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# No Decline in Skeletal Muscle Oxidative Capacity With Aging in Long-Term Calorically Restricted Rats: Effects Are Independent of Mitochondrial DNA Integrity

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We investigated if calorie restriction (CR) preserved skeletal muscle oxidative capacity with aging after accounting for life span extension by CR, and determined if mitochondrial content, mitochondrial DNA integrity, and peroxisome proliferator-activated receptor gamma coactivator-1α (PGC-1α) were involved. Ad libitum-fed (AL) and CR animals representing young adult, late middle age, and senescence were studied. Whereas citrate synthase and complex IV activities were lower in plantaris and gastrocnemius muscle of young adult CR animals, in contrast to the 15%–40% decline in senescent AL animals, there was no decline with aging in CR animals. There was no decline in citrate synthase protein in gastrocnemius with aging in either group, suggesting that CR preserves oxidative capacity with aging by protecting mitochondrial function rather than content. This protection was independent of mitochondrial DNA damage between groups. However, there was a slower decline in PGC-1α gene expression with aging in CR versus AL animals, suggesting a better maintenance of mitochondrial biogenesis with aging in CR animals.

A decline in skeletal muscle oxidative capacity with aging is well-known (1–3), although not without exception (4). The causes of declining oxidative capacity could include a loss of mitochondrial content and/or function. Although it remains unclear whether mitochondrial content is reduced with aging, with some studies showing no change (5,6) and others a decrease (7), an impairment of mitochondrial function is well-established. For example, aged human and rodent muscles exhibit characteristics of dysfunctional mitochondria, such as a greater decline in complex IV activity than other enzymes such as citrate synthase (8–10). Furthermore, oxidative capacity per mitochondrion declines with aging (7,8,10,11). The implications of declining oxidative capacity for aging muscles are far-reaching. For example, we recently showed that a decline in oxidative capacity was the primary factor accounting for a nearly 50% decline in mass-specific skeletal muscle aerobic function during muscular contractions with aging (12). In addition, single muscle fibers exhibiting complex IV deficiency have been implicated in the focal fiber atrophy and breakage that characterize muscles undergoing sarcopenia (13,14).

The likely causes of this mitochondrial dysfunction with aging include, but are not limited to, synthesis of abnormal electron transport chain (ETC) complexes secondary to damaged mitochondrial DNA (mtDNA) (14,15) and/or an accumulation of oxidatively damaged proteins (8,16). In regard to the significance of mtDNA damage with aging, it appears likely that a threshold of damage or mutation to the mitochondrial genome is required (60%–90%) before this damage can impact mitochondrial function (17–19). In support of the idea that mtDNA damage might precipitate mitochondrial dysfunction leading to muscle fiber atrophy and breakage with aging, a recent study observed that only a small fraction of fibers containing normal ETC function exhibited deletion mutations to the mtDNA, whereas all fibers exhibiting abnormal ETC function (deficient complex IV staining) exhibited high rates of mtDNA damage (20). Other studies have also shown that damage to the mitochondrial genome with aging in *Drosophila* (21), mouse (22), rat (12,14,20,23), and nonhuman primates (24–26), likely related to a lifetime of reactive oxygen species (ROS) production and damage, are strongly associated with abnormalities in ETC function with aging. Although the data relating to the impact of mtDNA deletions on mitochondrial oxidative capacity with aging seem convincing, the majority of these studies have investigated deletions in single fibers and, even if these events are important at the single cell level, the extent to which these deletion thresholds are exceeded across whole muscles (and the impact this might have on oxidative capacity) remains unclear. Indeed, some investigators (27,28) have refuted the involvement of mtDNA mutations and deletions altogether as a major contributing factor to the decline in skeletal muscle oxidative capacity with aging.

Calorie restriction (CR), which is the only known environmental intervention to consistently increase maximal life span in mammals (29–31), has impressive protective

Table 1. Absolute Age and Survival Rates for Young Adult, Late Middle-Aged, and Senescent Fischer 344 × Brown Norway F1-Hybrid (F344BN) Rats Following Ad Libitum (AL) or Calorie-Restricted (CR) Diets

| Relative Age | AL | CR | Survival Rate |
|---|---|---|---|
| Young adult | 8–10 months old | 8–10 months old | 100% |
| Late middle age | 30 months old | 35 months old | 75% |
| Senescent | 35 months old | 40 months old | 35% |

*Note*: Values for survival rate were extrapolated from survival curves published previously for this strain of rat (31).

effects for aging skeletal muscles. We recently showed that, in addition to preventing the age-associated decline in muscle peak force per cross-sectional area (11,32), CR completely prevented the nearly 50% decline in mass-specific skeletal muscle aerobic performance between young adulthood and senescence in male Fischer 344 × Brown Norway F1-hybrid (F344BN) rats (33). Furthermore, this protection of aerobic function was due to maintained skeletal muscle oxidative capacity with aging and superior mitochondrial function in CR animals (33). The current study aims to follow-up our previous findings to determine whether the protection of oxidative capacity by CR remains after accounting for the longer life span of CR animals. We hypothesized that CR would only delay the reduction in oxidative capacity with aging such that comparison of the decline with aging in AL versus CR animals over a similar relative fraction of the life span would show little or no difference between dietary treatments. In addition, because CR has also been shown to better preserve mtDNA integrity with aging (34), we hypothesized that the maintenance of oxidative capacity with aging by CR would be associated with a better preservation of mtDNA integrity.

## Experimental Procedures

### *Animals*

All experimental procedures were carried out with the approval of the University of Calgary Animal Care Committee. Male F344BN rats were obtained from the National Institute on Aging (NIA; Baltimore, MD) in two different groups: AL (8- to 10-month-old [$n = 10$], 30-month-old [$n = 5$], and 35-month-old [$n = 11$]) and CR (8- to 10-month-old [$n = 8$], 35-month-old [$n = 8$], and 40-month-old [$n = 6$]). Table 1 shows the relative age matches between dietary groups, based on survival curves for this strain of rat (31). CR entailed a 40% reduction in caloric intake, which was gradually imposed over a 2-week period at the NIA colony beginning at 14 weeks of age, and was maintained at this level until studied in our laboratory. Note that the feed of CR animals (NIH-31/NIA fortified; NIA, Baltimore, MD) is supplemented to match the nutritional intake of that of the AL animals. Upon arrival at the University of Calgary, AL and CR animals were housed in microisolation cages (12-hour light/dark cycle, 22°C) in the same room for at least 1 week prior to study. AL and CR rats were provided with water ad libitum, whereas feed (NIH-31 for AL group; NIH-31/NIA fortified for CR group) was provided ad libitum for AL animals and at 40% reduced caloric intake for CR animals. Necropsies were performed after each experiment to detect gross tissue abnormalities or lesions to limit the inclusion of diseased animals in the data set. No animals were excluded on this or any other basis.

### *Muscle Harvesting*

Animals were anesthetized with sodium pentobarbital (50–60 mg $kg^{-1}$ i.p). The distal hind-limb muscles were removed, trimmed free of fat and connective tissue, and weighed. The plantaris muscle and the mixed region of the gastrocnemius muscle were chosen for the analysis because both have similar fiber type content, they have demonstrated different rates of muscle atrophy (12,33,35), and they are the two major muscles that contribute to the overall force production of the gastrocnemius–plantaris–soleus muscle group, which we have previously studied for the functional impact of CR with aging (33). The muscles were pulverized under liquid nitrogen, and aliquots of muscle tissue were frozen and stored at −80°C until further biochemical and molecular analyses were performed. Following removal of the muscles, animals were killed by intracardiac injection of sodium pentobarbital.

### *Biochemistry*

Activities of citrate synthase and complex IV were determined spectrophotometrically, as was done previously by our group (33). Citrate synthase is an enzyme of the tricarboxylic acid cycle (TCA) cycle and thus provides a marker of changes in the capacity of this arm of the aerobic metabolic pathway. Complex IV is a component of the ETC and is the point at which $O_2$ is consumed in the pathway. Thus, not only does complex IV relate the capacity of the system to consume $O_2$, but in our prior study changes in complex IV activity with aging paralleled those of the maximal aerobic metabolic responses of the contracting muscles (33). Thus, we can infer likely functional outcome on the basis of changes in complex IV activity. The difference between the data published in our previous article (33) and the current data set for the 8-month-old AL, 8-month-old CR, 35-month-old AL, and 35-month-old CR groups is that we have added an additional two animals to each of the groups in the current study. The data for the 30-month-old AL and 40-month-old CR groups have not been published previously in whole or in part.

In brief, muscles were homogenized in phosphate buffer (pH 8.0) containing 0.05 M Tris HCl and 0.67 M sucrose in a ratio of 1 part muscle for every 20 parts of buffer (mass/volume) at 4°C. For citrate synthase, muscle homogenates were diluted with homogenizing medium to yield a final dilution of 1:400 before being used to determine, spectrophotometrically at 412 nm (Biochrom Ultrospec 2100 Pro; Fisher Canada, Nepean, Ontario, Canada), enzyme activity by virtue of the rate of Mercaptide ion production in assay buffer containing 0.3 mM acetyl coenzyme A, 100 mM Tris buffer (pH 8.0), 0.1 mM 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), and 0.5 mM oxaloacetate at 37°C, as described previously (33,36). Similarly, muscle homogenates were used to determined complex IV activity, spectrophotometrically at 550 nm, by assessing the rate of cytochrome *c*

oxidation in assay buffer (10 mM potassium phosphate buffer [pH 7.0] at 37°C, containing reduced cytochrome *c*), as previously described (33).

*Citrate Synthase Protein Expression*

Whole muscle protein extracts were made from the mixed gastrocnemius muscle samples only, because muscle tissue from plantaris was expended on other analyses. Protein extraction was achieved by mechanical homogenization of muscle tissue in an extraction buffer containing 20 mM Tris, 5 mM EDTA, 5 mM dithiothreitol (pH 7.0) containing 300 mM NaCl in a ratio of 1 part muscle tissue to 7 parts buffer, as done previously (37). Following homogenization, the samples were left on ice for 30 minutes, with occasional gentle vortexing, followed by the removal of cell debris by centrifugation for 20 minutes at 10,000 *g*. Supernatants were removed and replaced into an Eppendorf 1.5-ml tube. Protein concentration of these samples was determined using a Bradford assay (38). Samples were then diluted by half with 2X sample buffer containing glycerol, Tris (pH 6.8), sodium dodecyl sulfate (SDS), dithiothreitol, and bromophenol blue, and then further diluted with 1X sample buffer containing glycerol, Tris pH 6.8, SDS, bromophenol blue, and beta-mercaptoethanol to a final concentration of 5 μg $ul^{-1}$. Equal quantities of protein (20 μg total load) for each sample, and a common sample between all gels along with a prestained marker (Fermentas Canada Inc.; Burlington, ON, Canada), were loaded onto 10% SDS–polyacrylamide gel electrophoresis (PAGE) mini-gels and separated by electrophoresis at 100 V for 90 minutes. Proteins were then electrotransferred to nitrocellulose membranes in a transfer buffer containing 48 mM Tris, 39 mM glycine, 0.0375% SDS, and 20% methanol at 100 V for 80 minutes. Blots were blocked for 1 hour at room temperature with 5% nonfat milk solution, then incubated with a monoclonal antibody for citrate synthase (1:10,000 in blocking solution) overnight at 4°C. The following day, blots were washed three times for 15 minutes each with phosphate-buffered saline–0.5% Tween and probed with a HRP-conjugated goat-anti mouse secondary antibody (1:2000; Pierce) suspended in 5% nonfat milk solution for 1 hour at room temperature. Blots were washed three more times for 15 minutes each with phosphate-buffered saline–0.5% Tween and then treated with chemiluminescent developing solution (Pierce, Rockford, IL) for 5 minutes. Blots were then imaged with the Gel Doc ChemiGenius 2 (Syngene, Frederick, MD) system, and densitometry was determined using the Gene Tools for Syngene software package. Data were normalized to the expression of the common sample run on each gel to account for any potential differences in transfer efficiency between blots.

*Mitochondrial DNA Deletions*

Mitochondrial DNA deletions were assessed in plantaris and mixed gastrocnemius muscle by using a modified version of the method described previously by our group (12). In the current study, 40 thermocycles consisting of 30 seconds at 94°C, 25 seconds at 66°C, and 90 seconds at 72°C in both polymerase chain reaction (PCR) and nested PCRs were performed instead of 35 cycles, as previously described (12). This modification was used in both plantaris and mixed gastrocnemius muscles in an effort to resolve intact full-length mtDNA from the mixed gastrocnemius muscle samples; this effort proved to be unsuccessful, but did improve the resolution of the deletion fragments.

*Peroxisome Proliferator-Activated Receptor Gamma Coactivator-1α Gene Expression*

RNA was extracted from mixed gastrocnemius muscle tissue only (because plantaris tissue had been expended on other analyses) by using the RNeasy kit for fibrous tissue (Qiagen Inc. Canada, Mississauga, ON, Canada), according to manufacturer's instructions. Purified RNA was then subjected to reverse transcription to generate complementary DNA by using oligo-DT primer (Invitrogen) and a PCR kit (Qiagen Inc., Canada). Complementary DNA was then used in triplicate to determine the gene expression of peroxisome proliferator-activated receptor gamma coactivator-1α (PGC-1α) by real-time PCR (Bio-Rad iCycler; Hercules, CA) using a SYBR Green fluorescent dye PCR kit (Qiagen) and the following forward and reverse primers for PGC-1α: 5′-ATGAGAAGCGGGAGTCTGAA-3′ (forward) and 5′-GCGGTCTCTCAGTTCTGTCC-3′ (reverse). Melt-curves were performed after real-time PCR to demonstrate specific amplification of our single products of interest. Gene expression is expressed relative (set to 1000) to 18s ribosomal RNA (rRNA) expression (5′-AAACGGCTACCACATC-CAAG-3′ [forward]; 5′-CAATTACAGGGCCTCGAAAG-3′ [reverse]) using Δ-Δ Ct analysis. Note that we have previously determined that mixed gastrocnemius muscle 18s rRNA expression does not change with aging in this strain of rat (Baker DJ, unpublished observations, July 2005).

*Statistics*

Values are represented as means ± standard error. Two-way analysis of variance with Holm–Sidak post hoc analysis was used to determine differences between ages and dietary intervention for muscle mass, biochemical, and PGC-1α data. One-way analysis of variance and Holm–Sidak post hoc analysis was used for citrate synthase protein expression and mtDNA deletion products. The Holm–Sidak multiple comparison test uses a ''step-down'' critical *p* value approach in determining significance to maximize statistical power without compromising the risk of making a type I error (39).

## Results

*Descriptive Data*

Table 2 contains data relating to body and skeletal muscle masses of 8- to 10-, 30-, and 35-month-old AL animals, and of 8- to 10-, 35-, and 40-month-old CR animals. Body mass in CR rats was no different across ages, and the values were consistently lower than those of corresponding age-matched, AL controls ($p < .05$). Whereas CR rats exhibited smaller skeletal muscle masses compared with AL controls at each relative age comparison (except for the plantaris muscle at senescence), two other observations are noteworthy. First, the degree of muscle mass decline across the same absolute age range (between 8- to 10-month-old and

Table 2. Descriptive Data

| Group | Age | Body Mass (g) | Plantaris Mass (mg) | Gastrocnemius Mass (mg) |
|---|---|---|---|---|
| 8- to 10-mo-old AL | YA | 429 ± 9[a] | 399 ± 11[a] | 2081 ± 53[a] |
| 8- to 10-mo-old CR | | 320 ± 6[b] | 317 ± 7[b] | 1635 ± 53[b] |
| 30-mo-old AL | LMA | 576 ± 11[c] | 366 ± 12[c] | 1726 ± 58[c] |
| 35-mo-old CR | | 315 ± 7[b] | 242 ± 11[d] | 1235 ± 65[d] |
| 35-mo-old AL | SEN | 462 ± 14[d] | 199 ± 18[e] | 884 ± 77[e] |
| 40-mo-old CR | | 278 ± 16[b] | 189 ± 8[e] | 658 ± 89[f] |

*Notes*: Values are mean ± standard error.

Groups that do not share a superscript are significantly different ($p < .05$).

AL = ad libitum fed; CR = calorie restricted; YA = young adult; LMA = late middle-aged; SEN = senescent.

35-month-old) was markedly lower in CR rats (24% for both plantaris and gastrocnemius muscles) than in AL rats (50% for plantaris muscle, 58% for gastrocnemius muscle), showing that CR slows the loss of muscle mass with aging. Second, comparison of the degree of muscle atrophy across the same relative age range (between young adulthood and senescence) showed a similar decline in AL versus CR animals (40% for plantaris muscle and 60% for gastrocnemius muscle). Thus, CR slows muscle atrophy in proportion to the extension of life span.

### *Biochemistry*

Figure 1 shows citrate synthase and complex IV activities as determined from mixed gastrocnemius and plantaris muscle homogenates of AL and CR animals. The oxidative capacity of both plantaris and mixed gastrocnemius muscles was significantly lower in CR than in AL animals at 8–10 months of age. There was no decline between 8- to 10- and 30-month-old AL animals. However, by 35 months of age there was a significant decline in the oxidative capacity of both muscles in AL animals, with the extent of this decline varying between different enzymes and muscles. The decline in citrate synthase activity in the mixed gastrocnemius muscle was 23% in the AL group between the ages of

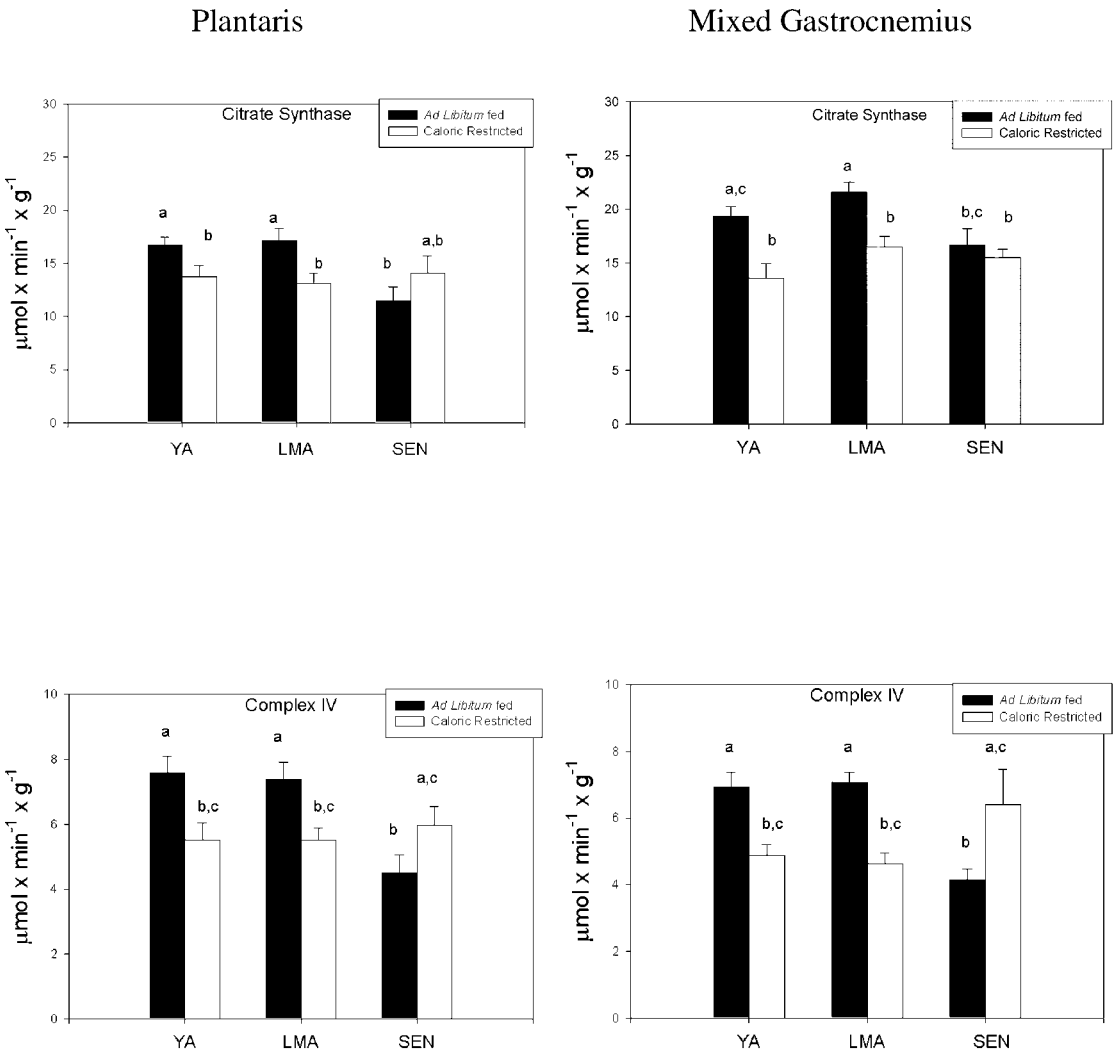


Figure 1. Citrate synthase and complex IV activities from plantaris and mixed gastrocnemius muscles of ad libitum fed or calorically restricted young adult, late middle age, and senescent Fischer 344 × Brown Norway F1-hybrid (F344BN) rats. Values are mean ± standard error. Groups that do not share a superscript are significantly different ($p < .05$). YA = young adult; LMA = late middle aged; SEN = senescent.

late middle age (30 months) and senescence (35 months), whereas the decline in citrate synthase activity in the plantaris muscle was 33% between the same ages ($p < .05$). Aging had no effect on the citrate synthase activity of either muscle group in the CR animals. The decline in complex IV activity of AL animals was 40% and 41% in mixed gastrocnemius and plantaris muscles, respectively. Similar to citrate synthase, complex IV activity did not decline with age in CR animals.

### *Citrate Synthase Protein Expression*

Densitometric analysis of chemiluminescent bands for citrate synthase protein expression revealed that no differences existed between dietary and age groups (Figure 2, $p = .480$). Citrate synthase protein expression was not conducted for 30-month-old AL animals because citrate synthase enzyme activity in those animals was no different from that in the 8- to 10-month-old AL animals. This decision was further justified given that no difference in citrate synthase protein expression existed between groups even when compared at senescence, despite a reduction in citrate synthase activity by senescence.

### *Mitochondrial DNA Fragmentation*

Figure 3 shows the frequency of mtDNA fragmentation with aging in both plantaris (Figure 3A) and mixed gastrocnemius (Figure 3B) muscles of AL and CR animals. Note that a 7591 bp region of the mitochondrial genome was amplified by nested PCR which relates to the portion of the mitochondrial genome referred to as the ''major arc.'' This region contains 10 of the 13 mtDNA-encoded peptides of the ETC, as we have detailed previously (12). In the plantaris muscle (Figure 3A) the sample for the 8- to 10-month-old AL group did not run on the gel successfully; however, based on our previous results with plantaris muscle, this group does not exhibit any mtDNA fragmentations (12). This issue aside, both muscle groups in young adult animals showed strong amounts of full-length mtDNA, with few deletions, which supports our previous findings in plantaris muscle (12). In the senescent AL group (35-month-old) a number of mtDNA fragments (occurrence of visualized bands on ethidium bromide–stained agarose gels at sizes smaller than the full length of the amplicon–7591 bp) was evident in both plantaris ($3.4 \pm 0.7$) and gastrocnemius ($7.8 \pm 0.9$) muscles, which were of similar frequency for CR animals at both the same absolute age (35 months; $3.3 \pm 1.0$ and $7.8 \pm 0.5$ for plantaris and gastrocnemius muscles, respectively) and same relative age (40 month; $1.0 \pm 0.0$ and $6.5 \pm 0.5$, respectively). Although there was a greater number of deletions in the gastrocnemius muscle than in the plantaris muscle ($p < .05$), the above noted values for the frequency of mtDNA deletion fragments were not different within either the plantaris ($p = .296$) or gastrocnemius ($p = .516$) muscles between dietary treatments at the same absolute or relative age. Note that numbers at the bottom of each lane in both Figure 3A and 3B reflect the number of bands detected within that lane.

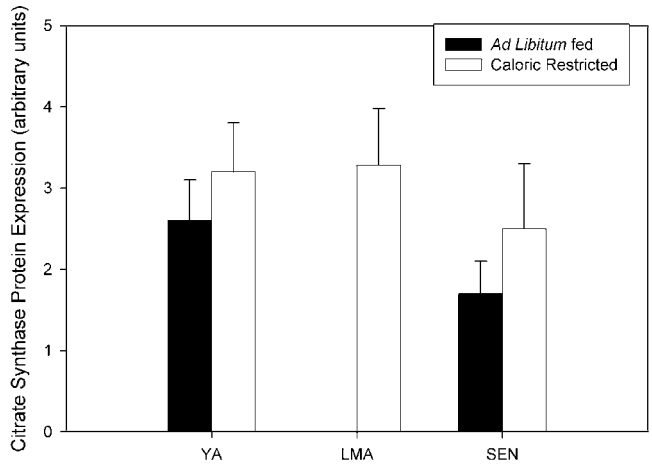


Figure 2. Citrate synthase protein expression for mixed gastrocnemius muscle of ad libitum fed and calorically restricted young adult (YA) and senescent (SEN) Fischer 344 × Brown Norway F1-hybrid (F344BN) rats. Values are mean ± standard error. LMA = late middle aged.

### *PGC-1α Gene Expression*

Figure 4 shows quantitative gene expression of PGC-1α, relative to 18s rRNA expression, in mixed gastrocnemius muscle of each group. Again due to tissue limitation with the plantaris muscle, PGC-1α was not measured in plantaris muscle. No difference in expression existed between diets in young adult animals. However, PGC-1α was reduced by 92% by late middle age (30 months old) in the AL group, whereas the decline was only 54% by late middle age in the CR group (such that the level of PGC-1α gene expression in late middle aged CR animals was greater than that in the relative age-matched AL group; $p < .05$). At senescence, AL animals demonstrated a 76% lower PGC-1α expression than that of young adults, which was no different from that of 30-month-old AL animals ($p = .335$). The magnitude of decline in PGC-1α gene expression in CR animals at senescence was no different from that observed for AL senescent animals (74% vs 76%).

Note that one animal from the 35-month-old CR group was excluded from this data set because it was deemed a statistical outlier, being more than 2 standard deviations away from the mean for this or any other group. The data for the 35-month-old CR group inclusive of this one outlier would be $286 \pm 129$ compared with $163 \pm 48$ following its exclusion, but irrespective of the inclusion of this outlier there was no effect on the data interpretation.

## Discussion

The purposes of this study were: (i) to determine if the previously observed maintenance of skeletal muscle oxidative capacity with aging by CR remained after accounting for the extension of life span by CR, and (ii) to gain insight into the mechanisms by which CR may be mediating its effects. A maintenance of skeletal muscle oxidative capacity by CR after accounting for the extension of life span would suggest that CR might actually prevent the decline in oxidative capacity with aging. In contrast, an age-associated

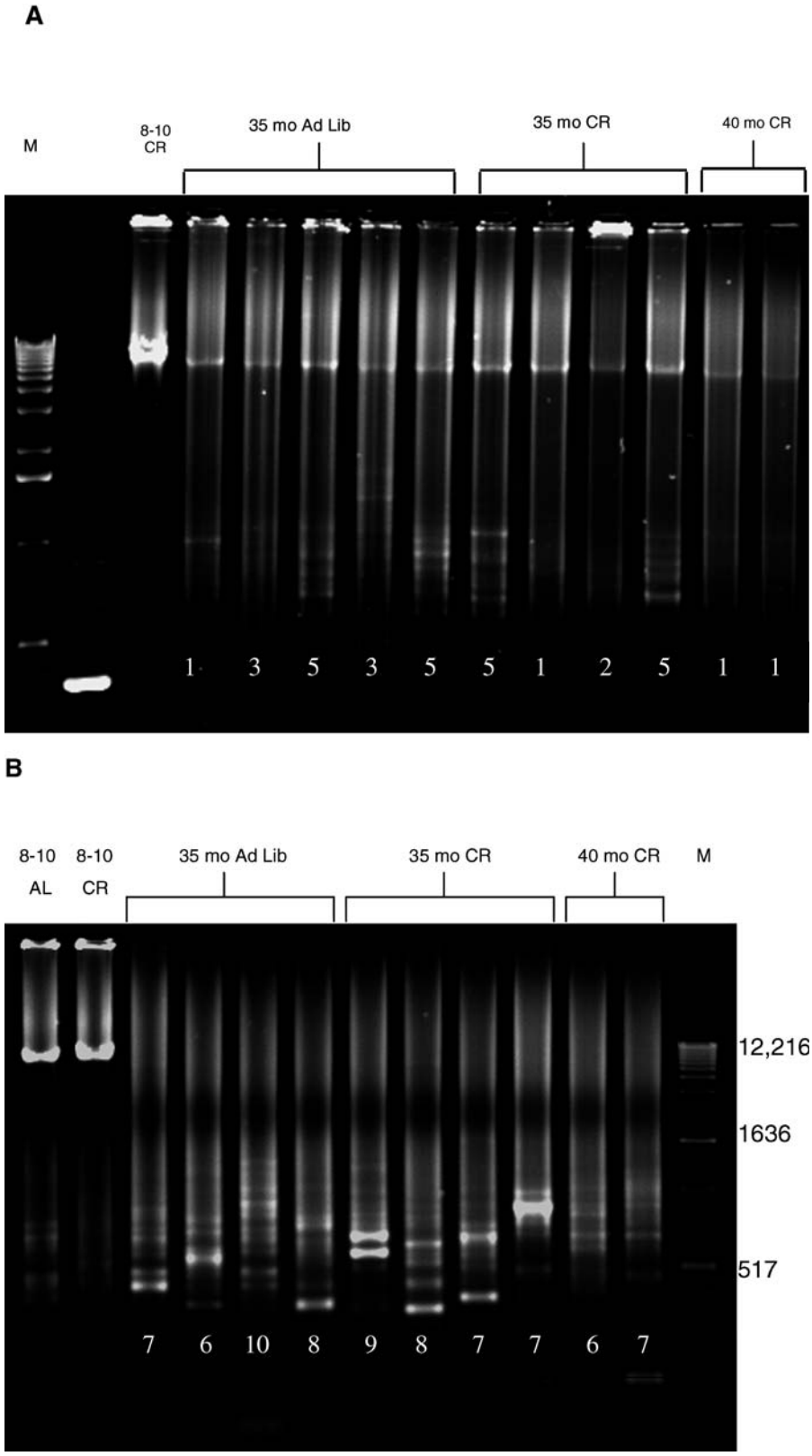


Figure 3. Ethidium bromide–stained agarose gels showing mitochondrial DNA fragmentation in plantaris (**A**) and mixed gastrocnemius (**B**) from young adult and aged ad libitum fed (Ad Lib) and calorie-restricted (CR) Fischer 344 × Brown Norway F1-hybrid (F344BN) rats. Numbers at the bottom of each lane represent number of bands detected within the lane.

decline in oxidative capacity in CR animals that parallels in magnitude that seen in AL animals after accounting for the life-span differences between the AL and CR animals would suggest that CR simply delays the decline in proportion to the extension of life span. In contrast to our hypothesis, CR was effective in completely preventing the decline in skeletal muscle oxidative capacity in both plantaris muscle and the mixed region of gastrocnemius muscle out to a survival rate of ≈35% in CR animals, which is a time when AL animals exhibit a 40% reduction in oxidative capacity. Although it is possible that even older CR animals might exhibit a decrease in oxidative capacity, our results show that CR does more than simply slow the decline of oxidative capacity in proportion to the extension of life span. This result likely translates to an extension of the health span by CR because the protection of oxidative capacity by CR has been shown to be important to the maintenance of skeletal muscle contractile function with aging (33). In explaining the means by which CR is acting to maintain oxidative capacity, because differences in citrate synthase protein content with aging and/or CR did not correlate with the observed differences in oxidative capacity with aging and/or CR, the results suggest that CR acts primarily to preserve mitochondrial function. In addition, differences in the accumulation of mtDNA deletion products with aging between AL and CR animals did not correlate with the protection of oxidative capacity by CR, suggesting that the accumulation of mtDNA deletion products in AL animals was not sufficient to contribute to the erosion of oxidative capacity with aging and that the protective actions of CR are independent of its effects on mtDNA integrity. In contrast, because one of the actions of CR is promotion of a higher rate of protein turnover, which in turn is thought to reduce the accumulation of oxidatively damaged proteins with aging, it is relevant that the gene expression levels of PGC-1α, a known stimulator of mitochondrial biogenesis, demonstrated a slower age-related decline in muscles of CR animals. This finding is consistent with a protection of oxidative capacity secondary to a higher rate of mitochondrial protein turnover with aging by CR. Thus, our results provide new insight into the duration of oxidative capacity protection with aging by CR and the means by which CR is achieving this protection.

### *CR Prevents the Decline in Skeletal Muscle Oxidative Capacity With Aging*

It is well established that skeletal muscle oxidative capacity declines with aging (1–3). Although some prior studies have suggested that CR does not maintain skeletal muscle oxidative capacity with aging, two of these studies did not include a young adult CR group (11,40) and a third study used every-other-day feeding with no supplementation of nutrient intake to make up that lost due to lower food intake (41). In opposition to these studies, Desai and colleagues (10) previously showed that CR prevented the age-related decline in oxidative capacity in gastrocnemius muscle of 20-month-old female B6C3F1 mice, and we recently showed that CR prevents the age-associated decline in skeletal muscle oxidative capacity at least out to 35 months of age in male F344BN rats (35% survival rate for AL animals) (33). These latter two studies both used young adult CR groups and nutritional supplementation of the feed to ensure adequate nutrition in the CR animals. In extending our understanding of the efficacy of CR in maintaining skeletal muscle oxidative capacity, the current results demonstrate that there is no decline in skeletal muscle oxidative capacity with aging in CR animals even after accounting for the extension of life span seen with this intervention. Specifically, we show here that in contrast to the ≈40% decline in oxidative capacity between 8 months (100% survival rate) and 35 months (35% survival rate) in AL animals, there was no decline in oxidative capacity between 8 months and 40 months (35% survival rate for CR) in CR rats. As such, these results lead to the remarkable conclusion that CR not only extends the absolute age span over which skeletal muscle oxidative capacity is maintained with aging, it actually maintains oxidative capacity over a greater proportion of the life span.

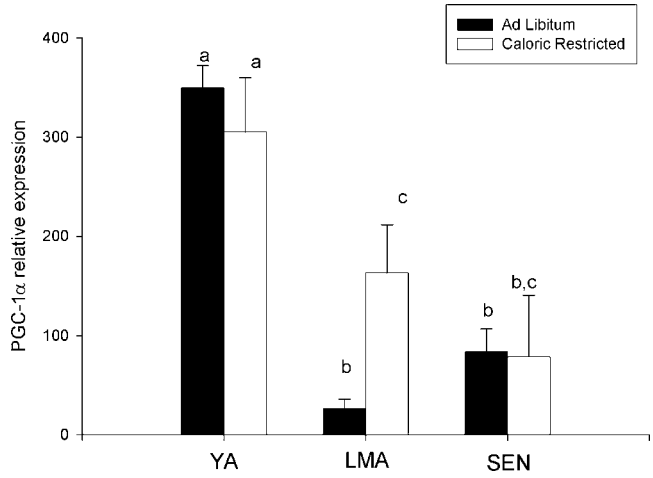


Figure 4. Peroxisome proliferator-activated receptor gamma coactivator-1α (PGC-1α ) gene expression in mixed gastrocnemius muscle as function of age in ad libitum fed or calorie-restricted Fischer 344 × Brown Norway F1-hybrid (F344BN) rats. Values are mean ± standard error. Groups that do not share a superscript are significantly different ($p < .05$). YA = young adult; LMA = late middle aged; SEN = senescent.

In contrast, both citrate synthase and complex IV activities were lower in CR than in AL young adult animals, as we have reported previously (33). This finding accounts for the disparate conclusions about the effects of CR on oxidative capacity with aging reached in two prior studies that did not include a young adult CR group in their study design (11,40). Related to the above point, it appears that skeletal muscles from CR animals have a lower oxidative capacity per mitochondrion, at least under in vitro conditions. Specifically Desai and colleagues (10) showed that the activities of complexes I, III, and IV per unit mass of mitochondrial protein were significantly lower in 10-month-old CR mouse gastrocnemius muscle than in the AL age-matched counterparts (10). Consistent with Desai and colleagues' prior results (10), in the current study we observed that, despite equal citrate synthase protein levels between young adult AL and CR animals, citrate synthase activities were lower in crude homogenates of mixed

gastrocnemius muscle of CR animals (implying that the in vitro citrate synthase activity per unit of citrate synthase protein was lower in CR animals). Despite a lower in vitro oxidative capacity per mitochondrion with CR, our previous study showed that AL and CR young adult skeletal muscles perfused in situ at matched rates of mass-specific oxygen delivery exhibit identical maximal aerobic performance during intense muscle contractions, revealing superior in situ mitochondrial function in CR animals (33). This latter finding, too, is similar to observations in the prior study of Desai and colleagues where superior sensitivity of complex IV to reduced cytochrome *c* was observed in mitochondria from CR animals (10). That there might be compensatory adjustments in mitochondrial oxidative capacity in young adult animals subject to CR should not be surprising, because Bevilacqua and colleagues (42) have shown previously that CR can induce alterations in mitochondrial function in as little as 2 weeks, and our young adult CR animals had been on the restricted diet for 4–6 months by the time of study. Thus, despite a lowering of oxidative capacity in young adult CR animals, which appears to be a down-regulation consequent to superior mitochondrial function (rather than a premature age-associated reduction), the maintenance of skeletal muscle oxidative capacity with aging by CR plays an important role in preserving skeletal muscle function with aging (33).

### *Effect of CR on Oxidative Capacity Is Independent of mtDNA Fragmentation*

Among the potential causes of the decline in oxidative capacity with aging is an accumulation of mtDNA damage. Many studies provide indirect evidence relating these events that shows that a reduction in oxidative capacity with aging correlates with an increase in mtDNA fragmentation in *Drosophila* (21), mouse (22,43), rat (12,14,20,23), non-human primates (24–26,44,45), and humans (46). The most direct evidence that mtDNA damage can contribute to mitochondrial dysfunction with aging comes from a study in which mice were engineered to express a dysfunctional mtDNA polymerase (15). That study used a knock-in of a proofreading-deficient version of PolyA, a nucleus-encoded catalytic subunit of mtDNA polymerase, in mice. The knock-in animals exhibited a 3- to 5-fold increase in point mutations and mtDNA deletions, which in turn did not affect steady-state levels of the mtDNA-encoded complex IV subunit 1 messenger RNA or the mtDNA-encoded complex IV subunit 2 polypeptide. Although this result provides evidence that the content of mtDNA-encoded subunits were unaffected by this deletion load, further analysis of respiratory chain enzyme activities and mitochondrial adenosine triphosphate (ATP) production rates showed a more rapid decline in the mutant mice, showing that accelerating the rate of mtDNA damage with aging can lead to an earlier appearance of a dysfunctional mitochondrial phenotype (15). Despite the convincing nature of this experiment (15), the extent to which mtDNA damage affects oxidative capacity under normal aging conditions remains unclear. Indeed, several studies report that the mtDNA deletion load with aging is not extensive enough to yield a reduction in oxidative capacity (27,28,47).

On the basis that CR reduces the rate of mtDNA fragmentation with aging (48), some researchers have implicated this effect in preserving mitochondrial function with aging (34,49). However, the current results do not agree with this view. Specifically, whereas CR prevented the age-associated decline in oxidative capacity in both plantaris and mixed gastrocnemius muscles, there was no difference in the frequency of mtDNA deletion products between AL and CR animals. Although the lack of difference in mtDNA deletion loads between CR and AL animals contrasts with some previous studies (49,50), the animals studied here are the oldest rats to have been reported in the literature for mtDNA deletions. Thus, although the rate of mtDNA insult by ROS with aging is reduced by CR, a lack of up-regulation of base excision repair mechanisms by CR noted previously (51) may explain why AL and CR animals exhibit similar accumulation of mtDNA deletions in very old age. Regardless of this point, it is clear from our data that the age-associated decline in oxidative capacity in AL animals (and the lack thereof in CR animals) is unrelated to mtDNA deletion load. Note, however, that these results in whole muscles may not apply to single skeletal myocytes in which focal damage may exceed that represented in a whole muscle.

### *CR Attenuates PGC-1α Gene Expression Decline With Aging*

There is strong evidence that CR evokes alterations in organism physiology that have important benefits for mitochondrial function with aging. For example, CR reduces ROS production, likely secondary to lower proton leak (42,52,53). In addition, CR augments ROS scavenging by up-regulating endogenous antioxidant capacity (40). Furthermore, CR is thought to enhance protein turnover (54,55). The overall effect of these alterations would be to decrease the accumulation of ROS-induced protein damage, consistent with previous results showing less oxidative protein damage in skeletal muscle mitochondria of CR aged mice (56). These effects likely contribute to the maintenance of oxidative capacity with aging by CR, because oxidative damage to mitochondrial proteins has been implicated in a reduction of mitochondrial aconitase activity (16).

Among the factors necessary to maintain a high rate of mitochondrial protein turnover, and thus maintained mitochondrial function with aging, is a maintained signaling within the mitochondrial biogenesis pathway. With this in mind, we evaluated the contribution of the drive on mitochondrial biogenesis in the CR-induced protection of muscle oxidative capacity with aging by determining the pattern of PGC-1α gene expression with aging. The direct involvement of PGC-1α in the signaling events involved with the drive on mitochondrial biogenesis was clearly defined by Wu and colleagues in 1999 (57), and has since been corroborated in studies using exercise training to increase skeletal muscle mitochondrial content (58–60) and in studies investigating mitochondrial regeneration following lipopolysaccharide-induced damage in cardiac tissue (61). As noted above, one mechanism by which CR prevents the decline in mitochondrial function with aging may be a decreased extent of oxidative damage accumulation and/or

rate of removal and turnover of damaged protein. This idea is consistent with reports showing higher rates of protein turnover (54,55) and lower levels of oxidative protein damage with aging in CR animals (55,56). On this basis one would expect that CR should demonstrate an enhanced turnover of mitochondrial protein, which should be reflected in a better maintained expression of PGC-1α with aging in these animals (i.e., because there is no difference in citrate synthase protein content between aged AL and CR animals, an elevated PGC-1α gene expression would be consistent with a higher rate of protein synthesis and degradation in CR animals). The data presented here show a marked decline in PGC-1α gene expression in AL mixed gastrocnemius muscle between 8 and 30 months of age, preceding the decline in oxidative capacity, and that PGC-1α gene expression remained at this low level into senescence, by which time oxidative capacity had declined by ~40%. In CR animals, however, in which there was no decline in oxidative capacity with aging, PGC-1α gene expression demonstrated a much more gradual decline with aging. This finding is significant in that the higher PGC-1α expression at late middle age in CR animals may facilitate better removal of oxidatively damaged protein, which, in conjunction with the lower levels of ROS-induced damage (see above), may explain the maintained activity at complex IV. Although PGC-1α expression by senescence in CR animals was similar to that observed for AL animals at an equivalent relative age, the reason that this may not have had the same impact on mitochondrial function in CR animals may relate to the fact that, even in AL animals (which do not have the additional benefit of reduced ROS-induced damage), a decline in PGC-1α temporally preceded the decline in oxidative capacity. Thus we provide evidence that the impressive maintenance of skeletal muscle mitochondrial function associated with CR may be promoted by a maintained drive on mitochondrial biogenesis, which, by facilitating a higher rate of mitochondrial protein turnover, likely contributes to a reduced accumulation of oxidatively damaged mitochondrial protein with aging in CR animals.

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