hormone, and thyroxine) (Evans, 2004). The consequences of sarcopenia and subsequent frailty include an estimated health care cost of \$130 billion by 2030 (Morley et al., 2002).

Muscle fiber atrophy and fiber loss are directly related to decrements in force production, and may be regulated by reduced satellite cell function, myonuclei loss, and mitochondrial dysfunction (Allen et al., 1999; Bua et al., 2002; Holloszy et al., 1991; Leeuwenburgh et al., 2005; Peterson, 1995; Siu et al., 2004; Siu et al., 2005; Song et al., 2006; Webster and Blau, 1990). Furthermore, the aging patterns of sarcopenia and increased connective tissue differ depending on the gender and muscle fiber type (Daw et al., 1988). For example, muscles with a high proportion of fast-twitch fibers display greater decrements in fiber number and fiber cross-sectional area with advancing age (Bua et al., 2002; Holloszy et al., 1991).

Lifelong moderate caloric restriction, ranging from 30%–40% less than *ad libitum* feeding without malnutrition, attenuates age-associated muscular atrophy (McKiernan et al., 2004) and enhances the life span of rodents and primates (Holloszy, 1997; Masoro, 1995, 2000; McCarter et al., 1997; Merry, 2002). McKiernan et al. (2004) found that lifelong 40% caloric restriction results in a significant decrease in the rate of muscle mass loss, and attenuates age-induced fiber loss. Recent research also indicates that caloric restriction prevents the age-induced decrease in muscle contractile force per cross-sectional area (Mayhew et al., 1998; Payne et al., 2003). Moreover, caloric restriction compensated for decrements in muscle mass specific $\text{VO}_{2\text{max}}$ (maximal aerobic capacity) and mitochondrial oxidative capacity that occur with aging (Hepple et al., 2005).

The effectiveness of caloric restriction in ameliorating the aging process in skeletal muscle is influenced by (a) onset (early in lifespan vs. late in lifespan), (b) duration (short-term vs. long-term), and (c) intensity (mild vs. moderate) of the restriction regimen. Weindruch et al. (1982, 1986, 2001) demonstrated that caloric restriction imposed before middle (12-month) age is necessary to elongate mean and maximum life span by 10–20% in mice. Moderate (e.g., 40%) caloric restriction for 12 or 18 months consistently reduces oxidative stress and increases lifespan in rodents (Hagopian et al., 2005; Lopez-Torres et al., 2002). Long-term caloric restriction attenuates the age-induced elevation in production of reactive oxygen species (ROS) by mitochondria and oxidative damage to mitochondrial DNA (mtDNA), while short-term caloric restriction does not (Gredilla et al., 2001, 2004). Short-term caloric restriction has only a modest to little effect on mitochondrial ROS production (Bevilacqua et al., 2005; Gredilla et al., 2002, 2004). Long-term, moderate caloric restriction is effective in increasing rodent and rhesus monkey lifespan and protecting against age-induced sarcopenia, but may be difficult to maintain long-term in humans due to adverse health effects (Dirks and Leeuwenburgh, 2006). Currently, it is unknown if a milder (8%) caloric restriction applied over the lifespan attenuates the affects of aging on skeletal muscle morphology.

While resistive exercise, but not endurance training, promotes muscle hypertrophy in young and middle-age subjects, both resistive exercise and endurance exercise may ameliorate sarcopenia and change in muscle morphology in aging populations (Brown et al., 1992; Evans,

1995; Starling et al., 1999; Tarpenning et al., 2004). However, the duration of longitudinal exercise studied is usually 15% or less of mean lifespan in studies using rodents or humans. Thus there is a paucity of longitudinal data examining the effects of lifelong exercise on muscle morphology. McCarter et al. (1997) attempted voluntary lifelong wheel running to protect skeletal muscle morphology. However, wheel running distance in rats fed *ad libitum* decreased with time, and indeed the rats stopped before a year in age, presumably because of a blunted foraging instinct. It has been shown previously that mild (8%) caloric restriction allows rats to continue voluntary wheel running until senescence (Holloszy et al., 1985; Judge et al., 2005; Seo et al., 2006). Holloszy et al. (1985) found that 8% caloric restriction ensures continuance of lifelong voluntary wheel running in rats without an initial reduction of body weight, unlike 30–40% caloric restriction.

Therefore, the purpose of our study was to investigate the influence of mild caloric restriction and wheel running on myofiber cross-sectional area, and extramyocyte space in plantaris muscle, which contains a high percentage (94%) of fast-twitch fibers (Delp and Duan, 1996). We hypothesized that lifelong mild caloric restriction and wheel running would attenuate the age-induced muscle atrophy, decrease in fiber cross-sectional area, and increase in connective tissue. We further postulated that lifelong exercise protection would be linked to lower oxidative stress and increased stress protection (superoxide dismutase and IGF-1).

METHOD

Animals

Male Fischer-344 rats were purchased from Harlan Sprague Dawley (Indianapolis, IN) at 10–11 weeks of age. All animals were housed in an isolated room within the University of Florida's Animal Care Services facilities until time of sacrifice at 6 and 24 months of age. Rats were housed one per cage in a ventilated room under pathogen-free conditions at a temperature $20 \pm 2.5^\circ\text{C}$ and kept on a 12h light: 12h dark diurnal cycle. All animals were given access to water and rat chow (Harlan Teklad Rodent Diet #8604) consisting of a high protein (24.5%), carbohydrate including hemicellulose (46.6%), and low fat (4.4%) mix including adequate vitamins and minerals to ensure that the caloric restricted groups were not malnourished. The animals used in this study showed no significant symptom of disease related distress prior to sacrifice. All experimental procedures had been previously approved by the University of Florida's Institute on Animal Care and Use Committee.

Experimental design and exercise protocol

To test the effects of mild caloric restriction (8%) and wheel running on fiber morphology of the skeletal muscle, rats were acclimatized at 10 weeks of age to the animal facilities for one week. Then rats were assigned to one of the following four groups: 1) 6-month-old sedentary *ad libitum* group (YAL; n=12), 2) 24-month-old sedentary *ad libitum* group (OAL; n=12), 3) long-term 8% caloric restriction from 11 or 12 weeks until 24 months (OCR; n=12), 4) lifelong 8% caloric restriction and voluntary wheel running from 11 or 12 weeks until 24 months (OExCR; n=12). Rats fed *ad libitum* tend to abruptly decrease their running activity by six months (McCarter et al., 1997). However, mild food restriction (8–10%) has been previously shown to prevent the decline in voluntary wheel running with age (Holloszy et al., 1985; Judge et al., 2005; Seo et al., 2006). Eight percent caloric restriction has been in minimum needed to encourage lifelong wheel running, and was chosen for this study. Food intake for both OCR and OExCR groups was therefore restricted by 8% below the *ad libitum* food intake of a separate group of sedentary, age-matched, male Fischer 344 rats. Throughout the duration of the study, the amount of food intake of OCR and OExCR group was modified weekly using direct comparison with food intake in the *ad libitum* group from the previous week. Wheel

running and caloric restriction regimens were commenced at 11 or 12 weeks of age, and continued through 24 months of age and time of sacrifice.

Diet and housing were maintained as previously described (Judge et al., 2005). All rats of sedentary group were housed in standard rodent cages (19.0 × 10.5 × 8.0 in) from Fisher Scientific (Pittsburgh, PA), and rats for the OExCR group were housed in the same cage assembled with Nalgene Activity Wheels purchased from Fisher Scientific, within the same facilities as the non-exercise groups. Rats in the exercise group were provided unrestricted access to the running wheels, which served as mild intensity exercise. Body weights were recorded weekly. Running wheels were 1.08 meters in circumference and fitted with a magnetic switch and LCD counter. The number of wheel revolutions per day was recorded for wheel running and the distance was calculated. The calculated average running distance for OExCR group was 666 ± 160 m/day during the first month of training, peaked at 2500 m/day at 6 months of age, leveled off, and slowly decreased to 943 ± 175m/day during the last month of training at 24 months of age (Seo et al., 2006). These data indicate that mild caloric restriction was capable of stimulating voluntary wheel running activity throughout the lifespan.

Tissue Preparation

Rats were anesthetized with 4% isoflurane 1L/min O₂ and maintained with 1.5–2.5% isoflurane 1L/min O₂ and body mass was measured. The plantaris muscles were removed, trimmed off fat and tendon tissue, weighed, and washed in PBS (4°C). Absolute plantaris mass, muscle mass/body mass, and muscle fiber cross-sectional area were measures as markers of sarcopenia and muscle integrity. The muscle mass to body mass ratio is used in rodents as a functional marker due to continued longitudinal growth throughout the lifespan of a rat. Muscle samples for cell morphological analysis were washed with PBS, weighed, snap frozen in liquid nitrogen (−180°C) at optimal length (L₀) in O.C.T., then stored at −80°C until subsequent analysis. Plantaris samples used in biochemical analyses of protein were snap frozen in liquid nitrogen and stored at −80°C until analyses. The plantaris muscle is a primarily fast-twitch muscle, consisting of 80% Type IIb and Type IIc, 14% Type IIa, and 6% Type I fibers (Delp and Duan, 1996).

Morphological analysis

Morphological analysis and tissue sectioning were conducted as described previously (7). Briefly, plantaris muscles (n=6/group) were placed to a cryostat (Shandon) pre-cooled to −20°C and cut into serial transverse cross-sections at 10 μm in thickness. The cross-sections were cut from the mid-belly of each muscle and placed on a slide. Plantaris cross-sections were then air-dried for 30 min. Then we assessed muscle fiber number, cross-sectional area, and % connective tissue via extramyocyte area on all discernible muscle fibers against a micrometer.

The muscle section staining procedure and analysis have been previously described by Kwak et al (2006). Briefly, we incubated each section with two drops of hematoxylin (HTX) to distinguish fibers from connective tissue, and incubated for 1 min at room temperature. Hematoxylin stains a bluish-purple color for myocytes and mitochondria, while nuclei stain dark blue. Connective tissue and fat are lightly stained except for nuclei. All samples were exposed to identical conditions among all 4 experimental groups. Stained sections were then rinsed with PBS and were air-dried for 30 min before mounting in Vectamount medium (Vector Laboratories: Burlingame, CA). Mounted muscle cross-sections were then dried for 1 hour prior to analyses.

Plantaris cross-sectional images were visualized and captured using a BioQuant TrueColor image analysis system (R&M Biometrics, BQTCW98, version 3.50.6) at an optical magnitude of 40X. For measurement of muscle fiber number, cross-sectional area, and % extramyocyte

area, muscle fiber membrane perimeters were traced and quantified using the NIH Image J analysis software program. Twelve to fifteen pictures per slide sample were analyzed. The total average area within the membrane outline were used to determine the cross sectional area per unit and expressed in μm^2 . Extramyocyte space, used as an indicator of connective tissue and fat, was calculated directly and also checked by calculating the difference between the total area of the image and total cross sectional area. Extramyocyte space was defined as space outside the myocytes consisting of connective tissue (i.e., collagen, laminae), blood vessels, and interstitial fluid (Hatakenaka et al., 2001). Muscle cell area and extramyocyte area were calibrated against images taken using a stage micrometer.

Extramyocyte connective tissue was estimated via collagen staining using an adaptation of the Masson's Trichrome technique. Briefly, 10 μm frozen plantaris cross-sections were cut at -16°C and placed on a slide. After a 20 min drying period, slides were placed in a Columbia jar and fixed overnight at room temperature in Bouin's solution. Slides were then rinsed in distilled water for 3 min, the running tap water for 5 min. Cross sections were then stained in Weigert's hematoxylin for 15 min, and washed in distilled water, then running tap water for 5 min. Muscle fibers were then stained with 1% Biebrich scarlet-acid fuchsin for 15 min, then washed in dH_2O for 5 min. After differentiation in 2.5% phosphomolybdic-phosphotungstic acid solution for 15 min, sections were transferred directly into 2.5% aniline blue solution for 12 min. Plantaris sections ($n=6/\text{group}$) were differentiated in 1% acetic acid solution for 3 min, dehydrated in 95% and 100% ethanol, then cleared in xylene. In this technique, skeletal muscle fibers stain a bright red and collagen fibers bold blue. 12–15 images/section were captured on a Zeiss Axioplan Vision-series microscope and software, and quantified using the NIH Image J analysis program.

Western Immunoblot Analysis

Protein expression for the Mn-isoform of superoxide dismutase (MnSOD), Cu,Zn isoform of superoxide dismutase (Cu,ZnSOD), and insulin-like growth factor-1 (IGF-1) was determined by Western immunoblot analysis. Separating gel [375 mM Tris-HCl; pH=8.8; 0.4% sodium dodecyl sulfate (SDS); 10% acrylamide] and stacking gel [125 mM Tris-HCl; pH=6.8; 0.4% SDS; 10% acrylamide monomer] solutions were made, and polymerization then was initiated by N,N,N',N' - tetramethylethylenediamine (TEMED) and ammonium persulfate (APS). Separating and stacking gels were then quickly poured into a Bio-Rad Protein III gel-box (Bio-Rad, Hercules, CA). Proteins (30 μg) from skeletal muscle homogenates in sample buffer (100 mM Tris-HCl, pH=6.8, 2% SDS, 30 mM dithiothreitol, 25% glycerol) were then loaded into the wells of the 10% polyacrylamide gels, and electrophoresed at 150V. The gels were then transferred (30V) overnight onto a nitrocellulose membrane (Bio-Rad, Hercules, CA). Membranes were blocked in 5% nonfat milk with 0.1% Tween-20 in PBS for 7 hours.

Following blocking, membranes were incubated at room temperature for 12 hours in PBS with the appropriate primary antibodies: MnSOD (1:50,000; Stressgen; Victoria, BC), Cu,ZnSOD (1:10,000; Santa Cruz Biotechnology; Santa Cruz, CA), and IGF-1 (1:1000; Upstate; Lake Placid, NY). Following three washings using PBS with 0.4% Tween-20, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies in PBS at room temperature for 90 min. Following 3 washes in PBS with 0.4% Tween-20, an enhanced chemiluminescence detection system (Amersham, Piscataway, NJ) was used for visualization. Densitometry and quantification were performed using a Kodak film cartridge, a scanner interfaced with a microcomputer, and the NIH Image J analysis software program. In order to ensure equal loading of protein, Ponceau-S-staining was performed for each membrane and the lane background reading was subtracted from each protein blot density. Protein expression was quantified as area times grayscale density relative to background per mg protein.

Citrate Synthase

Citrate synthase was measured as described previously (Lawler et al., 1993). It was used as a marker of oxidative capacity in skeletal muscle and is indicative of mitochondrial density and function.

Total Hydroperoxides

We measured total hydroperoxides as a marker of oxidative stress as described previously (Lawler et al., 2006). Briefly, lipid hydroperoxides were with FeSO_4 (250 μM) in sulfuric acid (25 mM), and a reactive dye (xylenol orange: 100 μM). The principle relies on the oxidation of Fe^{2+} to Fe^{3+} when hydroperoxides are reduced. Ionized sulfuric acid assists the reduction and prevents spontaneous reaction of Fe^{2+} with xylenol orange, unless oxidized by hydroperoxides first. The Fe^{3+} reacted with the xylenol orange to form a Fe^{3+} -xylenol complex purple in color. Absorbance was then read at 580 nm. Concentrations of hydroperoxides were quantified against a t-butyl hydroperoxide standard curve.

Statistical Analysis

The muscle mass to body mass ratio, fiber cross sectional area, fiber number, and percent extramyocyte space for 6 to 24 month AL rats as well as 24 month OCR, OExCR rats were analyzed by one-way ANOVAs using the SPSS software program. A Fisher's least significant difference test (LSD) was used for the post-hoc comparisons. Significance was established at $P < 0.05$.

RESULTS

Muscle Mass to Body Mass Ratio

Mean body mass in the OAL (389.7 $\pm$ 14g) was not significantly different ($P=0.34$) than YAL rats (374.3 $\pm$ 9.9g). In contrast, the body mass for the OExCR animals (341.3 $\pm$ 5.4g) was significantly lower than the YAL, OAL, and OCR (377.6 $\pm$ 5.4) (Table 1). Plantaris mass in the OAL was not significantly different from YAL. Absolute plantaris mass was less than 10% lower the 8% caloric restricted groups with or without wheel running, compared with young rats fed *ad libitum*. Thus both lifelong caloric restriction and lifelong wheel running protected absolute plantaris mass.

The plantaris mass to body mass ratio of OAL (1.67 $\pm$ 0.04 g/kg) exhibited a downward trend that was not statistically different ($P=0.12$) compared with YAL rats (1.74 $\pm$ 0.03 g/kg) (Fig. 1). In addition, the plantaris mass to body mass ratio of OCR (1.606 $\pm$ 0.024 g/kg) resulted in no significant difference compared with OAL rats. In contrast, the plantaris mass/body mass ratio was significantly higher for OExCR rats (1.80 $\pm$ 0.04 g/kg) compared with OAL ($P < 0.01$) and OCR ($P < 0.001$) groups (Fig. 1), indicating effectiveness of combined lifelong voluntary exercise and mild caloric restriction in preserving relative muscle mass.

Fiber Cross-Sectional Area and Morphology

Hematoxylin stained samples revealed qualitatively that plantaris muscle fibers were smaller with less angular, more rounded shape in the old *ad libitum* group compared with young *ad libitum* (Fig. 2). In contrast, the OCR and especially, OExCR groups exhibited plantaris muscle fiber shapes resembling more closely those observed in the YAL group, rather than OAL. Fibers in OCR and OExCR were more angular with 4–6 discrete sides. Large increases in extramyocyte space were observed in the OAL compared to YAL, which was attenuated in the OCR and OExCR groups (Fig. 2). Quantitative assessments of group mean differences in muscle morphology follow.

The cross-sectional area of plantaris muscle fibers is critical in the identification of muscular atrophy. As hypothesized, there was a significant decrease (-27%) in the mean cross-sectional area (μm^2) of plantaris muscle fibers from OAL rats ($1921 \pm 80\mu\text{m}^2$) compared with YAL rats ($2644 \pm 93\mu\text{m}^2$) (Fig. 3). In contrast, both lifelong caloric restriction alone ($2478 \pm 158\mu\text{m}^2$) and in combination with lifelong, voluntary wheel running ($2353 \pm 83\mu\text{m}^2$) were able to significantly attenuate the age-induced decreases in fiber cross-sectional area of the plantaris by $+29\%$ and $+22\%$, respectively. Moreover, mean fiber cross-sectional area was not significantly different among YAL, OCR, and OExCR. As a result, the number of plantaris fibers per field cross-sectional area was 28% higher in OAL rats than in the YAL rats ($P < 0.001$), simply reflective of reduced fiber cross-sectional area and not hyperplasia. As predicted from cross-sectional area data, fiber number/ mm^2 was not statistically different among YAL, OCR, and OExCR (data not shown).

Changes in Extramyocyte Space and Connective Tissue with Age

Aging increased the amount of connective tissue and fat in the plantaris muscles as quantified by extramyocyte area. The percentage of extramyocyte space was significantly higher in the OAL group ($9.59 \pm 1.60\%$) compared with plantaris muscles in YAL ($2.57 \pm 0.68\%$) (Fig. 4). Thus extramyocyte space was 3.7 fold greater with aging in the rat plantaris muscle. Conversely, plantaris muscles in old rats that underwent long-term mild (8%) caloric restriction exhibited significantly lower connective tissue area ($3.15 \pm 0.50\%$) than old *ad libitum* fed rats. In other words, accumulation of extramyocyte space was attenuated by $2/3$ as a result of mild caloric restriction. Similarly, lifelong voluntary wheel running + 8% caloric restriction resulted in a significant attenuation of extramyocyte space ($3.54 \pm 0.85\%$) compared with OAL ($9.59 \pm 1.60\%$). In addition, accumulation of nuclei in the extramyocyte space, outside muscle the sarcolemma and basal membrane, of the OAL group is consistent with proliferation of fibroblasts (Fig. 2). Noteworthy, caloric restriction, particularly when combined with lifelong wheel running, reduced extramyocyte nuclei proliferation. Attenuation of age-induced increases in extramyocyte space by lifelong caloric restriction and lifelong wheel running coupled with muscle fiber cross-sectional area similarly shown to young rats are indicative of preserved muscle morphology.

To determine if the effects of age, 8% CR and lifelong wheel running on extramyocyte space were a function of changes in connective tissue, we used the Masson's trichrome technique to stain differentially for collagen and muscle fibers. Representative sections are displayed (Fig. 5a). It is clear from blue staining outside muscle fiber cross-sections that far more collagen staining is present in muscles from the old rats that were fed *ad libitum* compared with the young *ad libitum* group. There was also strikingly less collagen staining in both the 8% CR group and the wheel running group of 24 months of age compared with old *ad libitum* rats. When connective tissue was quantified, based on collagen staining of the plantaris, we found that % connective tissue increased from 3.7% in the YA group to 9.7% in the OA group (Fig. 5b). Mild caloric restriction result in a reduced amount of connective tissue (5.4%) in old rats compared with age-matched rats fed *ad libitum*. Connective tissue levels in the plantaris were significantly lower in the wheel running group (3.2%) than the old *ad libitum* group. Interestingly, connective tissue training levels were also lower in plantaris sections with the wheel runners compared with 8% CR as well.

As an additional marker of connective tissue accumulation, fibroblast nuclei free in the extracellular spacer were counted and quantified per $100,000 \mu\text{m}^2$. The plantaris in the YAL group displayed an average of 5.0 fibroblasts $\pm 0.4/100,000 \mu\text{m}^2$. The number of fibroblasts in the plantaris samples was 224% higher or 22.0 ± 3.8 fibroblasts/ $100,000 \mu\text{m}^2$ in the old *ad libitum* group. Significant protection against accumulation of fibroblasts was observed with 8% CR, where 7.2 ± 1.3 fibroblasts/ $100,000 \mu\text{m}^2$ were counted or 67% lower than OAL. Lastly,

fibroblast number ($4.2 \pm 0.8/100,000 \mu\text{m}^2$) was also lower (-81%) in plantaris muscles from old wheel runners.

Lipid content was detected using an Oil Red O stain, counterstained with hematoxylin. However, little accumulation of fat in the extracellular space was observed in any of the groups, except for fascia outside the epimysium, regardless of age, feeding status, or exercise participation (data not shown).

Changes in Oxidative Capacity, Oxidative Stress, and Antioxidant Enzymes

In order to begin to understand the potential mechanisms by which lifelong wheel running and mild caloric restriction protected cell morphology in the aging plantaris, we tested the hypotheses that lifelong wheel running would increase oxidative capacity (i.e., citrate synthase), while decreasing oxidative stress (i.e., total hydroperoxide), and increasing antioxidant enzyme levels (i.e., Cu,Zn and Mn-isoforms of superoxide dismutase). Aging in *ad libitum* fed rats resulted in a significant decrease (-20%) in citrate synthase activity (Fig. 6a). Surprisingly, lifelong 8% caloric restriction had no effect on citrate synthase activity levels. Although citrate synthase activity in the wheel exercise group trended upwards, this trend was small ($+6\%$) and not statistically significant.

Total hydroperoxide levels were significantly higher ($+22\%$) in the *ad libitum* aging group compared with young *ad libitum* controls (Fig. 6b). Plantaris total hydroperoxides remained significantly higher ($+35\%$) than young controls in old rats that were exposed to lifelong mild caloric restriction only. However, lifelong wheel running blunted oxidative stress, with total hydroperoxide levels that were not significantly higher than young controls. MnSOD protein expression in the plantaris was not significantly altered by aging, mild caloric restriction, or wheel running (Fig. 7a). Plantaris Cu,ZnSOD protein levels exhibited small but significant increases in the old *ad libitum* ($+16\%$) and wheel running ($+16\%$) groups when compared with young *ad libitum* fed rats (Fig. 7b). However, mean Cu,ZnSOD protein expression was not significantly different between the old *ad libitum* and lifelong exercise groups.

Changes in IGF-1

Aging in *ad libitum* fed rats resulted in a significant decrease (-57%) in IGF-1 protein expression compared with young *ad libitum* controls (Fig. 8). Mild caloric restriction alone did not result in a significant improvement in IGF-1 protein expression compared with the *ad libitum* aging group, although an upward trend was evident. In contrast, wheel running resulted in a significant and substantial increase ($+51\%$) in IGF-1 protein levels in aging rats compared with old *ad libitum* fed rats. However, IGF-1 levels remained significantly below controls (-35%).

DISCUSSION

The major findings of the current study are as follows: mild (8%) caloric restriction attenuated the age-induced reduction in fiber cross-sectional area and increased extramyocyte space of the plantaris muscle. Lifelong wheel running combined with 8% caloric restriction resulted in a higher plantaris mass/body mass ratio than old rats fed *ad libitum* or 8% caloric restriction without exercise. Lifelong wheel running also attenuated age-induced alterations in fiber shape, cross-sectional area, and accumulation of extramyocyte space and connective tissue. To our knowledge, these are the first data to demonstrate that lifelong exercise or mild caloric restriction is able to ameliorate morphological changes that occur in a predominantly fast-twitch muscle as a result of aging. Lifelong voluntary exercise provided partial protection against age-related elevation in oxidative stress and depressed muscle IGF-1 protein expression.

We found higher muscle mass/body mass ratio and fiber cross-sectional area as a result of combined wheel running and mild caloric restriction compared with the old rats fed *ad libitum*. Interestingly, plantaris muscle mass/body mass ratio was not increased by mild (8%) caloric restriction in the current study unless combined with lifelong wheel running. Previous studies have demonstrated that moderate caloric restriction (30–40%) with adequate nutrition results in significant protection against accelerated loss of muscle mass/body mass ratio and fiber cross-sectional area with advancing age in rodents (Dirks and Leeuwenburgh, 2004; McKiernan et al., 2004). Several investigators have noted that the moderate caloric restriction protected against age-induced decreases in muscle weight to body weight ratio (Payne et al., 2003; Selman et al., 2002). Payne et al. (2003) showed that 40% caloric restriction resulted in greater muscle mass/body mass ratio of fast-twitch muscle (e.g., extensor digitorum longus), higher fiber cross-sectional area, and higher specific force (N/cm²), when compared with old rats fed *ad libitum*. Phillips and Leeuwenburgh (2005) also reported that 40% caloric restriction preserved muscle mass/body mass ratio in the superficial vastus lateralis muscle (fast-twitch).

While cage activity appeared to be greater in the caloric restricted groups, the body mass in the CR groups was not lower than old *ad libitum* groups unless mild caloric restriction was accompanied by voluntary wheel running. However, our observations may have an impact on caloric expenditure. Indeed, Judge et al. (2005) found that caloric expenditure was indeed significantly higher in a lifelong wheel running group compared with mild caloric restriction only. In combination with the current data, we postulate that attenuation of age-induced increases in body mass in the OExCR group, compared with the OAL group, requires voluntary wheel running in addition to mild caloric restriction. Higher plantaris mass/body mass ratios observed in old rats which had access to wheel running are consistent with preservation of lean mass and loss of fat mass compared with sedentary, old rats fed *ad libitum*.

It should be noted that in the initial stages of 30–40% caloric restriction, there is significant body mass and muscle mass loss (Dirks and Leeuwenburgh, 2004). After this initial atrophy, muscle mass tends to remain fairly steady in rodents. For example, absolute soleus and EDL mass was reduced by moderate (40%) caloric restriction compared with the *ad libitum* (Payne et al., 2003). Since rats continue to grow longitudinally throughout their lifespan, it can be argued that muscle mass/body mass ratio is a more meaningful marker of muscle preservation and function than absolute mass, and may indicate that both 8% CR and mild exercise could be effective in reducing sarcopenia. Indeed, changes in muscle mass/body mass ratio mirror changes in muscle specific force in rodent studies (Jubrias et al., 1997; Payne et al., 2003).

The ability of mild 8% caloric restriction alone to attenuate age-induced changes in muscle fiber morphology, fibrosis, and cross-sectional area are striking and novel findings. A reduction in fiber cross-sectional area has been associated with a decrease in force generation in response to unloading, aging, and pathological models including denervation and cachexia (Evans, 1997; McDonald and Fitts, 1995; Song et al., 2006; Vasilaki et al., 2003). Previously, 40% caloric restriction was found to protect fiber cross-sectional area in fast-twitch muscle with aging, and lifetime caloric restricted rats were compared with aging rats fed *ad libitum* (Payne et al., 2003; Phillips and Leeuwenburgh, 2005). In the current investigation, fibers in the old *ad libitum* group were consistently misshapen and intersections with two adjoining cells often rounded. In addition, while a few fibers in the plantaris cross-sections rounded and/or ribbon-like morphology, none were detected in old rats that underwent 8% caloric restriction. It is possible that cell shape changes and rounding may be a precursor to apoptosis (Flusberg et al., 2001), although this remains unknown in aging skeletal muscle. Recently, our laboratory demonstrated that 12 weeks of exercise training reduced markers of apoptosis in the white gastrocnemius of old rats (Song et al., 2006). Aspnes et al. (1999) and Bua et al. (2002) also reported that lifelong moderate caloric restriction attenuated apoptosis and fiber loss in aging fast-twitch muscle.

Previously, Payne et al. (2003) established that moderate (40%) caloric restriction attenuated age-related decline in both muscle mass/body mass and force generation as well as increased connective tissue in fast-twitch, but not slow twitch muscle. A direct relationship existed between preservation of fiber cross-sectional area and protection of muscle specific force generation by 40% caloric restriction in fast-twitch muscle (Payne et al., 2003). Thus caloric restriction may have therapeutic and clinical value in preserving specific force in skeletal muscle. Mild caloric restriction is particularly attractive in preserving skeletal muscle morphology and function as it is unlikely to have the potential compromises on health in humans including menstrual irregularities, osteopenia, slower wound healing, loss of stamina, and depression associated with moderate (30–40%) caloric restriction (Dirks and Leeuwenburgh, 2006).

The combination of wheel running with 8% caloric restriction may preserve muscle mass, fiber cross-sectional area and possibly strength, since reduction in muscle mass and muscle strength/body mass have been shown to occur with aging (Payne et al., 2003; Tarpenning et al., 2004). In addition, muscle fiber shape was better preserved in the aging exercise group compared with 24 mo. rats fed *ad libitum*. Differences in morphology clearly indicate that far less mass of an aging muscle is made of myocytes, and that both mild caloric restriction and lifelong wheel running preserve the proportion of muscle made of myofibers. Given that the combination of wheel running and 8% caloric restriction was more effective than 8% caloric restriction alone, mechanical and metabolic stresses of exercise appear to be important in optimizing muscle mass preservation in aging rats. In aging patients, both chronic resistive exercise and endurance exercise appear to attenuate loss of muscle atrophy (Brown et al., 1992; Seynnes et al., 2004; Tarpenning et al., 2004). However, the ability of lifelong wheel running to preserve muscle mass/body mass ratio suggests that even mild, voluntary exercise may be beneficial in protecting against age-induced sarcopenia, if the training period covers a significant portion of lifespan.

Lifelong exercise also protected against accumulation of extramyocyte space and connective tissue in the plantaris. Increased extramyocyte space with aging may be caused by increased connective tissue, increased fat, and increased fluid volume in both rodents and humans (Hatakenaka et al., 2001; Kandarian et al., 1991; Lexell et al., 1988; Payne et al., 2003). While increased connective tissue, water, and fat are all potential contributors in increased extramyocyte space, the large degree (3.9 fold) of elevated extramyocyte space in OAL compared with YAL suggests a potential contribution of connective tissue including the endomysium and perimysium. We found using a Masson's trichrome stain for collagen, that aging did indeed increase connective tissue content in the plantaris (Fig. 5). In addition, 8% CR and wheel running attenuated age-induced increases in connective tissue, although wheel running was more effective. Indeed, extracellular space and connective tissue levels in the plantaris expressed as a percentage of total area in old rats with the combination of lifelong wheel running and 8% CR was similar to that of young rats. Furthermore, the presence of a significant number of extramyocyte nuclei, consistent with fibroblast location (Fig. 2), when quantified was substantially higher in OAL than YAL rats, but reduced in the OCR and especially OExCR groups. We also postulate that large accumulation of extramyocyte space and connective tissue observed in the current study may explain why absolute plantaris mass was not reduced in OAL as expected.

Fat is usually associated with perimysium and epimysium rather than endomysium. Oil Red O staining found no alterations with age, 8% CR or wheel running in the endomysium. Thus large reductions in muscle fiber cross-sectional area (Fig. 3) were accompanied by significant increases in extracellular space and connective tissue (Fig. 4), but not fat. In addition, our data indicate that attenuation of increased extramyocyte space by wheel running and caloric restriction were a more of a function of decreased connective tissue rather than fat.

The use of lifelong voluntary exercise and mild caloric restriction to reduce connective tissue accumulation are significant findings that have potential therapeutic significance. Maximal force generated declines by 1.5% per annum between 65–80 yrs in human patients with about 40% explained by a decline in whole muscle cross-sectional area and specific muscle force, or muscle quality (Jubrias et al., 1997). Increased fat and connective tissue in the extramyocyte space are postulated as significant contributors to specific force reduction with aging (Jubrias et al., 1997; Payne et al., 2003). While connective tissue in the extramyocyte space can contribute to passive (i.e., recoil) tension, it cannot contribute to active tension (Payne et al., 2003). Thus, increased connective tissue elevates internal work decreasing mechanical efficiency, while a reduction in age-related connective tissue could theoretically reduce internal work, improve efficiency, and increase muscle quality. Clearly further study is warranted in this exciting area.

Although the mechanisms are not fully understood, aging may increase connective tissue via higher cytokine levels, oxidative stress, and increased mechanical stress between fibers as they atrophy (Ahmed et al., 2005; Fulle et al., 2004; Kjaer, 2003; Song et al., 2006). Recently, our laboratory found that 3 months of treadmill exercise retarded age-induced fiber atrophy in the tibialis anterior muscle (Song et al., 2006). In addition, extramyocyte space was also reduced in old rats that completed 12 weeks of treadmill exercise (Song et al., 2006). While data from this recent publication are consistent with the current study, it is possible that the mild caloric restriction, necessary to maintain activity through the lifespan, influenced the effects of the wheel running group. Some protection against age-induced changes in fiber morphology and connective tissue in fast-twitch muscle has been previously noted with moderate caloric restriction (Aspnæs et al., 1997; Payne et al., 2003).

Protection against age-related changes in muscle fiber morphology and fibrosis by mild caloric restriction and lifelong wheel running did not appear to be related to changes in oxidative capacity. While citrate synthase activity was reduced with age, 8% caloric restriction and the wheel running surprisingly had little positive benefit on citrate synthase activity. The lack of an exercise effect for citrate synthase in the plantaris is indicative of a relatively low/mild exercise intensity and/or limited recruitment of fast-twitch fibers. Thus protection of muscle mass and morphology with lifelong exercise and mild caloric restriction was not dependent on high intensity exercise such as resistive training. This indicates that lifelong mild caloric restriction and voluntary low-intensity or mild exercise can significantly protect relative muscle mass and muscle fiber morphology without protection of oxidative capacity. These data suggest an alternative explanation, including reduced oxidative stress and upregulation of stress response proteins, such as IGF-1. In addition, these data indicate that high intensity exercise is not a firm requirement for protection against age-induced sarcopenia. Therefore, we postulate that the cell signaling mechanisms leading to sarcopenia are not simply the inverse of those that cause muscle hypertrophy. Indeed, our data imply that if exercise spans a large % of lifespan, that moderate or high intensity training is not requisite in reducing sarcopenia and/or some systemic, humoral factors regulate protection of aging muscle.

Thus we explored potential upstream mediators of protection by wheel running and mild caloric restriction against age-induced changes in muscle mass and muscle morphology in aging *ad libitum* fed rats. Total hydroperoxide levels were higher in old *ad libitum* rats than young controls. However, mean hydroperoxide levels in the wheel running group were not different from young controls, indicating some protection against elevated oxidative stress. Partial protection of oxidative stress with lifelong wheel running appears to be unrelated to antioxidant protection by isoforms of superoxide dismutase. Indeed, protein expression for MnSOD was not altered by age, caloric restriction, or wheel running. Cu,ZnSOD protein expression was elevated by age, but not reduced by lifelong exercise or mild caloric restriction. Thus protection

against oxidative stress in the plantaris was indicated in rats that were in the lifelong wheel running group, but was not due to an upregulation in superoxide dismutase.

Reduction in IGF-1 has been directly related to age-induced sarcopenia (Winn et al., 2002). IGF-1 indeed decreased in the aging plantaris in *ad libitum* fed rats (Fig. 7). Lifelong wheel running provided significant, partial protection against age-induced depression of IGF-1. Therefore, protection of relative muscle mass and muscle morphology against age-induced changes in the wheel running group may be in part a function of partial protection against reduced IGF-1 protein with aging. Although the intermediate mechanisms for IGF-1 protection against age-induced sarcopenia remain uncertain, previous aging studies indicate that IGF-1 could protect muscle mass and morphology via increased protein synthesis, muscle regeneration, increased number of dihydropyridine sensitive voltage-dependent Ca^{2+} channels (DHPRs), and increased protection against cell necrosis and apoptosis of myonuclei and satellite cells (Musaro et al., 2001; Siu et al., 2004, 2005; Song et al., 2005; Winn et al., 2002). For example, IGF-1 stimulation by lifelong exercise could protect against muscle fiber atrophy and stimulate cell growth through a cascade involving Akt phosphorylation and downstream elevation of mTOR, NF- κ B (nuclear factor-kappaB), and heat shock proteins. In addition, attenuation of age-induced depression of IGF-1 protein could inhibit pro-apoptotic foxo and Bad (Bcl-2 antagonist of cell death) phosphorylation (Datta et al., 1997; Dudek et al., 1997; Guttridge, 2004; Sandri et al., 2004). This is a focus of future studies designed to elucidate the mechanisms by which lifelong exercise protects skeletal muscle mass and morphology.

While we cannot extrapolate our results directly to human populations, the strategy of long-term mild caloric restriction may be an attractive alternative to moderate (30–40%) caloric restriction as preventative measure for sarcopenia and fiber loss. Mild caloric restriction may provide protection of muscle mass and function (Payne et al., 2003; Visser et al., 1998) without the health and compliance issues possible in humans who undergo chronic 30–40% caloric restriction (Dirks and Leeuwenburgh, 2006). Additional longitudinal animal and human studies are clearly needed to determine if the combination of mild caloric restriction and daily voluntary exercise attenuates age-induced sarcopenia in human patients.

In addition, a limitation of the present study design is that a group of rats subject to voluntary wheel running fed *ad libitum* could not be included. Unfortunately, rats fed *ad libitum* will diminish and stop voluntary wheel running activity after 6 months of age (Holloszy et al., 1985; Judge et al., 2005; McCarter and McGee, 1987; Seo et al., 2006). Daily observed cage activity was greater in the 8% CR group, but was not quantified by an activity monitor. Thus we are not able to determine from the current data set whether the effects of lifelong exercise and caloric restriction are additive or interactive.

In summary, this study is the first to demonstrate that lifelong mild (8%) caloric restriction retards age-induced changes in fast-twitch muscle (plantaris) morphology including fiber atrophy, increased extramyocyte space, and accumulation of connective tissue. In addition, lifelong voluntary exercise protected plantaris mass/body mass as well as ameliorating fiber atrophy, altered myocyte morphology, and increased connective tissue. These findings provide direct evidence supporting the notion that long-term primary prevention strategies in adults to address age-induced sarcopenia should include lifelong mild caloric restriction and daily, continuous voluntary exercise. Protection against age-induced changes in relative muscle mass and cell morphology in the lifelong wheel running group was related to attenuation against oxidative stress and depressed IGF-1 levels.

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Figure 1.

Plantaris muscle mass per body mass (g/kg) in young (6 mo) Fischer-344 rats fed *ad libitum* (YAL), old (24 mo) rats fed *ad libitum* (OAL), old rats that underwent lifelong 8% caloric restriction (OCR), and old rats that participated in lifelong voluntary wheel running plus 8% caloric restriction. (OExCR). Values are means $\pm$ SEM. (a) indicates significant difference from YAL group. (b) indicates significantly different from OAL group. (c) indicates significant difference from OCR group.

Figure 2.

Plantaris hematoxylin-stained cross-sections from young (6 mo) Fischer-344 rats fed *ad libitum* (YAL), old (24 mo) rats fed *ad libitum* (OAL), old rats that underwent lifelong 8% caloric restriction (OCR), and old rats that participated in lifelong voluntary wheel running plus 8% caloric restriction. (OExCR). Standard bar = 100 μ m.

Figure 3.

Plantaris muscle fiber cross-sectional area in young (6 mo) Fischer-344 rats fed *ad libitum* (YAL), old (24 mo) rats fed *ad libitum* (OAL), old rats that underwent lifelong 8% caloric restriction (OCR), and old rats that participated in lifelong voluntary wheel running plus 8% caloric restriction (OExCR). Values are means $\pm$ SEM. (a) indicates significant difference from YAL group. (b) indicates significant difference from OAL group.

Figure 4.

Percent (%) extramyocyte space in the plantaris muscle from young (6 mo) Fischer-344 rats fed *ad libitum* (YAL), old (24 mo) rats fed *ad libitum* (OAL), old rats that underwent lifelong 8% caloric restriction (OCR), and old rats that participated in lifelong voluntary wheel running plus 8% caloric restriction. (OExCR). Values are means $\pm$ SEM. (a) indicates significant difference from YAL group. (b) indicates significant difference from OAL group.

Figure 5.

Plantaris cross sections stained with Masson's trichrome in Fig 5a. Calculated percent (%) connective tissue in the plantaris in Fig. 5b from young (6 mo) Fischer-344 rats fed *ad libitum* (YAL), old (24 mo) rats fed *ad libitum* (OAL), old rats that underwent lifelong 8% caloric restriction (OCR), and old rats that participated in lifelong voluntary wheel running plus 8% caloric restriction. (OExCR). Values are means $\pm$ SEM. (a) indicates significant difference from YAL group. (b) indicates significant difference from OAL group. (c) indicates significant difference from OCR group.

Figure 6. Citrate synthase activity (6a) and total hydroperoxide levels (6b) in young (6 mo) Fischer-344 rats fed *ad libitum* (YAL), old (24 mo) rats fed *ad libitum* (OAL), old rats that underwent lifelong 8% caloric restriction (OCR), and old rats that participated in lifelong voluntary wheel running plus 8% caloric restriction (OExCR). Values are means $\pm$ SEM. (a) indicates significant difference from YAL group.

Figure 7. Superoxide dismutase protein expression for the Mn-isoform (7a) and the Cu,Zn-isoform (7b) in young (6 mo) Fischer-344 rats fed *ad libitum* (YAL), old (24 mo) rats fed *ad libitum* (OAL),

old rats that underwent lifelong 8% caloric restriction (OCR), and old rats that participated in lifelong voluntary wheel running plus 8% caloric restriction. (OExCR). Values are means $\pm$ SEM. (a) indicates significant difference from YAL group.

Figure 8.

IGF-1 protein expression in young (6 mo) Fischer-344 rats fed *ad libitum* (YAL), old (24 mo) rats fed *ad libitum* (OAL), old rats that underwent lifelong 8% caloric restriction (OCR), and old rats that participated in lifelong voluntary wheel running plus 8% caloric restriction (OExCR). Values are means $\pm$ SEM. (a) indicates significant difference from YAL group. (b) indicates significant difference from OAL group.

Table 1

Body mass (kg) and plantaris muscle mass (g) in young (6 mo) Fischer-344 rats fed *ad libitum* (YAL), old (24 mo) rats fed *ad libitum* (OAL), old rats that underwent lifelong 8% caloric restriction (OCR), and old rats that participated in lifelong voluntary wheel running plus 8% caloric restriction (OExCR). Values are means $\pm$ SEM. (1) indicates significant difference from YAL group. (b) indicates significantly different from OAL group. (c) indicates significant difference from OCR group.

	YAL (n=12)	OAL (n=12)	OCR (n=12)	OExCR(n=12)
Body Mass (kg)	374.3 $\pm$ 9.9	389.7 $\pm$ 14	377.6 $\pm$ 5.4	341.3 $\pm$ 5.4 <i>abc</i>
Plantaris Mass(g)	0.653 $\pm$ 0.012	0.648 $\pm$ 0.041	0.607 $\pm$ 0.148	0.613 $\pm$ 0.014