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Cellular adaptation contributes to calorie restriction-induced preservation of skeletal muscle in aged rhesus monkeys

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Abstract

We have previously shown that a 30% reduced calorie intake diet delayed the onset of muscle mass loss in adult monkeys between ~16 and ~22 years of age and prevented multiple cellular phenotypes of aging. In the present study we show the impact of long term (~17 years) calorie restriction (CR) on muscle aging in very old monkeys (27–33yrs) compared to age-matched Control monkeys fed *ad libitum*, and describe these data in the context of the whole longitudinal study. Muscle mass was preserved in very old calorie restricted (CR) monkeys compared to age-matched Controls. Immunohistochemical analysis revealed an age-associated increase in the proportion of Type I fibers in the VL from Control animals that was prevented with CR. The cross sectional area (CSA) of Type II fibers was reduced in old CR animals compared to earlier time points (16–22 years of age); however, the total loss in CSA was only 15% in CR animals compared to 36% in old Controls at ~27 years of age. Atrophy was not detected in Type I fibers from either group. Notably, Type I fiber CSA was ~1.6 fold greater in VL from CR animals compared to Control animals at ~27 years of age. The frequency of VL muscle fibers with defects in mitochondrial electron transport system enzymes (ETS^{ab}), the absence of cytochrome c oxidase and hyper-reactive succinate dehydrogenase, were identical between Control and CR. We describe changes in ETS^{ab} fiber CSA and determined that CR fibers respond differently to the challenge of mitochondrial deficiency. Fiber counts of intact *rectus femoris* muscles revealed that muscle fiber density was preserved in old CR animals. We suggest that muscle fibers from CR animals are better poised to endure and adapt to changes in muscle mass than those of Control animals.

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Keywords

rhesus monkey; calorie restriction; sarcopenia; aged

1. Introduction

1.1

Sarcopenia is one of a number of geriatric syndromes that contributes to morbidity in the aged (Cruz-Jentoft et al., 2010). While not directly responsible for mortality, grave impairment to health and well-being is associated with significant muscle mass loss. Sarcopenia is simply defined as the age-related loss of skeletal muscle mass and size. A refined definition of sarcopenia has developed over the years to include the loss of strength and/or function (Rosenberg 1997; Morley et al., 2001; Roubenoff, 2001). Recent studies in mice and humans have pointed to the importance of skeletal muscle in overall metabolic homeostasis including glucoregulatory function (Lira et al., 2010). This raises the possibility that sarcopenia has a systemic impact and places renewed emphasis on understanding the factors that contribute to it and how to prevent it.

1.2

Calorie restriction (CR), the reduction of caloric intake without malnutrition, delays aging in diverse species and prevents the onset of numerous age-associated pathologies (McCay et al., 1935; Weindruch and Walford, 1982). CR improves survival and reduces morbidity in rhesus monkeys (*Macaca mulatta*), demonstrating that CR's anti-aging effect is conserved in primates (Colman et al., 2008). Rhesus monkeys have a lifespan of several decades and share many human characteristics including the spectrum of age-associated diseases, the onset of sarcopenia in middle age (~16yrs for rhesus monkeys) and the trajectory of muscle mass decline. Importantly, CR significantly reduces sarcopenia in rhesus monkeys (Colman et al., 2005) and delays aging-induced cellular phenotypes in skeletal muscle in adult animals 16–22yrs of age (McKiernan et al., 2011).

1.3

The constituent fibers of skeletal muscle are heterogeneous in terms of metabolism and functional capacity (Bassel-Duby and Olson, 2006). Type I fibers are more reliant on oxidative metabolism and express a specialized isoform of the structural protein myosin that is associated with endurance. Type II fibers, also known as “fast twitch”, tend to rely less on oxidative metabolism and express distinct myosin isoforms that are associated with greater contractile force and velocity. We have previously shown fiber type distribution shifts with age in rhesus monkey *vastus lateralis* (VL), where the proportion of Type I fibers is higher in muscle from ~22 year old Control (fed an *ad libitum* diet) monkeys (McKiernan et al., 2009) compared to muscle from ~22 year old CR (caloric intake restricted by 30% for ~12 years) monkeys (McKiernan et al., 2011). This shift was not simply due to the loss of Type II fibers, but an actual increase in the absolute number of Type I fibers (McKiernan et al., 2004). In addition, cross sectional area of Type II fibers declined with age, coincident with the decline in estimated muscle mass (McKiernan et al., 2009). In CR animals Type II fiber atrophy was not observed at ~22 years of age (McKiernan et al., 2011).

1.4

The mitochondrion is unique among cellular organelles in that it carries genomic material encoding several genes essential to oxidative phosphorylation. Mitochondrial DNA (mtDNA) deletion mutations occur stochastically in aging muscle tissue, but once

established the dysfunctional mitochondria replicate and eventually expand intracellularly along a muscle fiber (Cao et al., 2001; Gokey et al., 2004). These deletion mutations lead to defects in the activities of Electron Transport System enzymes (ETS^{ab}) which can be detected histologically (Herbst et al., 2007). The age-dependent accumulation of mitochondrial deletion mutations in skeletal muscle of the rat contributes to muscle fiber loss and is linked to dysfunctional cellular phenotypes and muscle fiber atrophy, breakage and loss (Herbst et al., 2007).

1.5

The extent to which the beneficial effects of CR on sarcopenia are still observed in old age is unknown. The average lifespan for rhesus monkeys in captivity is about 27yrs (Colman et al., 1998). In this study we investigated the impact of age and diet on the remaining aged animals from our longitudinal study. The monkeys in this now smaller cohort were on average 27 years old for both the Control and CR groups. CR animals had been on the 30% reduced calorie diet for an average of 17 years. At the whole body level we determined body weight, percent body fat and muscle mass of the upper legs. Cellular aspects of muscle aging were measured in biopsy sample of the VL muscle from both Control and CR monkeys. Age and diet associated changes in muscle composition were determined, including the relative proportion of Type I and Type II fibers, and the degree of fibrotic infiltration. To measure the extent of muscle fiber atrophy, Type I and Type II fiber cross-sectional area (CSA) were measured. The percentage of fibers that presented abnormal mitochondrial enzyme activities were determined and further analyzed for localized fiber atrophy. To determine the impact of age and diet on muscle fiber loss we used whole *rectus femoris* (RF). The RF has a similar fiber type composition to VL. Finally, we conducted statistical analyses of data collected over the entire longitudinal study. This included previously published data from the same animals at ~16 years, ~18 years and ~22 years of age (McKiernan et al., 2009; McKiernan et al., 2011), as well as the ~27 year data reported here.

We show that CR continues to confer a positive impact on rhesus monkey skeletal muscle in old animals of ~27yrs. Fiber loss and fiber atrophy are attenuated in CR animals compared to age-matched Controls. The impact of both age and CR is fiber type specific. Type I fibers significantly increase in cross-sectional area in aging CR animals, an increase that may offset the eventual atrophy of Type II fibers. The incidence of fibers with ETS^{ab} was the same between diet groups, however, ETS^{ab} fibers from CR monkeys were resistant to atrophy. Our data suggest muscle fibers from old Control and CR animals are distinct, and that adaptation of Type I muscle fibers plays a role in conserving skeletal muscle mass.

2. Materials and Methods

2.1 Animals and diets

All animal procedures were performed at the Wisconsin National Primate Research Center (WNPRC) under approved protocols from the Institutional Animal Care and Use Committee of the Graduate School of the University of Wisconsin, Madison. This work is part of an ongoing longitudinal study on aging and CR in non-human primates at the WNPRC (Colman et al., 2008; Kemnitz et al., 1993; Ramsey et al., 2000). Briefly, 30 male rhesus monkeys (*Macaca mulatta*) between 8- and 14-years of age, were monitored for baseline food intake and randomly assigned to either Control (n=15) or CR (n=15) diets. Control animals were provided *ad libitum* access to food (purified lactalbumin based diet containing 10% fat and 15% protein [Teklad #85387, Madison, WI]). For CR animals (Teklad diet #93131, enriched by 30% in vitamins and minerals), a 30% restriction was applied in 10% increments over a 3-month period at the outset of the study.

Although the study began with 15 monkeys in the Control group and 15 in the CR group, here we report data from nine Control and 11 CR monkeys with average age of ~27ys for both groups (26–33 yrs) and average time on the study of 17yrs. Numerical values calculated for the longitudinal analyses reported here were not equivalent to our previous reports (McKiernan et al., 2009; McKiernan et al., 2011) in that data shown here includes these current animals only and is presented in years of age and not years on the study. Five of the Control monkeys died between 2003 and 2008. Age and cause of death were: 30.13y – peritonitis/septicemia; 25.93y – accidental; 26.11y renal and cardiac disease; 25.91y – adenocarcinoma; 27.66y – adenocarcinoma. Of the 11 CR monkeys two died; 25.76y – disseminated intravascular coagulation and 25.92y – peritonitis. Age, length of time in the study, as well as whole body and muscle characteristics of these animals were consistent with the live monkeys in the study.

2.2 Body composition

Body weight, appendicular lean mass and fat mass were measured biannually using whole body dual energy x-ray absorptiometry (DEXA) (Model DXP-L, GE/Lunar Corp., Madison, WI). Estimated skeletal muscle mass (ESM) of the upper leg was determined by summing the lean mass from the thigh region of both limbs. Upper leg muscle mass for each individual animal was determined and expressed as a proportion of its upper leg lean mass at ~27 years of age compared to its maximum upper leg lean mass attained during the study. On average, maximum upper leg muscle mass was realized at 16 years of age for the Control monkeys and at 18 years of age for the CR monkeys [8]. DEXA measurement of fat mass was used to calculate percent body fat (%BF = [fat mass / body weight] × 100).

2.3 Tissue collection and processing

Upper leg skeletal muscle biopsies of the vastus lateralis (VL) were collected at year 19 of the study. The specimen was bisected and one half was flash frozen in liquid nitrogen. The other half was embedded in Optimal Cutting Temperature Medium (OCT, Sakura Inc., Torrance, CA) and frozen in liquid nitrogen. Samples were stored at –80°C until use. Frozen muscle biopsies were sectioned using a cryostat. For each biopsy, 200 consecutive 10µm-thick sections were cut and stored at –80°C.

2.4 Immunohistochemistry

Muscle fiber types were identified using standard immunohistochemical detection of the Type II isoform of myosin heavy chain (monoclonal antibody MY32, Sigma St. Louis, MO) followed by the 3,3'-Diaminobenzidine tetrachloride (DAB) reaction for visualization (Sheehan and Hrapchak, 1980). Type I fibers were not immunoreactive to MY32 and appear as unstained fibers. Digital images of stained, cross sections of the VL biopsy were obtained using a Nikon Supercool Scan 9000. Muscle fibers were annotated on digital images using PhotoShop CS and the total number of fibers was determined using Image-Pro Plus (MediaCybernetics Inc. Silver Spring, MD). Type I muscle fibers were counted and totaled, similarly. The percentage of Type I or Type II muscle fibers was calculated from the number of fibers of a specific type divided by the total number of fibers in the biopsy.

Muscle fiber size was analyzed by measuring the cross-sectional area (CSA) of 200 Type I and 200 Type II muscle fibers from each Control and CR VL biopsy. Measurements were made by tracing the outer perimeter of the cell using the measurement feature of ImagePro software calibrated to the magnification of the digital image.

2.5 Histochemistry

Muscle fibrosis was visualized using Masson's Trichrome stain (Sheehan and Hrapchak, 1980), where muscle fibers stain red and fibrotic tissue (collagen, specifically) stains blue. Stained sections from each animal were scanned and digital images were produced. All blue stained areas of the cross-section were measured using ImagePro. The proportion between the sum total of the blue areas and the total cross-sectional area of the biopsy ($\times 100$) provided the percent fibrosis.

Mitochondrial electron transport system (ETS) enzyme activities of cytochrome C oxidase (COX, Complex IV) and succinate dehydrogenase (SDH, Complex II) were performed on air-dried VL cryo-sections according to Seligman et al. (1968) and Dubowitz (1985), respectively. Twenty slide triplicates (the 1st, 2nd and 3rd, the 11th, 12th and 13th etc.) from the 200 slide sections from each biopsy (representing 2000 μ m of muscle) were stained for COX or SDH or both COX and SDH. Fibers negative for COX and hyper-reactive for SDH were considered fibers with abnormal ETS enzyme activities (ETS^{ab}) (Aiken et al., 2002). These cells were identified in each muscle sample and the percentage of ETS^{ab} fibers was determined. To measure atrophy specifically associated with ETS^{ab} regions, cross-sectional area ratios were determined for ETS^{ab} fibers where the minimum CSA of the ETS abnormal region was divided by the mean CSA of the normal region of the same fiber to give a minimum CSA ratio. Cross-sectional areas were measured at 100 μ m intervals for 2000 μ m for 48 ETS^{ab} and 48 normal fibers from the Control monkeys and for 54 ETS^{ab} and 54 normal fibers from the CR monkeys. The length of the ETS abnormality within a fiber was also measured.

2.6 Whole muscle fiber counts

Whole quadriceps muscles were obtained from WNPRC aging colony monkeys and from monkeys in the present study within 3 hours of euthanasia. The *rectus femoris* (RF) was isolated, weighed, bisected at the mid-belly, placed in OCT, and frozen in liquid nitrogen.

Cryostat sections were stained with hematoxylin and eosin (H&E) (Sheehan and Hrapchak, 1980) for muscle morphology and muscle fiber counts. Digital images of whole RF cross-sections were obtained using a Nikon Supercool Scan 9000. Muscle area was measured using Image-Pro Plus (MediaCybernetics Inc. Silver Spring, MD) image processing software. Muscle fibers were annotated on digital images using PhotoShop CS and muscle fibers count obtained using Image-Pro Plus.

Due to the large number of animals and the use of 5 distinct staining approaches (My32, Masson's trichrome, COX, SDH, H&E,) specimens were processed in batches. None of the measures was dependent on the intensity of the stain. For fiber type determination, My32 stain was either present (Type II) or absent (Type I). A subset of the specimens across batches were stained and analyzed, and confirmed that the data were not affected by batch to batch differences.

2.7 Statistics

Differences between Control and CR group means were assessed using independent samples t-tests for unequal variances with Welch-Satterthwaite adjustment for the degrees of freedom where appropriate (Zimmerman, 2004). Several nonparametric tests were conducted to assess whether distributions of minimal cross-sectional area ratios of normal and ETS abnormal fibers were different between Control and CR VL biopsies. To test distributional differences between normal and abnormal fibers within each diet condition, Sign Tests and Wilcoxon Signed Rank Tests were calculated. Kruskal-Wallis (KW) tests were conducted to test between group distributional differences for each fiber class (normal

and ETS^{ab}). A linear mixed model approach was used to estimate longitudinal trends in the data while accounting for the dependency in the data due to multiple observations per subject. SAS PROC Mixed was used to estimate the correlation among the repeatedly measured outcomes. All tests were performed at the 0.05 level of significance. SAS version 9.2 was used to perform all analyses.

3. Results

3.1 Body weight and body composition

CR had a significant impact on body weight and body composition of rhesus monkeys. After 19 years on the study (and an average of 17 years of dietary intervention), when Control animals were $27.2 \pm 1.3y$ ($n = 9$) and CR animals were $27.6 \pm 2.2y$ ($n = 11$), significantly lower body weight ($p = 0.03$) and percent body fat ($p = 0.04$) were observed in CR (Table 1, Figure 1a,b)). Body weight of the CR monkeys was 20% lower than Control monkeys and body fat was 35% lower.

Estimation of lean muscle mass using DEXA analysis provided a measure of sarcopenia in individual animals over the study period. By ~27 years of age the estimated skeletal muscle mass of the upper leg of Control monkeys was $1218 \pm 67.7g$, while that of the CR monkeys was $1347 \pm 59.8g$ ($p < 0.18$), when normalized to body weight the upper leg muscle mass of CR monkeys was significantly greater ($p < 0.01$; Table 1) compared to that of Controls. To determine the extent of muscle mass loss we expressed the change in upper leg muscle mass of each monkey as a proportion of the maximum mass attained over the course of the experiment. The Controls retained only $56.80 \pm 4.09\%$ of their maximum upper leg muscle mass, significantly less than the $73.03 \pm 2.40\%$ retained for the CR monkeys ($p < 0.01$, Table 1, Figure 1c).

3.2 Skeletal muscle fibrosis

To determine the impact of CR on fibrotic infiltration of aging skeletal muscle, VL biopsy and necropsy sections were treated with Masson's Trichrome, a stain that distinguishes normal muscle tissue (muscle fibers stain red) from fibrotic tissue (collagen stains blue). Quantitative image analysis revealed that fibrotic material represented a significantly larger percentage of total area in Control samples ($13.98 \pm 2.29\%$) compared to CR ($6.44 \pm 1.04\%$). CR significantly reduced fibrotic infiltration in VL skeletal muscle ($p = 0.02$, Table 1, Figure 2).

3.3 Fiber type distribution and fiber size

Muscle fiber types are distinguished on the basis of myosin heavy chain isoform expression. Using antibodies specific to Type II myosin heavy chain, Type II fibers were detected immuno-histochemically. Type I fibers fail to react with the antibody and were identified as unstained cells in the VL sections. By ~27yrs of age, the percentage of Type I fibers in Control monkey VL biopsy samples was 36% and was significantly higher ($p = 0.015$, Table 1, Figure 1d) than CR VL biopsy samples which contained 18% Type I fibers. Conversely, the percentage of Type II fiber in the Controls was 64% and 82% in the CR.

Muscle fiber size was determined by measuring the cross-sectional areas of the individual fibers from Control and CR biopsies (minimum 200 of each fiber type per animal). Type II muscle fiber CSA from both Control ($7,791 \pm 1081 \mu m^2$) and CR animals ($8,260 \pm 530 \mu m^2$) was not significantly different. The average CSA of Type I muscle fibers from Control monkeys was $7,811 \pm 1,173 \mu m^2$. CSA of Type I muscle fibers from CR was $12,619 \pm 778 \mu m^2$ and significantly larger than Controls ($p < 0.01$, Table 1, Figure 1e,f).

3.4 Mitochondrial Electron Transport System abnormalities

The number of unique ETS^{ab} muscle fibers was determined for each of the Control and CR VL biopsies along 2000µm of muscle tissue. There was no difference in the percentage of ETS^{ab} fibers between Control ($3.13 \pm 1.2\%$) and CR ($3.09 \pm 0.84\%$, $p = 0.974$), and the length of the abnormality within a fiber was not significantly different between Control ($1,006 \pm 480\mu\text{m}$) and CR ($900 \pm 550\mu\text{m}$).

To determine whether the ETS^{ab} contributes to localized fiber atrophy the cross-sectional area ratio for ETS^{ab} and ETS normal fibers from Control and CR monkeys was measured. Overt intra-fiber atrophy within the ETS abnormal region (≤ 0.5 CSA ratio) was observed in 20% of the ETS^{ab} fibers from Control animals. Only 6% of the ETS^{ab} fibers from CR monkeys showed this degree of atrophy. The distribution of minimum CSA ratios indicates the degree of atrophy (or hypertrophy) among groups of fibers. Normal fibers from Control and CR monkeys had similar CSA ratio distributions ($p = 0.57$; Figure 3a, b). There was also no difference in distribution between ETS normal and ETS^{ab} fibers from the Control monkeys ($p = 0.30$; Figure 3a, c). In contrast, there were significant differences in the minimum CSA ratio distributions between ETS^{ab} fibers of the Control monkeys and the ETS^{ab} fibers of CR monkeys ($p = 0.022$; Figure 3c, d) and between ETS normal and ETS^{ab} fibers from CR animals ($p < 0.001$; Figure 3b, d). In ETS^{ab} fibers from CR monkeys, fiber CSA was maintained or increased.

3.5 Muscle fiber number

The loss of muscle mass with age is due to a combination of both fiber atrophy and fiber loss. The contribution of fiber loss to sarcopenia in rhesus monkeys cannot be adequately assessed in biopsies where only a small portion of the whole muscle can be investigated. To determine the effect of age and CR on fiber number in aged rhesus monkey muscle we used cross-sections of *rectus femoris* (RF), a quadriceps muscle that had not been biopsied and is one of the quadriceps muscles of the upper leg with similar fiber type distribution. Intact RFs from three adult male monkeys (14y–17y) from the WNPRC Aging Colony, four old male Controls (26–30y) and four old male CR monkeys (26–33y) were analyzed. Wet weight of the RF was not different between the adult and old Control monkeys, and, the old CR RF weighed significantly less than the old Control ($p = 0.01$, Table 2). Cross-sectional area of the RF muscle was statistically the same among adult, old Control and old CR. Fiber number in the adult RF was higher than both old Control ($p = 0.01$) and old CR ($p = 0.04$). When fiber number was normalized to muscle area, however, there was a significant difference in fiber density between adult (132 ± 22 fibers/mm²) and old Control (92 ± 16 fibers/mm², $p < 0.01$). Old CR monkeys had a significantly higher density of muscle fibers (124 ± 15 fibers/mm²) compared to old Control animals ($p = 0.03$) and were not different from the adult Controls ($p = 0.65$).

3.6 Longitudinal analysis of sarcopenia in the aging rhesus monkey

To get a broader sense of the impact of age and CR on skeletal muscle mass we conducted statistical analysis of data collected over the entire period of the longitudinal study. This includes previously published data from the same animals at ~16 years, ~18 years and ~22 years of age [10, 12] as well as the data at ~27 years of age shown above.

We determined that the upper leg muscle mass in both Control and CR animals declined over time ($p < 0.01$), however, the rate of decline in muscle mass was significantly slower for CR animals ($p < 0.01$). Average peak upper leg muscle mass occurred later in the CR monkeys (~18 years of age), two years later than for Control animals [10]. Based on this peak, CR monkeys had lost 20% of their upper leg muscle mass by ~27 years of age. In contrast, Control monkeys had already lost 20% 5 years earlier at ~22 years, and by ~27

years of age, a 44% loss was observed (Figure 4a). The CR regimen employed in this study had a significant effect on muscle mass by both delaying and attenuating loss.

In Control animals at ~27 years of age Type II fiber CSA in VL was not different from CR; however, the trajectory of fiber atrophy over time was significantly different between groups (Figure 4b; $p = 0.037$). For Control animals, a 30% reduction in Type II fiber CSA was observed over the period from ~16 years of age ($11,858 \pm 1,236\mu\text{m}$) to ~22 years of age ($8,401 \pm 1,276\mu\text{m}$). At ~27 years of age Type II fiber CSA was $7,791 \pm 1,082\mu\text{m}$ and not different from the previous time point. The CSA of Type II fibers from CR animals remained relatively constant between ~16 and ~22 years of age but underwent a 25% reduction in CSA by ~27 years of age indicating that atrophy of Type II fibers, although delayed, was not prevented in aged CR animals (Figure 4b).

Type I fibers were not susceptible to age-associated atrophy in rhesus VL (Figure 4c), in fact, with age Type I fiber CSA increased in both Control and CR. The age-associated increase in CSA in Type I fibers from CR animals was considerably larger ($p < 0.01$) and the rate of increase greater ($p < 0.01$) than Type I fibers from age matched Controls. The average CSA of Type I fibers from CR animals was larger at ~27 years than at any other time during lifespan ($p < 0.01$).

Over the course of the study the age-dependent increase in the number ETS^{ab} fibers was identical in both Control and CR VL muscle (Figure 4d) despite significant differences in the extent of fiber atrophy and muscle mass loss.

4. Discussion

4.1

Rhesus monkeys have an average lifespan of 27 years and a maximum life span of 40 years (Colman et al., 1998). Monkeys progress through stages of life in a similar fashion to humans, however, at a rate 2.5 – 3 times faster. Estimated skeletal muscle mass in the male monkey on a chow diet peaks at ~16 years of age (Colman et al., 2005). Similarly, male human lean mass stabilizes by the mid- 40's (Roubenoff and Hughes, 2000), and typical muscle mass loss over the next 4 decades is between 15 – 42% (Lexell et al., 1988). As shown in this study, Control rhesus monkey upper leg muscle mass declined an average of 44% over a comparable lifespan period. The ingestion of 30% fewer calories over ~17 years resulted in a 20% reduction in body weight, a 34% reduction in body fat compared to Controls and only a 27% decline in upper leg muscle mass from peak upper leg muscle mass in CR monkeys. In addition, monkeys on the CR diet had a more youthful appearance at their average lifespan compared to age-matched Control monkeys (Colman et al., 2009).

4.2

Until this time point in our longitudinal study we were unable to assess muscle fiber loss as a function of age or whether CR had an impact on fiber loss because such measures require whole muscles that become available only upon the death of animals on the study. Here we examined whole RF muscles from colony and experimental animals to determine whether there was age-dependent fiber loss in rhesus monkeys. RF is one of four muscles in the upper leg quadriceps and is of similar fiber type composition to VL, the muscle group that our biopsies have been derived from. We observed no differences in wet muscle weight and whole muscle cross-sectional area in RF from adult and old Control monkeys; however, we observed a significantly lower number of fibers (~30% less) in the old animals. A similar decrease in fiber number was observed in skeletal muscle from aged rats (Wanagat et al., 2001). When fiber number was normalized to muscle area, density of fibers was lower in the old Control monkeys compared to the adults. Although the total number of fibers in RF from

old CR animals was not different from old Control animals, this may be explained by the fact that muscles from CR animals were smaller in size. Fiber density was significantly higher in muscles from old CR animals compared to old Controls and was not different from adult animals, indicating that CR attenuates fiber loss.

The maintenance of RF wet muscle weight in Control animals with age was somewhat unexpected. A possible explanation is that a change in muscle composition masks any loss in mass due to loss of fiber number. Analysis of VL muscle composition indicated that age is associated with fibrotic infiltration, suggesting that fiber loss and fiber atrophy triggers collagen deposition within the tissue. Collagen is deposited around and within muscle bundles, filling in spaces created as fibers shrink and are lost. Importantly, both fiber loss and fibrosis were attenuated in CR animals.

4.3

Analysis of the impact of age on skeletal muscle at the cellular level indicates that Type II fiber atrophy is an early contributor to sarcopenia. The decline in muscle mass that occurs between ~16 and ~22 years of age in Control animals is coincident with a decline in the Type II fiber CSA. There was no further decline in Type II CSA after ~22 years of age for Control animals indicating that the loss of muscle mass in the later stages of sarcopenia is not explained by fiber atrophy. A possible explanation comes from our cross-sectional investigation of fiber density in RF. The 30% loss of fibers from old animals compared to adults indicates that perhaps fiber loss is driving the later decline in muscle mass in rhesus monkeys.

We also observed that the impact of age is fiber type specific. Our data show that unlike Type II fibers, Type I fibers do not undergo age-associated atrophy. Consistent with this, Type I fiber CSA is also not diminished with age in humans (Hortobagyi et al., 1995; Proctor et al., 1995). We have shown (Figure 4c) that there was a modest increase in the CSA of Type I fibers from Control animals with age; however, the CSA of Type I fibers from aged CR animals was ~62% larger than that of Control animals. The adaptation of increased Type I fiber CSA to preserve muscle mass is not unprecedented. Human studies have previously identified a possible role for Type I fibers in prevention of muscle mass loss, where endurance exercise prevented the onset of sarcopenia, and was coincident with an increase in the CSA of Type I fibers (Proctor et al., 1995; Harber et al., 2009). Type I fibers rely preferentially on oxidative metabolism and have a higher density of mitochondria raising the possibility that this difference in metabolism protects against age-induced atrophy. At this stage it is unclear how CR induced an increase in Type I fiber CSA or whether the mechanisms involved are linked to the decline in Type II CSA.

4.4

Aging is associated with an increase in the number of ETS^{ab} fibers in skeletal muscle from rodents (Herbst et al., 2007; Wanagat et al., 2001) and rhesus monkeys (Lee et al., 1998; Lopez et al., 2000). In rodents, sarcopenia onset occurs relatively late in the lifespan and the accumulation of ETS^{ab} is concurrent with significant muscle mass loss (Lushaj et al., 2008). Furthermore, ETS^{ab} are associated with localized atrophy within individual fibers (Herbst et al., 2007; Wanagat et al., 2001). The rodent data suggested that ETS^{ab} play a causative role in fiber atrophy and loss; however, we do not observe a clear relationship between ETS^{ab} and fiber atrophy in rhesus monkeys. In monkeys, the onset of sarcopenia occurs earlier (mid-way through life) and progresses more gradually. There was evidence of modest localized intrafiber atrophy in cross-sections where ETS^{ab} were detected; however, the fiber minimum CSA ratio distribution for Control animals was not different between ETS normal and ETS^{ab} fibers. These data are consistent with previous smaller-scale studies in monkeys

(Lee et al., 1998; Lopez et al., 2000) and in humans (Bua et al., 2006) where overt changes in fiber CSA were detected in a small percentage of ETS^{ab} fibers from very old individuals. These data suggest that there may be a greater tolerance of abnormal mitochondria in the in the skeletal muscle fibers of primates compared to rodents.

CR monkeys responded to ETS^{ab} differently than Control animals. In CR animals the distribution of fiber minimum CSA ratios for abnormal fibers is statistically distinct from that of CR normal fibers and from Control fibers in general, with the distribution curve shifted towards larger girth with ETS^{ab}. The underlying basis for this response to ETS^{ab} is not clear, but the fact that it is unique to CR suggests that muscle fibers from CR animals are functionally different from those of Control animals in how they adapt to changes in mitochondrial fidelity.

4.5

Our data suggest that there are multiple factors contributing to the preservation of muscle mass in CR animals: first, CR prevents muscle fiber loss, a key component of muscle mass loss; second, atrophy of Type II fibers is delayed and attenuated in muscle from CR monkeys; third, the CSA of Type I muscle fibers increased with CR in old animals. These findings indicate that muscle fibers from CR animals are better poised to endure and adapt than fibers from Control animals. Intrinsic differences in plasticity may explain why muscle fibers from Control and CR monkeys differ in their tolerance of ETS^{ab}. Elevated levels of abnormal mitochondria were associated with aging in muscle from Control animals but presented independent cellular aging phenotypes in CR animals.

Highlights

- Calorie restriction preserved upper leg muscle mass in very old (27y) rhesus monkeys.
- Muscle fiber density was maintained and fibrotic infiltration was attenuated with calorie restriction.
- Age-dependent Type II fiber atrophy was delayed by ~10 years with calorie restriction.
- Type I fibers increase in cross-sectional area with age, both the size and rate of increase of Type I fibers was greater with calorie restriction.
- Preservation of fiber density, delayed Type II fiber atrophy and an increase in Type I fiber cross-sectional area in the vastus lateralis muscle of calorie restricted monkeys contributed to muscle mass maintenance in the aged primate.

Abbreviations

CR	calorie restriction
VL	vastus lateralis
RF	rectus femoris
DEXA	dual energy x-ray absorptiometry
ESM	estimated skeletal muscle mass
CSA	cross-sectional area
COX	cytochrome c oxidase

SDH	succinate dehydrogenase
mtDNA	mitochondrial DNA
ETS^{ab}	electron transport system enzyme abnormalities where COX is absent and SDH hyper-reactive in muscle fibers

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Figure 1.

Body and VL muscle composition of aged (~27 y) rhesus monkeys. Vertical bars represent the mean values for control (black) and restricted (white) and small square boxes represent individual monkeys: **a**). body weight, $p=0.035$; **b**). body fat, $p=0.038$; **c**). percent upper leg muscle mass expressed as a proportion of estimated upper leg mass at ~27 years of age over maximum upper leg muscle mass observed, $p=0.015$; **d**). percentage of Type I muscle fibers, $p=0.02$; **e**). Type I fiber cross-sectional area (CSA), $p < 0.01$ and **f**). Type II fiber CSA, $p=0.70$

Control VL

CR VL

Figure 2. Masson's trichrome stain for collagen on aged rhesus monkeys' VL. Muscle stains red, collagen stains blue. Scanned images (2x) and close ups (20x).

Figure 3.

Histogram of the frequency of normal and ETS^{ab} abnormal muscle fibers based on minimum cross-sectional area (CSA) ratio (the proportion between the minimum CSA of a fiber within the ETS^{ab} region and the mean CSA of the normal region of the fiber). Significant differences in CSA ratio distributions were observed between Control ETS^{ab} fibers and CR ETS^{ab} fibers ($p = 0.022$) and between CR normal fibers and CR ETS^{ab} fibers ($p < 0.001$).

Figure 4.

Longitudinal analysis of Control and CR rhesus monkey VL muscle after an average of 17 years in the study. **a).** Upper leg muscle mass / maximum upper leg muscle mass. Both Control (n=9) and CR (n=11) undergo significant declines with age ($p < 0.001$), CR were significantly higher overall ($p = 0.04$) and Controls have a significantly sharper rate of loss compared to Controls ($p < 0.001$); **b).** Type II fiber CSA. Significant reduction in Type II fiber CSA in Control VL (n=8) occurs between ~16- and ~22-years of age, CR VL (n=10) Type II fiber CSA is relatively constant between the same years and begins to decline at ~27 years of age, Type II fibers from Controls have a significantly different trend for age-dependent atrophy compared to CR ($p = 0.04$); **c).** VL Type I fiber CSA significantly increased with age in both Control (n=8) and CR (n=10; $p < 0.001$), Type I fiber CSA of CR monkeys was significantly higher than Control ($p < 0.001$) and the rate of increase was significantly higher in CR ($p < 0.001$) and **d).** Percentage of ETS^{ab} fibers in 2000µm of VL tissue. A significant increase with age was observed in both Control (n=8) and CR (n=11). Graphs represent data accumulated between years 6 and 19 of the CR study with animals between the ages of ~16y and ~27y.

Table 1

Analysis of whole body, upper leg muscles and *vastus lateralis* muscles of Control and Calorie Restricted monkeys.

	Condition		Mean ^a Difference	p-value ^a
	Control (± se) (n)	CR (± se) (n)	(95% CI)	
Age at Bx (yrs)	27.22 ± 0.44 (9)	27.55 ± 0.65 (11)	-0.33 (-1.98 – 1.33)	0.68
Body Weight (g)	12091 ± 914 (9)	9723 ± 311 (11)	2368 (212–4523)	0.03
Percent Fat	27.85 ± 3.67 (9)	18.34 ± 1.73 (11)	9.51 (0.63–18.36)	0.04
Upper Leg Lean MM ^b (g)	1218 ± 67.7 (9)	1347 ± 59.8 (11)	129.2 (-323–65.11)	0.18
Percent MM/BW	10.42 ± 0.78 (9)	13.84 ± 0.43 (11)	-3.24 (-3.35–1.49)	<0.01
Percent MM/Max MM	56.80 ± 4.09 (9)	73.03 ± 2.40 (11)	-16.23 (-26.45–6.02)	<0.01
Percent Fibrosis ^c	13.98 ± 2.29 (7)	6.44 ± 1.04 (10)	7.54 (1.80–13.28)	0.02
Percent Type I ^c	36.24 ± 5.77 (9)	17.50 ± 3.46 (11)	18.74 (4.25–33.23)	0.02
Percent Type II ^c	63.76 ± 5.77 (9)	82.5 ± 3.46 (11)	-18.74 (-33.23–(-4.25))	0.02
CSA Type II (μm ²) ^c	7791 ± 1081 (8)	8260 ± 530 (10)	-468.80 (-3110–2173)	0.70
CSA Type I (μm ²) ^c	7811 ± 1173 (8)	12619 ± 778 (10)	-5423 (-9176–(-1670))	<0.01
Percent ETSab ^c	3.13 ± 1.2 (8)	3.09 ± 0.84 (11)	0.05 (-3.10–3.20)	0.97

^a Confidence Intervals, t-tests and p-values are based on Satterthwaite adjustment to degrees-of-freedom for unequal sample sizes and variances.

^b MM, muscle mass of the upper leg as determined by DXA

^c Analysis on vastus lateralis (VL)

Table 2

Intact *rectus femoris* (RF) muscle from adult colony, old control and old calorie restricted monkeys.

Monkey	n	Age	Diet	RF Weight (g)	RF Fiber Number	RF Area (mm ²)
Adult	132 ^a	15.33	AL	34.25 ^{ac}	50735 ^a	387 ^a
	±22	±1.53		±1.95	±6426	±61
Old	92 ^b	27.25	C	37.68 ^a	33989 ^b	384 ^a
	±16	±1.89		±3.66	±2149	±93
Control	124 ^a	29.00	R	29.44 ^{bc}	37919 ^b	299 ^a
	±15	±3.54		±2.33	±3493	±45
Restricted						

^{abc} Within a column values with different superscripts were significantly different (p < 0.05).