

Caloric restriction of rhesus monkeys lowers oxidative damage in skeletal muscle

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ABSTRACT In laboratory rodents, caloric restriction (CR) retards several age-dependent physiological and biochemical changes in skeletal muscle, including increased steady-state levels of oxidative damage to lipids, DNA, and proteins. We used immunogold electron microscopic (EM) techniques with antibodies raised against 4-hydroxy-2-nonenal (HNE)-modified proteins, dinitrophenol, and nitrotyrosine to quantify and localize the age-dependent accrual of oxidative damage in rhesus monkey *vastus lateralis* skeletal muscle. Using immunogold EM analysis of muscle from rhesus monkeys ranging in age from 2 to 34 years old, a fourfold maximal increase in levels of HNE-modified proteins was observed. Likewise, carbonyl levels increased ~ twofold with aging. Comparing 17- to 23-year-old normally fed to age-matched monkeys subjected to CR for 10 years, levels of HNE-modified proteins, carbonyls, and nitrotyrosine in skeletal muscle from the CR group were significantly less than control group values. Oxidative damage largely localized to myofibrils, with lesser labeling in other subcellular compartments. Accumulation of lipid peroxidation-derived aldehydes, such as malondialdehyde and 4-hydroxy-2-alkenals, and protein carbonyls were measured biochemically and confirmed the morphological data. Our study is the first to quantify morphologically and localize the age-dependent accrual of oxidative damage in mammalian skeletal muscle and to demonstrate that oxidative damage in primates is lowered by CR.—Zainal, T. A., Oberley, T. D., Allison, D. B., Szweda, L. I., Weindruch, R. Caloric restriction of rhesus monkeys lowers oxidative damage in skeletal muscle. *FASEB J.* 14, 1825–1836 (2000)

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BY UNKNOWN MECHANISMS, caloric restriction (CR) without essential nutrient deficiency extends maximum life span and retards the development of a

broad spectrum of pathophysiological changes in laboratory rodents (1). One possibility being investigated is that CR lowers age-dependent increases in oxidative stress and damage (1–3). With regard to human health, investigation of the ability of CR to retard aging and diseases in a long-lived primate species may provide data more relevant than that derived from rodent studies. In response, a long-term CR study in adult (8- to 14-year-old) male rhesus monkeys was initiated in 1989 at the Wisconsin Regional Primate Research Center (WRPRC) (4). The maximum life span of rhesus monkeys is thought to be ~40 years. This ongoing study, along with another (5), provides opportunities to investigate the ability of adult-onset CR to influence longevity, disease patterns, and other expressions of biological aging in a nonhuman primate species.

The loss of skeletal muscle mass during aging, often referred to as sarcopenia, is of great public health significance (6) but of unknown etiology (7). The accumulation of oxidative damage in skeletal muscle with age as a result of increased oxidative stress has been hypothesized to contribute to the development of sarcopenia (8). Age-dependent increases in markers of oxidative damage to DNA, lipids, and proteins determined biochemically in mammalian skeletal muscle have been widely reported (9–19), thereby supporting a role of oxidative damage in the etiology of sarcopenia. To date, however, the accumulation of oxidative damage in mammalian skeletal muscle during aging has not been investigated histologically, so that the localiza-

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tion of the accrual of oxidative damage is not well defined.

Caloric restriction provides a tool to investigate the importance of oxidative damage in the development of sarcopenia. In rats, CR imposed at late middle age (17 months) opposed the age-dependent loss of muscle fibers and the development of mitochondrial abnormalities in rats studied at 30–32 months of age (20). In mice, CR started early in life largely prevents the age-dependent accumulation of oxidative damage in skeletal muscle, as measured biochemically (13, 15, 17). Therefore, investigation of the mechanism by which CR delays these physiological and biochemical changes in skeletal muscle is important because it may resolve the etiology of sarcopenia. In an attempt to reveal the subcellular localization of the inhibition by CR and to see whether it occurs in primates, we used immunogold light and electron microscopic (EM) techniques, using well-characterized antibodies raised against 4-hydroxy-2-nonenal (HNE)-modified proteins (21–23), dinitrophenol (DNP) (24–25), and nitrotyrosine (NT) (26–28) to compare vastus lateralis biopsies obtained from normally fed vs. calorie-restricted rhesus monkeys. Biochemical assays were used to investigate correlations among biochemical and morphological techniques, as shown previously in a model of iron-induced oxidative stress (23).

MATERIALS AND METHODS

Animals and experimental groups

This study involved 52 male rhesus monkeys (*Macaca mulatta*), born and housed indoors at the WRPRC with complete clinical and experimental histories available. Age-dependent accrual of oxidative damage was measured in 27 monkeys ranging in age from 2 to 34 years. For the dietary study, normally fed ($n=13$) and calorically restricted ($n=12$) monkeys from a long-term CR study initiated in 1989 (4) were examined. These monkeys (ranging in age from 17 to 23 years) had been on CR for 10 years when biopsies of vastus lateralis were obtained for this study. No animal had any clinical or experimental history expected to differentially affect skeletal muscle. This protocol was carried out with the approval of the Institutional Animal Care and Use Committee of the University of Wisconsin.

For the aging study, animals were fed standard monkey laboratory chow (No. 5038, Purina, St. Louis, Mo.). For the long-term CR study, both control and calorie-restricted animals were fed a semipurified diet (No. 85387, Teklad, Madison, Wis.) as described previously (4, 29). The CR animals had ~30% lower food intakes than the controls. This restriction was achieved by randomly assigning animals to a treatment group after a 3- to 5-month period of baseline assessment, during which food intake of the experimental diet was determined for individual animals. Food intake of the animals assigned to CR was reduced from their baseline period averages by 10% per month for 3 months and then maintained at this 30% restriction level. The controls continued to have free access to food. In 1994, the CR monkeys were

switched to a modified Teklad diet (No. 93131), which is enriched by 30% in vitamins and minerals. These animals were housed individually for accurate measurement of daily food intake, but had extensive auditory and visual contact with other monkeys housed in the same room. For all monkeys, temperature was maintained at ~21°C with average relative humidity of 50–65%. Room lighting was automatically controlled to provide alternating 12 h periods of light and darkness.

Animals consuming the standard laboratory chow were often not allowed true *ad libitum* access to food. These animals were fed in the morning, and by afternoon many of the animals had consumed all of their food. In contrast, the normally fed animals on the Teklad diet always had food remaining in their cages by late afternoon. Recent measurements of total body percent fat tissue mass (%FTM) using dual-energy X-ray absorptiometry confirmed that animals fed standard laboratory chow *ad libitum* had %FTM comparable to CR animals rather than normally fed (data not shown). Therefore, the animals of diverse ages consuming standard lab chow were in fact mildly restricted calorically in comparison to the normally fed animals given a semipurified diet.

Tissue preparation

A small portion (200 mg) of the vastus lateralis was removed by the veterinary staff of the WRPRC under aseptic conditions and general anesthesia. A portion of each skeletal muscle biopsy was diced and either snap-frozen in liquid nitrogen and stored for subsequent biochemical analyses or processed for ultrastructural analysis and immunogold EM. The remainder of the muscle biopsy was processed for histological analysis and immunogold light microscopy (LM).

Antibody specificity

The cross-reactivity of polyclonal antibody raised to HNE-modified keyhole limpet hemocyanin was tested toward compounds structurally similar to HNE such as malondialdehyde (MDA), N_{α} -acetylcysteine derivatives of acrolein, *trans*-2-pentenal, and *trans*-2-nonenal. Competitive Western blot experiments with these potential competitors were performed. Results indicate that the anti-HNE antibody is highly specific to HNE-derived modifications to protein, exhibiting no binding to Michael adducts such as N_{α} -acetylcysteine derivatives of acrolein, *trans*-2-pentenal, and *trans*-2-nonenal, or to MDA (22). Antibody binding is thus dependent on the presence of the 4-hydroxyl group and is sensitive to the chain length of the modifying HNE (22). Furthermore, as judged by competitive enzyme-linked immunosorbent assay experiments, antibody binding is not affected by the amino acid portion of the adducts (21). Therefore, the epitope recognized by the antibody is the hemiacetal form of the HNE-derived portion of protein-HNE adducts (21). The specificity of both the DNP (24–25) and NT (26–28) antibodies has been well characterized by other groups. Preincubation of NT with anti-NT blocked labeling of specific bands in Western blot analyses of kidney tissue homogenates (T. A. Zainal and T. D. Oberley, unpublished observations).

Biochemical assays

Colorimetric assay for lipid peroxidation

Concentrations of MDA and 4-hydroxy-2-alkenals (4-HDA) were determined using a colorimetric assay kit (LPO-586, OXIS International, Portland, Oreg.) according to the manufacturer's instructions with one exception: sufficient 2,[6]-

di-tert-butyl-p-cresol (Sigma, St. Louis, Mo.) was added immediately prior to homogenization to achieve a 5 mM final concentration in order to inhibit further oxidation. The protein concentration of each sample was measured using a BCA protein assay reagent (Pierce Chemical, Rockford, Ill.).

Protein carbonyl assay

Protein carbonyl concentrations were measured using the 2,4-dinitrophenylhydrazine (DNPH) procedure of Levine et al. (30), as modified by Sohal et al. (31). The protein concentration of each sample was measured using a BCA protein assay reagent (Pierce).

Histology

Routine histology was performed as described previously (23).

Ultrastructural analysis

Routine ultrastructural analysis was performed as described previously (23).

Immunogold LM

Immunogold LM was performed as described previously (23) with the following exceptions. For carbonyl analysis, sections were incubated for 1 h in 0.01% DNPH, followed by extensive rinsing with Tris-buffered saline (TBS: 0.05 M Tris, 0.9% NaCl, pH 7.6). Sections were incubated with anti-HNE-modified proteins (1:60), anti-DNP (1:40; LO-DNP-2, Zymed, San Francisco, Calif.), or anti-NT (1:100; 06-284, Upstate Biotechnology, Lake Placid, N.Y.) primary antibody overnight at 4°C. The slides were incubated with diluted (1:100) gold-conjugated goat anti-rabbit immunoglobulin G (IgG) (LM.GAR5, Goldmark Biologicals, Phillipsburg, N.J.) or goat anti-rat IgG (LM.GAT5, Goldmark Biologicals) for 1 h at room temperature.

Immunogold LM quantitation

For quantification using image analysis, black and white prints for each specimen were prepared using a 2× objective lens. Black and white images were then obtained as PICT files with a Microtek ScanMaker V300 flatbed scanner. Image analysis was performed using NIH Image freeware (version 1.61, available for download from <http://rsb.info.nih.gov/nih-image>). The PICT files were opened in gray scale mode by NIH Image. To make a manual area measurement, a region of interest (0.3 mm²) was outlined using the rectangular selection tool. The measure command that would compute the area, mean gray value, and the minimum and maximum gray value was then selected. Ten regions were randomly selected for each PICT file. Mean gray value for each PICT file was obtained by taking the mean value of the 10 measurements.

Immunogold EM

Immunogold EM was performed as described previously (23) with the following exceptions. For carbonyl analysis, sections were incubated for 1 h in 0.01% DNPH, followed by extensive rinsing with TBS (0.05 M Tris, 0.9% NaCl, pH 7.6). Sections were incubated with anti-HNE-modified proteins (1:60), anti-DNP (1:400; LO-DNP-2, Zymed), or anti-NT (1:1000; 06-284, Upstate Biotechnology) primary antibody overnight at 4°C.

The grids were incubated with diluted (1:75) gold-conjugated goat anti-rabbit IgG (EM.GAR15, Goldmark Biologicals) or goat anti-rat IgG (EM.GAT15, Goldmark Biologicals) for 90 min at room temperature.

Immunogold EM quantitation

For quantification using counting of gold beads, a magnification of 25,000× was used. To take a measurement, a region of interest (0.1 μm²) was outlined using the picture field. Gold beads within the region were then counted. The total value for each region was calculated by adding myofibrillar, sarcoplasmic, and mitochondrial counts. However, there was no mitochondrial labeling with antibody to HNE-modified proteins. For all antibodies, labeling in nuclei was rare in comparison to other subcellular compartments. Using low magnification so that beads were not discernible, 10 regions were randomly selected for each sample. Mean bead count for each sample was obtained by taking the mean value of the 10 measurements.

Data analysis

For biochemical assays, statistical analyses were performed using the unpaired *t* test, which was modified for unequal variances when necessary. Data are reported as mean ± standard error. For morphological studies, preliminary and primary data analyses were conducted as described below.

Preliminary data analysis

For measurements of myofibrillar, sarcoplasmic, and mitochondrial labeling at the EM level, 10 measurements for each subcellular category were made for each animal, allowing us to evaluate whether or not the total 30 measurements were the result of a single factor or, in contrast, more than one factor. Nuclear labeling was not measured, because random sampling of cells with cytoplasm as large as skeletal muscle cells only infrequently contained nuclei in the grid sections. We subjected the 30 measurements to principal components analysis. Based on examination of the scree plot (32) and the rotated factor pattern matrices, it seemed that there were three relatively independent components explaining variation among the 30 measurements. These components were clearly the myofibrillar, sarcoplasmic, and mitochondrial measurements. Therefore, in addition to pooling all 30 measurements together, separate analyses were conducted for the myofibrillar, sarcoplasmic, and mitochondrial measurements. However, all 10 measurements for each subcellular category were averaged to increase the measurement reliability.

Primary analysis

For EM, four primary analyses were conducted: one for the myofibrillar measurement, one for the sarcoplasmic measurement, one for the mitochondrial measurement, and one for the integrated response. Last, total values were also analyzed. For each analysis, we used ordinary least squares (OLS) regression to assess simultaneously the independent effects of age and diet on the dependent variable, gold beads/0.1 μm². Independent variables included in the model were age and diet. In each case, interactions between age and diet condition were tested. However, in no case were such interactions even close to being statistically significant; therefore, they were not retained in the model. Assumptions of the parametric statistical analysis (i.e., normality of residuals and ho-

moscedasticity) were tested. At most, minor departures were detected. To evaluate the extent to which this might be producing false positives, we recondacted the analyses using ranked data per the recommendation of Puri and Sen (33). In no case did the results change in terms of statistical significance or general magnitude of effect, which gives substantial confidence in the significance levels we report. Therefore, we report only the parametric statistical analyses here. Pairwise differences were tested following significant omnibus tests of the group effect using the Fisher LSD approach, which appropriately holds the family-wise alpha level to the nominal alpha level when there are three, and only three, groups as in the present study (34).

RESULTS

Biochemical assays

The concentration of lipid peroxidation products (MDA and 4-HDA) in rhesus monkey vastus lateralis skeletal muscle displayed an 80% maximal age-dependent increase when comparing 4-, 15-, and 26-year-old animals (Fig. 1). Levels of MDA + 4-HDA in muscle from both adult (15 years, 54.6 ± 1.6 nmol/100 mg protein) and old (26 years, 64.1 ± 0.9 nmol/100 mg protein) monkeys were significantly greater than in young (4 years, 35.9 ± 1.1 nmol/100 mg protein) animals. As shown in Fig. 2, protein carbonyl content, determined spectrophotometrically, increased progressively and significantly with age. A maximum age-dependent increase of twofold was observed. Protein carbonyl content in muscle from both adult (4.71 ± 0.01 nmol/mg protein) and old (5.58 ± 0.05 nmol/mg protein) animals was significantly greater than in young (2.61 ± 0.04 nmol/mg protein) animals. Furthermore, both adult and old groups differed significantly ($P < 0.05$) from each other.

Histology

Regardless of age or diet, no intramuscular fibrosis was noted in hematoxylin- and eosin-stained sections

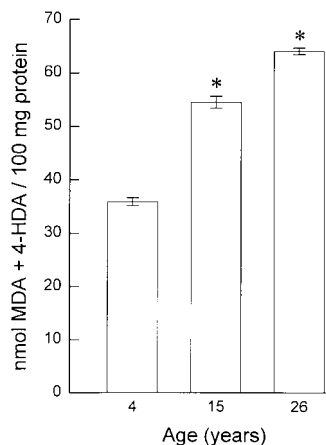


Figure 1. Quantitation of MDA and 4-HDA in rhesus monkey vastus lateralis skeletal muscle. Values represent mean \pm SE ($n=2$). * $P < 0.05$ vs. 4 years.

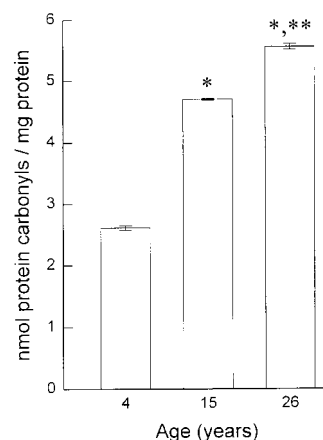


Figure 2. Quantitation of protein carbonyls in rhesus monkey vastus lateralis skeletal muscle. Values represent mean \pm SE ($n=2$). * $P < 0.05$ vs. 4 years. ** $P < 0.05$ vs. 15 years.

(data not shown). However, starting at age 16, minimal to moderate deposition of mature adipose tissue commenced, replacing normal muscle fibers (data not shown). Calorically restricted animals had a slight tendency toward less fat deposition (data not shown).

Ultrastructural analysis

EM examination of rhesus monkey vastus lateralis skeletal muscle showed normal cellular architecture, regardless of age or diet (data not shown).

Immunogold LM

Labeling in rhesus monkey skeletal muscle was negative when normal rabbit serum was used in place of primary antibody raised in rabbit (data not shown). Using a polyclonal antibody raised against HNE-modified proteins, skeletal muscle from a 4-year-old animal gave a weak antibody reaction (Fig. 3A). However, staining in tissue from a representative 26-year-old animal showed much greater reactivity (Fig. 3B). These data are representative of the age-dependent increase in labeling observed when monkeys fed standard lab chow and ranging in age from 2 to 34 years were compared. In all cases, labeling specifically localized to the sarcolemma, the sarcoplasm, and nuclei. The staining in the sarcoplasm was granular in nature. When comparing skeletal muscle from animals in the long-term CR study, the age-dependent accumulation of HNE-modified proteins in rhesus monkey skeletal muscle was shown to be attenuated by CR. To illustrate, labeling in a 17-year-old calorically restricted animal (Fig. 3C) was less than that observed in a normally fed animal of the same age (Fig. 3D). Using the OLS regression model with age and diet as predictors and mean gray value as the dependent variable (Fig. 3E), both

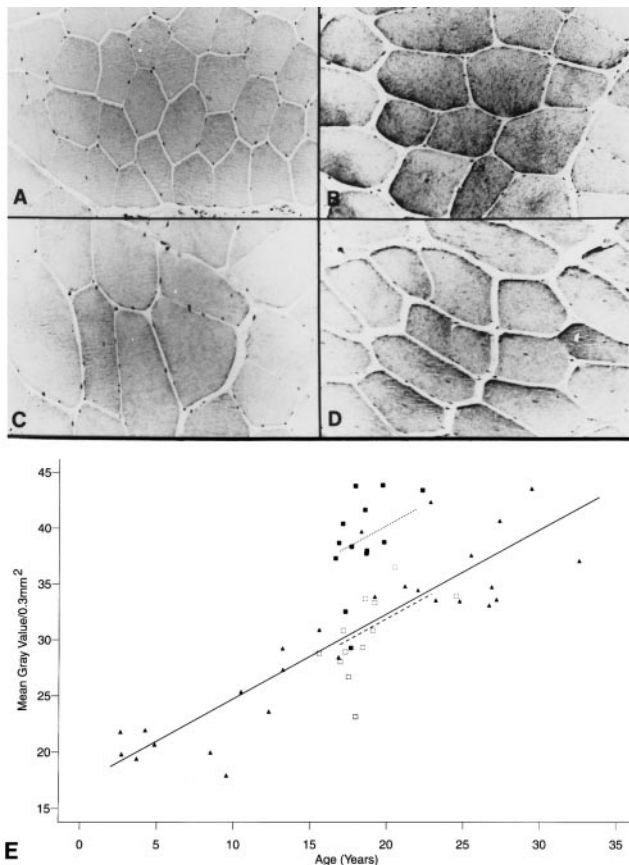


Figure 3. Immunogold light microscopy of rhesus monkey vastus lateralis skeletal muscle with antibody raised against HNE-modified proteins ($\times 200$). *A*) A 4- and *B*) 26-year-old fed standard monkey lab chow. 17-year-old *C*) calorically restricted and *D*) control animals consuming semipurified diets. *E*) Scatterplot of mean gray value vs. age by group with least-squares regression lines. Filled triangles and continuous line, normally fed monkeys (lab chow). Filled squares and thin-spaced line, control fed monkeys (semipurified diet). Open squares and thick-spaced line, calorically restricted monkeys (semipurified diet).

explanatory variables had statistically significant regression coefficients ($P < 0.001$), with no interaction of age and group. Furthermore, pairwise comparisons revealed that the normally fed purified diet group was statistically different ($P < 0.001$) from each of the other two groups.

Incubation of tissue with DNPH results in derivatization of carbonyl groups leading to DNP adduction to carbonyl-containing biomacromolecules. The specificity of this method to detect carbonyl groups was demonstrated by chemical and immunochemical controls. In the former case, reduction of carbonyl groups with NaBH_4 blocked DNPH binding (data not shown). Immunochemical validation was demonstrated by omission of DNPH treatment or substitution of normal rat serum for anti-DNP primary antibody raised in rat. In both cases, labeling was negative (data not shown). Using a monoclonal antibody raised against DNP, muscle from a 4-year-

old animal (**Fig. 4A**) showed considerably less reactivity than tissue from a 26-year-old animal (Fig. 4B). These data exemplify the age-dependent increase in labeling observed when monkeys fed standard lab chow and ranging in age from 2 to 34 years were compared. In all cases, labeling specifically localized to the sarcolemma, the sarcoplasm, and nuclei. The staining in the sarcoplasm was granular in nature. When comparing muscle from control vs. CR animals, carbonyl levels were not significantly different. For example, labeling in a 17-year-old CR animal (Fig. 4C) was less than that observed in a normally fed animal of the same age (Fig. 4D). Using OLS regression with age and diet as predictors and mean gray value as the dependent variable (Fig. 4E), only age had a statistically significant regression coefficient ($P < 0.001$). No interaction of group and age was observed.

Using a polyclonal antibody raised against NT, skeletal muscle from both a 4-year-old (**Fig. 5A**) and

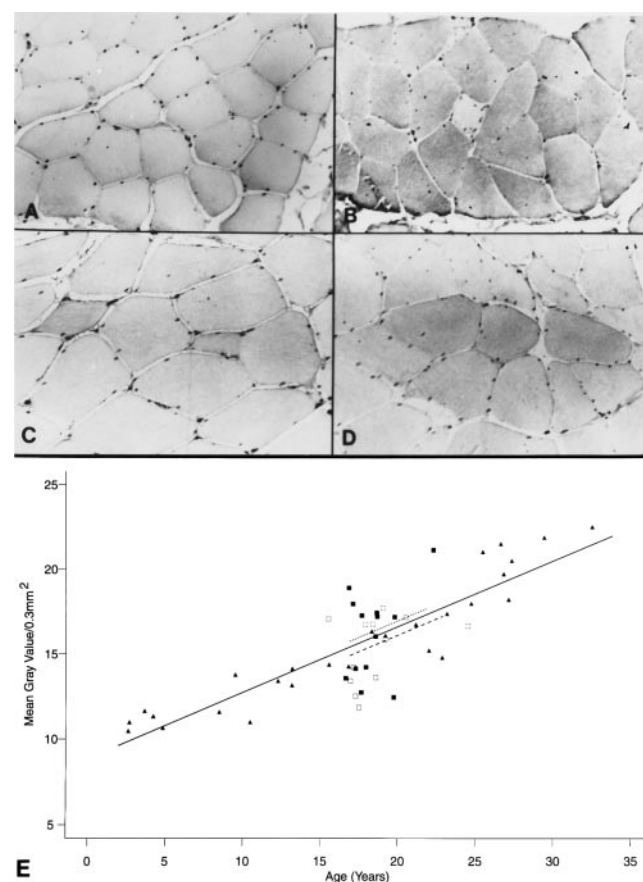


Figure 4. Immunogold light microscopy of rhesus monkey vastus lateralis skeletal muscle with antibody raised against DNP ($\times 200$). *A*) A 4- and *B*) 26-year-old fed standard monkey lab chow. 17-year-old *C*) calorically restricted and *D*) control animals consuming semipurified diets. *E*) Scatterplot of mean gray value vs. age by group with least-squares regression lines. Filled triangles and continuous line, normally fed monkeys (lab chow). Filled squares and thin-spaced line, control fed monkeys (semipurified diet). Open squares and thick-spaced line, calorically restricted monkeys (semipurified diet).

a 26-year-old (Fig. 5B) animal gave similar moderate antibody reactions. Previous results from our laboratory (T. A. Zainal and T. D. Oberley, unpublished observations) showed that preincubation of nitrotyrosine with the anti-NT polyclonal antibody blocked binding in Western blot studies of kidney homogenates. Labeling localized to the sarcolemma, the sarcoplasm, and nuclei. The staining in the sarcoplasm was granular in nature. When comparing skeletal muscle from animals in the long-term CR study, nitrotyrosine reactivity was lowered by CR. For instance, labeling in a 17-year-old CR animal (Fig. 5C) was less than that observed in a normally fed animal of the same age (Fig. 5D). Labeling was negative when normal rabbit serum was used in place of anti-NT primary antibody (data not shown). Using OLS regression (Fig. 5E), both age ($P=0.015$) and diet ($P<0.001$) had statistically significant regression coefficients using mean gray value as the dependent variable. OLS regression analysis showed

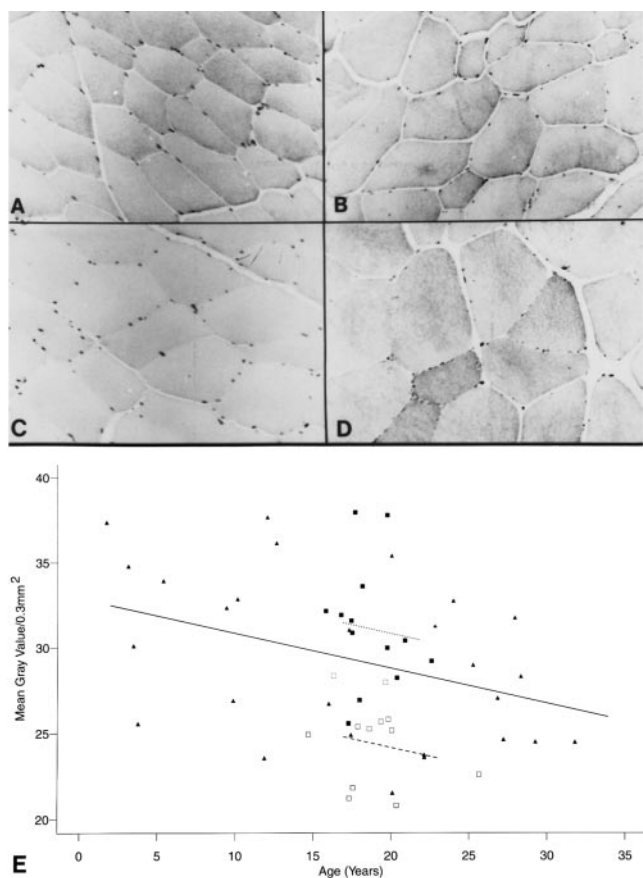


Figure 5. Immunogold light microscopy of rhesus monkey vastus lateralis skeletal muscle with antibody raised against NT ($\times 200$). A) A 4 and B) 26-year-old fed standard monkey lab chow. 17-year-old C) calorically restricted and D) control animals consuming semipurified diets. E) Scatterplot of mean gray value vs. age by group with least-squares regression lines. Filled triangles and continuous line, normally fed monkeys (lab chow). Filled squares and thin-spaced line, control fed monkeys (purified diet). Open squares and thick-spaced line, calorically restricted monkeys (semipurified diet).

that NT levels decreased significantly with age. Pairwise comparisons revealed that the CR group was statistically significantly different ($P<0.001$) from each of the normally fed groups.

Immunogold EM

Labeling in normal rabbit serum-stained skeletal muscle was negative (data not shown). Weak labeling using antibody raised against HNE-modified proteins was present in skeletal muscle from a 4-year-old rhesus monkey (Fig. 6A). As displayed in Fig. 6B, increased antibody labeling was detected in a 26-year-old animal. These data are representative of the age-dependent increase in labeling observed when rhesus monkeys fed standard lab chow and ranging in age from 2 to 34 years were compared. In all cases, staining was localized to myofibrils, nuclei, and the sarcoplasmic reticulum. Labeling was primarily localized to myofibrils. No mitochondrial labeling was observed. When comparing biopsies from animals in the long-term CR study, the age-dependent accumulation of HNE-modified proteins in rhesus monkey skeletal muscle was shown to be attenuated by CR. To illustrate, labeling in a representative 17-year-old CR animal (Fig. 6C) was considerably less than that observed in a normally fed animal of the same age (Fig. 6D). Using an OLS regression model with age and diet as predictors and myofibrillar, sarcoplasmic, or total gold bead count as the dependent variable (Fig. 6E), both explanatory variables had statistically significant regression coefficients ($P<0.001$), with no interaction of age and group. Furthermore, pairwise comparisons revealed statistically significant differences ($P<0.001$) between all three diet groups regardless of count classification.

For immunogold EM analysis of carbonyl groups, labeling was negative when the controls described in the immunogold LM results section were used (data not shown). Using anti-DNP antibody, skeletal muscle from a 4-year-old animal (Fig. 7A) displayed lower reactivity than tissue from a representative 26-year-old animal (Fig. 7B). These data exemplify the age-dependent increase in labeling observed when animals fed standard lab chow and ranging in age from 2 to 34 years were compared. In all cases, staining was primarily localized to myofibrils, but was also found in mitochondria, nuclei, and the sarcoplasmic reticulum. The age-dependent accrual of carbonyl groups in rhesus monkey skeletal muscle was shown to be attenuated by CR (e.g., 17-year-old CR animal [Fig. 7C] vs. control of the same age [Fig. 7D]). Using OLS regression (Fig. 7E), both age and diet had statistically significant regression coefficients ($P<0.001$) when using myofibrillar or total gold bead count as the dependent variable. For sarcoplasmic counts, both age ($P<0.001$) and diet

($P < 0.05$) also had statistically significant regression coefficients. However, coefficients for age and diet were not statistically significant for mitochondrial measurements. Pairwise comparisons revealed that the normally fed semipurified diet group was statistically significantly different ($P < 0.001$) from each of the other two groups for the myofibrillar and total gold bead count classifications. For sarcoplasmic counts, both normally fed groups were statistically significantly different (< 0.01) from each other. No differences were observed for mitochondrial measurements.

Skeletal muscle from both a 4-year-old (Fig. 8A) and a 26-year-old (Fig. 8B) animal gave similar moderate antibody reactions when anti-NT was used. Generally, no age-dependent change in labeling was observed when animals fed lab chow and ranging in age from 2 to 34 years were compared. In all cases,

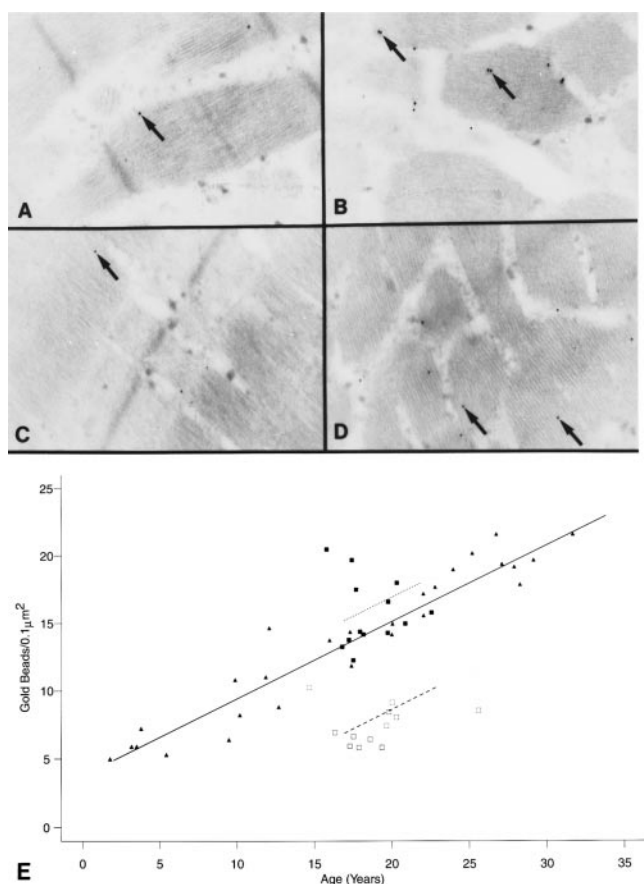


Figure 6. Immunogold EM of rhesus monkey vastus lateralis skeletal muscle with antibody raised against HNE-modified proteins ($\times 35,000$). A) A 4 and B) 26-year-old fed standard monkey lab chow. 17-year-old C) calorically restricted and D) control animals consuming semipurified diets. Arrow, myofibrillar labeling. E) Scatterplot of total (myofibrillar and sarcoplasm) gold beads vs. age by group with least-squares regression lines. Filled triangles and continuous line, normally fed monkeys (lab chow). Filled squares and thin-spaced line, control fed monkeys (semipurified diet). Open squares and thick-spaced line, calorically restricted monkeys (semipurified diet).

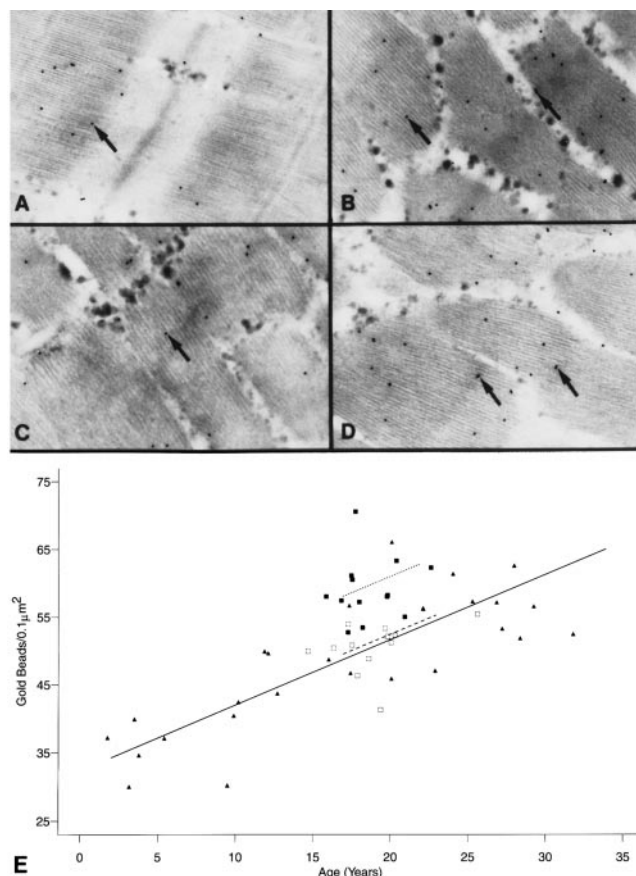


Figure 7. Immunogold EM of rhesus monkey vastus lateralis skeletal muscle with antibody raised against DNP ($\times 35,000$). A) A 4 and B) 26-year-old fed standard monkey lab chow. 17-year-old C) calorically restricted and D) control animals consuming semipurified diets. Arrow, myofibrillar labeling. E) Scatterplot of total (myofibrils, sarcoplasm, and mitochondria) gold beads vs. age by group with least-squares regression lines. Filled triangles and continuous line, normally fed monkeys (lab chow). Filled squares and thin-spaced line, control fed monkeys (semipurified diet). Open squares and thick-spaced line, calorically restricted monkeys (semipurified diet).

staining was localized to mitochondria, myofibrils, nuclei, and the sarcoplasmic reticulum. Labeling was primarily localized to myofibrils. When comparing skeletal muscle from animals in the CR study, nitrotyrosine reactivity was lowered by CR. For instance, labeling in a 17-year-old CR animal (Fig. 8C) was considerably less than that observed in an age-matched control (Fig. 8D). Using OLS regression (Fig. 8E), diet but not age had a statistically significant regression coefficient ($P < 0.001$) using myofibrillar, sarcoplasmic, or total gold bead counts as the dependent variable. Mitochondrial measurements showed no statistical significance. It should be noted that the influence of age approached statistical significance for the myofibrillar ($P = 0.073$) and total ($P = 0.079$) measurements. Pairwise comparisons revealed statistically significant differences ($P < 0.001$) between all three diet groups for myofibrillar and

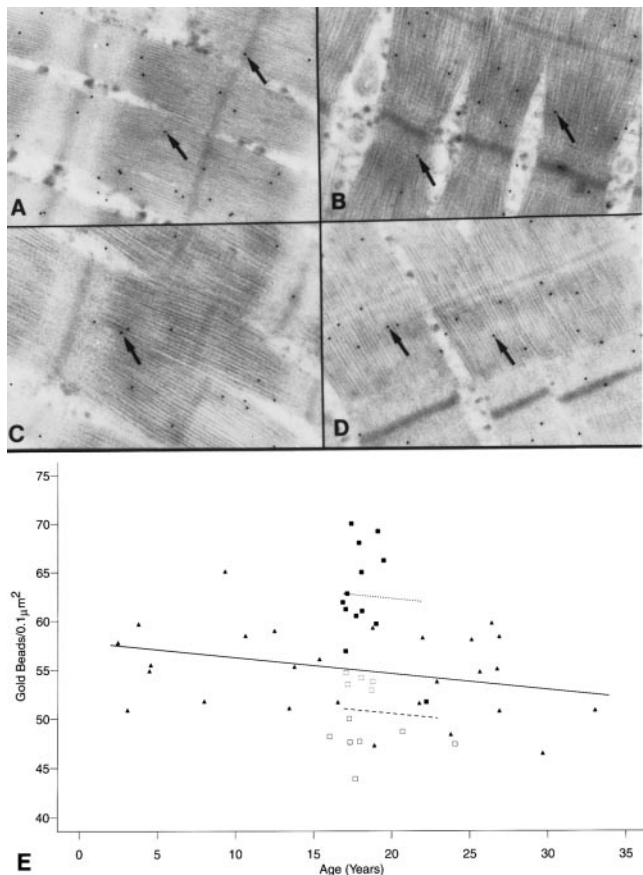


Figure 8. Immunogold EM of rhesus monkey vastus lateralis skeletal muscle with antibody raised against NT ($\times 35,000$). *A*) *A* 4 and *B*) 26-year-old animal fed standard monkey lab chow. 17-year-old *C*) calorically restricted and *D*) control animal consuming semipurified diets. Arrow, myofibrillar labeling. *E*) Scatterplot of total (myofibrils, sarcoplasm, and mitochondria) gold beads vs. age by group with least-squares regression lines. Filled triangles and continuous line, normally fed monkeys (lab chow). Filled squares and thin-spaced line, control fed monkeys (semipurified diet). Open squares and thick-spaced line, calorically restricted monkeys (semipurified diet).

total counts. For sarcoplasmic counts, the lab chow group was statistically significantly different ($P < 0.05$) from each of the other groups. Again, mitochondrial measurements showed no statistical significance.

DISCUSSION

Although many studies have measured biochemically age-dependent accumulation of oxidative damage, our study is the first to localize morphologically and quantify oxidative damage in aging mammalian skeletal muscle. Furthermore, this is the first demonstration that CR of rhesus monkeys lowers oxidative damage in skeletal muscle. Another finding reported here is the subcellular localization of oxidative damage primarily to myofibrils using immunogold EM

techniques and antibodies raised against HNE-modified proteins, DNP, and NT.

In general, mitochondria are presumed to be the primary cellular sites of reactive oxygen species production during aging; however, our study demonstrates that oxidative damage does not localize primarily to that subcellular compartment in rhesus monkey skeletal muscle. Enhanced antioxidant protection relative to other subcellular compartments and the diffusion of free radicals out of mitochondria to cause damage elsewhere may explain these results. Note that mitochondria did label with anti-DNP and anti-NT antibodies, but not with anti-HNE modified proteins. The explanation for this is not clear, though we have identified strong mitochondrial labeling of mitochondria with antibody to HNE-modified proteins in other systems, including kidney and cardiac muscle. One possible explanation is that HNE performs a specific physiological function in skeletal muscle mitochondria, with enzymes designed to cleave it specifically from that location. Future studies will be necessary to test this hypothesis.

In the present study, biochemically measured lipid peroxidation-derived aldehydes, such as MDA and 4-HDA, were significantly elevated in muscle from rhesus monkeys aged 15 and 26 years vs. 4-year-old animals. These data on lipid peroxidation agree with previous studies (9–12, 17–19) demonstrating that tissue content of thiobarbituric acid reactive substances (TBARS), a marker of endogenous lipid peroxidation, increases with age in mammalian skeletal muscle. Comparing the concentration of TBARS in the mitochondrial fraction isolated from upper hind limb skeletal muscle of mice ranging in age from 7 to 29 months, Lass et al. (17) showed a sigmoid increase with age. A rapid increase occurred between 12 and 14 months of age with no additional elevation thereafter. However, Lass et al. (17) also reported that the concentration of TBARS in mitochondria from mice subjected to CR was unaltered during an equivalent period of the life span. When vastus medialis or lateralis from 66 humans aged 25 to 93 years was compared, there was a significant age-dependent increase in levels of MDA (18). Also, examination of vastus lateralis, rectus abdominis, and gluteus maximus muscles from 117 humans aged 17 to 91 years revealed a significant increase in MDA and 4-HNE levels when comparing 66- to 75- and 76- to 85-year-old subjects with those aged 17–25 years (19).

Our biochemical data on protein carbonyl content concur with previous reports (17–18) of age-dependent increases in mammalian skeletal muscle. Using mitochondria isolated from upper hind limb skeletal muscle, Lass et al. (17) observed an age-dependent increase in mitochondrial carbonyl content when

comparing mice from 7 to 29 months of age. In contrast, during a comparable range of age, Lass et al. (17) also reported that mitochondria from mice on CR exhibited no discernible increase in carbonyl content. When vastus medialis or lateralis from 66 humans aged 25 to 93 years was compared, there was a significant age-dependent increase in protein carbonyl content (18). In this study, Mecocci et al. (18) found that skeletal muscle protein carbonyl levels from humans aged >60 years were statistically significantly greater than subjects <50 years in age. Although not statistically significant, Viner et al. (16) also reported that carbonyl groups per mole of Ca-ATPase in sarcoplasmic reticulum (SR) vesicles isolated from hind limb muscles of male Fisher 344 rats aged 5 or 28 months increased with aging.

As a result of its high reactivity with biological molecules (particularly protein), HNE appears to be an important aldehyde generated by the peroxidation of cellular membrane lipids. In this study, an approximate twofold maximal increase in MDA and 4-HDA was measured biochemically in vastus lateralis from animals aged 4, 15, and 26 years. The biochemical assay we used measures free 4-HDA, of which HNE is a major species, whereas the antibody used for histological analysis was raised specifically against HNE-modified proteins. A comparison between biochemical and morphological measurements therefore is limited since free HNE and HNE adducts have distinct chemical properties. Immunogold LM analysis of skeletal muscle from monkeys ranging in age from 2 to 34 years old revealed a twofold maximal increase in levels of HNE-modified proteins as well. However, a fourfold maximal increase was observed using immunogold EM analysis. At the light level, HNE-modified protein labeling in skeletal muscle from calorically restricted animals was 20% less than in tissue from normally fed animals, whereas immunogold EM revealed a 50% inhibition. Increased sensitivity due to enhanced antigen preservation with immunogold EM and inherent discrepancies in software analysis with immunogold LM may explain these differences.

Incubation of tissue with DNPH results in derivatization of free carbonyls on macromolecules, leading to DNP adduction to the carbonyl-containing molecule. Therefore, unlike the antibody used to detect HNE-modified proteins, a direct comparison can be made between biochemical and morphological analysis of carbonyl groups. In our investigation, an approximate twofold maximal increase in protein carbonyl content was measured biochemically in muscle from animals aged 4, 15, and 26 years. In agreement, a similar increase was observed using both immunogold light and EM analysis and an antibody raised against DNP. Using immunogold LM, carbonyl labeling in muscle from animals on

long-term CR was only 5% less than in tissue from normally fed animals, whereas immunogold EM revealed a 15% inhibition. In regard to the NT antibody, immunogold LM demonstrated an age-dependent decrease in NT levels whereas immunogold EM analysis showed no change with age. As previously mentioned, increased sensitivity due to enhanced antigen preservation with immunogold EM and inherent discrepancies in software analysis with immunogold LM may explain these differences. Nitrotyrosine levels were lowered by 20% by CR at both the light and EM level.

It should be noted that animals consuming the standard laboratory chow were not allowed true *ad libitum* access to food. These animals were fed in the morning and, by the afternoon, many had consumed all of their food. In contrast, the normally fed animals on the semipurified diet always had food remaining in their cages by late afternoon. Measurements of total body %FTM using dual-energy X-ray absorptiometry confirmed that animals fed standard laboratory chow had a total body %FTM comparable to CR and significantly different from normally fed animals (data not shown). Therefore, the animals consuming standard lab chow were, in fact, calorically restricted in comparison to the normally fed animals given semipurified diet, thus providing a likely explanation for the difference in accrual of oxidative damage with age.

The present histological data demonstrate that the subcellular localization of oxidative damage in rhesus vastus lateralis is generally myofibrillar in nature, regardless of age or diet. For all antibodies, staining was largely localized to myofibrils, nuclei, and the sarcoplasmic reticulum. However, staining was localized primarily to myofibrils. The bulk of skeletal muscle intracellular protein is comprised of myofibrillar proteins (35), which are necessary for the generation and transmission of contractile force. The sheer abundance of myofibrillar proteins may explain in part the subcellular localization of oxidative damage to myofibrils observed in this study. On the other hand, turnover of individual proteins in skeletal muscle occurs at widely differing rates. For example, myofibrillar protein turnover is different from that found in the mitochondrial, nuclear, and sarcoplasmic reticulum compartments. The myofibrillar proteins actin and myosin are continuously degraded and replaced at rates that vary according to muscle fiber type and the age of the animal (36). However, the myofibrillar proteins actin and myosin are long-lived, with a turnover of 1–2% per day, hence a half-life of 30–60 days (37). In contrast, a recent study (38) using dual isotope analysis of rat skeletal muscle has shown that calcium regulatory proteins of the SR, such as Ca-ATPase, the ryanodine receptor (RyR), and calsequestrin, and

the abundant SR protein—the 53 kDa glycoprotein—display half-lives of less than 14 days. These investigators report that turnover rates among individual SR proteins differed, thereby suggesting individual protein susceptibility to accrual of oxidative damage. Therefore, in contrast to other intracellular proteins in skeletal muscle, myofibrillar proteins likely pose a target for accumulation of oxidative damage due to their sheer abundance and slower turnover.

An age-dependent decrease in mammalian skeletal muscle protein turnover appears to occur (39). The decline in skeletal muscle protein turnover in rats from weaning to old age has been carefully described (40, 41). Goldspink et al. (42) found that between weaning and senescence, rat diaphragm and extensor digitorum longus skeletal muscles exhibit progressive decreases in their fractional rates of growth, protein synthesis, and protein turnover. Also, using measurement of urinary excretion of 3-methylhistidine (3-MH), an index of myofibrillar protein breakdown, and rate of L-[¹⁴C]-tyrosine incorporation into gastrocnemius skeletal muscle, Fruhbeck et al. (43) demonstrated an age-dependent reduction in the rate of skeletal muscle protein turnover in male Wistar rats. In that study, degradation rates were slightly faster than synthesis reduction. No significant change with age in the urinary 3-MH-creatinine ratio was observed in an investigation comparing human subjects 18–25 years of age with those 67–91 years (44). However, Yarasheski et al. (45) reported a significant age-dependent decrease in postabsorptive muscle protein synthesis when subjects 23–25 and 60–73 years old were compared. Myofibrillar protein synthesis was also shown to decrease with age in skeletal muscle from humans aged 21–31 vs. 62–81 years (46). Balagopal et al. (47) reported a progressive decline in myosin heavy-chain, but not sarcoplasmic, protein synthesis rate with advancing age in humans. CaATPase and the RyR exhibit significantly slower turnover than other SR proteins in aged rat skeletal muscle (38). These authors postulate that these two sarcoplasmic proteins are prone to greater accumulation of modifications with age. This is of note since the myofibrillar proteins actin and myosin have longer half-lives than CaATPase and the RyR. An age-dependent decrease in skeletal muscle protein turnover could account, in part, for the age-dependent accrual of HNE-modified proteins and carbonyls reported here.

Nitric oxide (NO) performs many regulatory functions in skeletal muscle (48, 49). Along with other reactive nitrogen species generated as a result of the interaction between oxygen and NO, peroxynitrite catalyzes the nitration of protein tyrosine residues to form NT adducts. Our immunogold light and EM

NT results are consistent with a report (50) describing no accumulation of nitrotyrosine adducts with advancing age. Using isotope dilution gas chromatography-mass spectroscopy, Leeuwenburgh et al. (50) observed no change in 3-nitrotyrosine levels when comparing vastus lateralis from rats aged 9 and 24 months. These authors speculated that their analysis of whole tissue homogenates yielded an average value for all proteins, thereby failing to detect a possible selective increase in the oxidation of specific proteins. This is a significant point since differential accumulation of NT has been shown to occur in isoforms of sarcoplasmic reticulum Ca-ATPase in rat skeletal muscle during aging (14). Specifically, Viner et al. (51) demonstrated that only the SERCA2a isoform contained significant amounts with approximately one and four NT residues per young and old Ca-ATPase, respectively. Nitrotyrosine adduct formation is dependent on equivalent production of both superoxide radical and NO. Since NO has numerous regulatory functions, we suggest that our NT findings may simply indicate that production of NO does not increase with age along with superoxide radical.

Accrual of protein oxidative damage is a function of protein synthesis, turnover, and repair. Our histological data illustrate that regardless of antibody, CR lowers oxidative damage in rhesus monkey vastus lateralis. We speculate that, to some extent, CR may do so by altering regulation of protein turnover rate. Comparing gastrocnemius muscle from 30-month-old CR and normally fed C57BL/6 mice, Lee et al. (52) used high-density oligonucleotide arrays, representing 6347 genes, to demonstrate that 16% of transcripts highly induced by CR encode proteins involved in protein synthesis and turnover. Measurements of heart (53) and whole body (54) protein turnover rates in calorically restricted rats have been shown to be age-for-age higher than for normally fed animals, despite slower growth. In contrast, muscle sizes and total, but not fractional, synthetic rates were shown to be consistently decreased by chronic CR (42, 55). Furthermore, urinary excretion of 3-MH has recently been observed to be unaffected by CR (56, 57). However, the effect of CR on turnover rates of individual skeletal muscle proteins during aging has yet to be investigated, thus entertaining the possibility that increased turnover of a specific protein may be induced by CR.

In summary, this is the first report to localize and quantify the age-dependent accumulation of oxidative damage in mammalian skeletal muscle and to characterize its attenuation by CR. Also new is the finding that CR lowers oxidative damage in aging primates. The data suggest that if an age-dependent increase in oxidative stress/damage is pivotal for the development of sarcopenia in mammalian skeletal

muscle, the target of oxidative stress is more crucial than the subcellular site of free radical production. Similar to previous studies that biochemically measured markers of oxidative damage in whole tissue homogenates, our study yields an average value for all proteins; therefore, our techniques would fail to detect a selective increase in the oxidation of specific proteins. It appears timely that future studies should not only identify individual oxidized skeletal muscle proteins, but should also attempt to demonstrate precise localization of oxidative damage. **FJ**

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