Effects of aging and caloric restriction on mitochondrial energy production in gastrocnemius muscle and heart


Mitochondria are chronically exposed to reactive oxygen intermediates. As a result, various tissues, including skeletal muscle and heart, are characterized by an age-associated increase in reactive oxidant-induced mitochondrial DNA (mtDNA) damage. It has been postulated that these alterations may result in a decline in the content and rate of production of ATP, which may affect tissue function, contribute to the aging process, and lead to several diseases. We show that with age, ATP content and production decreased by ~50% in isolated rat mitochondria from the gastrocnemius muscle; however, no decline was observed in heart mitochondria. The decline observed in skeletal muscle may be a factor in the process of sarcopenia, which increases in incidence with advancing age. Lifelong caloric restriction, which prolongs maximum life span in animals, which increases in incidence with advancing age. Lifelong caloric restriction, which prolongs maximum life span in animals, attenuates the age-related decrease in ATP content or rate of production in skeletal muscle and had no effect on the heart. 8-Oxo-7,8-dihydro-2’-deoxyguanosine in skeletal muscle mtDNA was unaffected by aging but decreased 30% with caloric restriction, suggesting that the mechanisms that decrease oxidative stress in these tissues with caloric restriction are independent from ATP availability. The generation of reactive oxygen species, as indicated by H2O2 production in isolated mitochondria, did not change significantly with age in skeletal muscle or in the heart. Caloric restriction tended to reduce the levels of H2O2 production in the muscle but not in the heart. These data are the first to show that an age-associated decline in ATP content and rate of ATP production is tissue specific, in that it occurs in skeletal muscle but not heart, and that mitochondrial ATP production was unaltered by caloric restriction in both tissues.

reactive oxygen species; 8-oxo-7,8-dihydro-2’-deoxyguanosine; hydrogen peroxide; oxidative stress

MITOCHONDRIA produce ~90% of the required ATP necessary for cellular function during oxidative phosphorylation. A decline in mitochondrial oxidative function and an increase in the incidence of mitochondrial DNA (mtDNA) oxidative damage have been shown to occur in various tissues with age, and there is strong support that reactive oxygen species (ROS), such as superoxide anion (O2•−) and H2O2, play a prominent role in these processes (7, 12, 15, 17, 26, 43, 49, 52). Moreover, mtDNA is more susceptible to damage by ROS than nuclear DNA due to the lack of protective histones, relative deficiency in repair mechanisms, and the proximity of mtDNA to the source of ROS, the inner mitochondrial membrane (15, 17, 43, 47, 49). The accumulation of mtDNA modification and mutations with age can interfere with the synthesis of proteins and enzymatic pathways responsible for the transfer of electrons along the respiratory chain, as well as with the production of ATP (38). These processes, particularly a decreased energy production (43, 48, 49), have been implicated in the reduction of cell viability as well as an increase in cell necrosis and/or apoptosis (13, 34, 35, 39) with age, resulting in tissue dysfunction and ultimately disease pathologies (27, 49). It has been suggested that mitochondrial dysfunction, resulting from an increase in oxidative stress, may be involved in both sarcopenia (1, 13), i.e., the age-related decrease in muscle mass and function, and the decrease in cardiomyocytes with age (32). In contrast, caloric restriction, which significantly increases the maximum life span in several mammalian species, attenuates the age-associated increase in ROS production and ROS-associated damage to proteins, lipids, and DNA (16, 20, 24, 42, 43, 52). Caloric restriction has also been shown to decrease proton leak in skeletal muscle and maintain mitochondrial and microsomal membrane order and fluidity during aging in several tissues, including skeletal muscle and heart (20, 22, 52).

In the present study, we determined the effects of aging and lifelong caloric restriction on ATP content...
and the rate of ATP production in rat skeletal muscle and heart mitochondria and relate these effects to the production of reactive oxidants (4). We chose skeletal muscle and heart because they are postmitotic tissues and exhibit tissue-specific aging effects (13, 34, 35, 39), including differences in the accumulation of mutant mtDNA (15). Additionally, the heart has a chronically high aerobic requirement throughout life (3, 40), whereas skeletal muscle is active only intermittently throughout life. We compared these findings to those observed after long-term caloric restriction, which is known to attenuate the effects of oxidative stress (16, 20, 24, 42, 52) and reduce the incidence of mtDNA deletions (2, 43). We hypothesized that an age-associated decline in mitochondrial ATP content and ATP production would be observed in both tissues resulting from a perturbation in mitochondrial function and that these effects would be attenuated by long-term caloric restriction.

METHODS

Animals and experimental design. Ad libitum-fed and caloric-restricted male Fischer 344 rats were obtained from the National Institute of Aging colony (Harlan Sprague Dawley, Indianapolis, IN) several weeks before being killed. We used 12-mo-old ad libitum-fed (12AD, n = 14), 26-mo-old ad libitum-fed (26AD, n = 9), and 26-mo-old caloric-restricted animals (26CR, n = 12). Caloric restriction was started at 3.5 mo of age (10% restriction), increased to 25% restriction at 3.75 mo, and maintained at 40% restriction from 4 mo throughout the individual animal’s life (see Table 1 for additional information). Animals were anesthetized with an intraperitoneal injection of pentobarbital sodium (5 mg/100 g body wt). Mitochondria were subsequently isolated from the right and left ventricle of the heart and the gastrocnemius muscle as described previously (13, 17, 34). The gastrocnemius is a mix of type II and type I fibers although it is predominately type II and contains a mix of type II subtypes (i.e., types IIa, IIx, and IIb). The gastrocnemius was chosen because it shows significant atrophy with age, and one complete muscle yields sufficient mitochondria for our experiments. All treatment of animals throughout this study conformed fully with the “Guiding Principles for Research Involving Animals and Human Beings” of the American Physiological Society and in addition received local institutional animal care and use committee approval.

ATP content and production. Mitochondria were isolated from gastrocnemius muscle and heart ventricles as previously described (13, 17, 34) and used immediately to determine mitochondrial ATP content and rate of ATP production. During the isolation procedure, no protease was used; therefore the mitochondria obtained were primarily subsarcolemmal rather than interfibrillar. ATP production was determined using a luminometer (model TD-20/20, Turner Designs, Sunnyvale, CA), employing an assay that utilizes firefly luciferase, which fluoresces in proportion to the presence of ATP and d-luciferin. In brief, to determine ATP content, freshly isolated mitochondria were added to a cuvette containing 1 mM pyruvate, 1 mM malate, and a luciferin-luciferase ATP monitoring reagent (ATP Determination Kit A-22066, Molecular Probes, Eugene, OR). This was immediately followed by the addition of 2.5 mM ADP to determine ATP production. A blank cuvette containing no sample was assayed to account for nonspecific ATP production, and known concentrations of ATP were used to establish a standard curve. All mitochondrial samples were performed in triplicate, and an average of these results was used in quantifying ATP content and rate of production. All mean values were normalized to the 12AD rats.

Oxidant production. H2O2 production was measured at 37°C using a method developed by Barja and colleagues (4, 17). Fluorescence was determined using a fluorescent microplate reader (GeminiXS, Molecular Devices, Sunnyvale, CA), and a standard curve was generated for each analysis using glucose-glucose oxidase.

Measurement of oxidized bases. 8-Oxo-7,8-dihydro-2′-deoxyguanosine (8-oxoG) and deoxyguanosine (dG) concentrations were measured by HPLC with online electrochemical and ultraviolet detection, respectively, using the method described in Lopez-Torres et al. (26). For quantification, peak areas of dG standards and of three-level calibration of pure 8-oxoG standards (Sigma, St. Louis, MO) were analyzed during each HPLC run. Comparison of areas of 8-oxoG standards injected with and without simultaneous injection of dG standards ensured that no oxidation of dG occurred during HPLC analyses.

Statistical analysis. Unpaired Student’s t-tests were used for comparisons between groups using a statistical package from Prism (GraphPad, San Diego, CA). All data are expressed as means ± SE. A P value of <0.05 was considered statistically significant.

RESULTS AND DISCUSSION

ATP content and rate of ATP production declines in skeletal muscle, but not in heart, with age. The rate of ATP production depends on the synthesis of ATP by intact mitochondria as protons reenter the mitochondrial matrix through the rotary motor channels in the ATP synthase enzyme complex (F1F0-ATPase) (46). In the gastrocnemius muscle, ATP content (Fig. 1A) and rate of ATP production (Fig. 1C) declined by ~50% with age. Our findings correspond to those of an in vivo study, employing NMR, which estimated that the mean ATP production in the quadriceps muscle of old human subjects (mean age 69 yr) was ~50% that of younger subjects (mean age 39 yr) (9). The ATP results for the heart were at variance with our initial expectations, as we found no significant decline with age in ATP content (Fig. 1B) or rate of ATP production (Fig.

Table 1. Body mass and gastrocnemius and heart muscle weight in 12AD, 26AD, and 26CR male Fischer 344 rats

<table>
<thead>
<tr>
<th></th>
<th>12AD</th>
<th>26AD</th>
<th>26CR</th>
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<tbody>
<tr>
<td>Body mass</td>
<td>411 ± 11.9</td>
<td>384 ± 16.2</td>
<td>266 ± 6.3</td>
</tr>
<tr>
<td>Gastrocnemius</td>
<td>1.36 ± 0.03</td>
<td>1.03 ± 0.05</td>
<td>0.93 ± 0.05</td>
</tr>
<tr>
<td>Heart</td>
<td>1.08 ± 0.03</td>
<td>1.12 ± 0.04</td>
<td>0.88 ± 0.02</td>
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Values are means ± SE. Groups: 12-mo-old ad libitum-fed (12AD), 26-mo-old ad libitum-fed (26AD), and 26-mo-old caloric-restricted rats (26CR). Body mass did not change significantly with age (12AD vs. 26AD), although caloric restriction (26CR) resulted in a significant reduction in body mass compared with 26AD (tP < 0.0001). Furthermore, the mass of the gastrocnemius muscle declined significantly with age (tP < 0.0001; 12AD vs. 26AD) but not with caloric restriction. The heart mass was unaffected with age (12AD vs. 26AD), while caloric restriction decreased heart muscle mass significantly (tP < 0.001; 26AD vs. 26CR).
Table 2. Values are means ± SE. *P ≤ 0.05, ***P ≤ 0.001.

1D). Aged cardiac muscle exhibits smaller increases in mtDNA deletions compared with skeletal muscle (15, 40); therefore, it is feasible that ROS-induced damage is relatively lower in heart compared with skeletal muscle and may explain why there was no significant decline in ATP content or production with age.

Our data are in agreement with current literature on mtDNA deletions, as it appears that mtDNA deletions may interfere with respiratory chain ATP production (8, 36) and are tissue specific (3, 15, 40). Barazzoni et al. (3) found that a reduction in mtDNA copy number, transcription of mitochondrially encoded cytochrome c oxidase subunits, and oxidative capacity were significantly higher in aging skeletal muscle compared with aging heart. They reported that both the mtDNA copy number and mtDNA transcript levels of cytochrome c oxidase I and III were significantly reduced during aging by 23–25% and 17–22%, respectively, in the gastrocnemius muscle of male Fischer 344 rats but that no differences in these two parameters were observed in the heart (3) in parallel with the reduction in oxidative capacity for the gastrocnemius muscle compared with age relative to the heart. This disparity was attributed to the differences in adaptive mechanisms that may have evolved in response to the chronically high aerobic requirement of the heart to prevent and/or repair oxidative damage (3, 40). Additionally, Liu et al. (25) examined the relationship between the accumulation of mtDNA4977, the most common mtDNA deletion accounting for 30–50% of all deletions (49), in skeletal muscle and heart from humans ranging in age from 1 h to 90 yr old. This study (25) found a strong positive correlation between age and mtDNA4977 accumulation. Moreover, the level of mtDNA4977 deletions was significantly higher in skeletal muscle (0.015–0.3% of mtDNA) compared with heart (0.0003–0.004%) (25).

The mtDNA4977 deletion contains a portion of the mitochondrial genome that encodes for 7 of the 13 proteins that become subunits in the respiratory chain complexes, including the two subunits for the ATP synthase enzyme (8) responsible for ATP production (49). Therefore, the relatively low accumulation of mtDNA deletions in the heart, compared with the skeletal muscle, may help explain the absence of an age-associated decline in heart ATP content or production compared with the gastrocnemius muscle. In addition, regional differences in the subcellular location of mitochondria, both in skeletal muscle and heart tissue, exist (14, 18, 29, 33, 34). The subsarcolemmal mitochondria population is located below the plasma membrane and is metabolically distinct from the intermyofibrillar mitochondria, which are located between the myofibers. In our study, only the subsarcolemmal fraction was isolated, and it is not well established as to what extent the rate of ATP production in each subcellular mitochondrial population is affected by increasing age. However, recent evidence suggests that there is a selective decrease in the rate of oxidative phosphorylation in the intermyofibrillar population of cardiac mitochondria but not in the subsarcolemmal mitochondria (19, 28). These studies also used Fischer 344 rats as a model for studying the effects of aging on cardiac mitochondria but not in the subsarcolemmal mitochondria (19, 28).

Lifelong caloric restriction has no effect on ATP content or ATP production. Caloric restriction was unable to attenuate the decline in oxidative metabolism observed in skeletal muscle and had no effect whatsoever in the heart. Caloric restriction has been shown to...
decrease the production of reactive oxygen intermediates and oxidative stress with increasing age in heart and skeletal muscle (17, 20, 42, 43). We initially hypothesized that lifelong caloric restriction would attenuate any age-associated decline in ATP concentration and ATP production, assuming that ROS-induced damage to mitochondria leads to mitochondrial energy dysfunction. However, no differences were observed in ATP content or rate of production in either gastrocnemius muscle or heart in 26CR animals compared with the 26AD group due to caloric restriction (Fig. 1). Very recently, Sreekumar et al. (45) also showed that mitochondrial ATP production and citrate synthase activity in the gastrocnemius muscle were unaltered by long-term caloric restriction, supporting our findings in skeletal muscle. It is perhaps surprising that caloric restriction did not counteract the decrease in either ATP content or ATP synthesis in skeletal muscle. Caloric restriction initiated during late middle age has previously been shown to retard the age-associated fiber loss and fiber type changes, decrease the number of skeletal muscle fibers showing mitochondrial enzyme abnormalities, and lead to a decline in the accumulation of mtDNA deletions (2). Apparently, the requirement and demand for ATP were in balance with caloric restriction. In addition, the amount of free ATP available appears minimal, and therefore the rate of ATP production probably better reflects the in vivo situation. Additional studies are required to investigate variations in ATP production between the different mitochondria populations within different tissues and fiber types.

No effect of age on body mass was observed, but caloric restriction reduced body weight by 31% (Table 1). Moreover, we observed a significant decline (24%) in gastrocnemius muscle mass with age, similar to other studies (1, 13). The caloric-restricted rats had a similar decline in muscle mass compared with 12AD rats, and therefore, the leaner CR animals had a greater muscle mass relative to body weight compared with the ad libitum-fed animals. With age there was no change in heart weight, but caloric restriction reduced heart weight significantly, as found by others (50). Muscle-composition changes with age, such as increases in inner connective tissues, could have profound effects on function, but it is unknown if changes in organ weight could have an effect on the parameters determined in this study, such as ATP production.

Of interest are the differences between the ATP content and rate of production between the gastrocnemius and heart muscle (Table 2). Skeletal muscle is active only intermittently throughout an organism’s lifetime. Hence because of the immediate force placed on the gastrocnemius when the muscle is activated, it is possible that it may require a higher reserve of free ATP as opposed to the heart, which is constantly active. In contrast, the heart is capable of producing ~2.5–7 times more ATP than the gastrocnemius due to the persistent high-energy demand of the active heart (Table 2).

The oxidant production and oxidative damage. Age had no effect on H$_2$O$_2$ production in mitochondria isolated from skeletal muscle (Fig. 2A) or heart (Fig. 2B), whereas caloric restriction tended to reduce oxidant production in mitochondria isolated from gastrocnemius muscle (A) and heart mitochondria (B; Refs. 16, 17). Age had no significant effect on H$_2$O$_2$ production in the gastrocnemius muscle. Caloric restriction tended to reduce the levels of H$_2$O$_2$ (26AD vs. 26CR, $P = 0.0661$). B: additionally, H$_2$O$_2$ production in the heart was unaffected by age and caloric restriction. Values are means ± SE.

Table 2. ATP content and rate of production in gastrocnemius and cardiac muscle

<table>
<thead>
<tr>
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<th>Skeletal Muscle</th>
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<th>Cardiac Muscle</th>
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<tr>
<td></td>
<td>12AD</td>
<td>26AD</td>
<td>26CR</td>
<td>12AD</td>
</tr>
<tr>
<td>ATP content</td>
<td>1.21 ± 0.18</td>
<td>0.78 ± 0.18</td>
<td>1.10 ± 0.21</td>
<td>0.23 ± 0.04</td>
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<tr>
<td>ATP production</td>
<td>7.29 ± 1.01</td>
<td>3.17 ± 0.89</td>
<td>2.77 ± 0.53</td>
<td>18.31 ± 1.46</td>
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Values are means ± SE. ATP content is expressed as nmol ATP/mg protein, and ATP production is expressed as nmol ATP·mg protein$^{-1}$·min$^{-1}$. These data represent the average of 2 separate batches of Fischer 344 rats analyzed at different times. The mean from each batch was normalized to the 12 AD group, and results are shown in Fig. 1.
production. There is still a significant amount of controversy as to whether oxidant production increases with age. Recently, Barja and colleagues (5, 17) have carefully reviewed this topic and concluded that there is little evidence of an increase in H2O2 production in isolated mitochondria with age. Bejma et al. (6) found increases in mean cellular oxidative stress with age in skeletal muscle using the 2''',7''''-dichlorodihydrofluorescein method (4, 21), which indicates intracellular oxidant levels. Conversely, our method measured the steady-state extracellular H2O2 production, which appears less prone to scavenging by matrix antioxidants. Superoxide dismutase has been found in the inner membrane space (31); however, it is unknown if glutathione peroxidase is located in the inner membrane space of the mitochondria (I. Fridovich, personal communication). Consequently, it is unclear whether age-related changes in antioxidant defenses in these two tissues with age may explain our findings. We recently showed that isolated rat heart mitochondria upregulate superoxide dismutase (+50%) and glutathione peroxidase (+25%) significantly with age (34), suggesting a possible mechanism that leads to the increased removal of H2O2 with age. Studies examining changes in antioxidant enzyme activities in skeletal muscle also tend to support an increase in enzymatic activities with age (23, 30). This indicates that upregulation of antioxidant enzymes may be due to chronic lifelong exposure to oxidants. In any case, a lack of increase in H2O2 production with age in vivo would be consistent with the fact that aging is a progressive phenomenon, and thus aging rate is roughly similar at different adult ages (4, 5).

Previously, Gredilla et al. (17) found that 8-oxodG levels in heart mtDNA did not increase with age, suggesting that mechanisms to remove oxidized bases are very effective in the heart, consistent with the observation that 8-oxodG endonuclease activity in rat heart mitochondria increased with age (44). Due to the reduction in ATP production with age in skeletal muscle, we also measured 8-oxodG levels in mtDNA isolated from the gastrocnemius muscle and found no change with aging (Fig. 3), consistent with the results observed for H2O2 production. Levels of 8-oxodG in skeletal muscle mtDNA (Fig. 3) decreased significantly by 30% (26AD vs. 26CR, P = 0.006), indicating that lifelong caloric restriction may lead to mechanisms that inhibit the oxidation of DNA bases that lead to mtDNA damage. While the decline in H2O2 production was not statistically significant, it did show a strong trend toward a reduction (−28%; P = 0.0661) in oxidant production with caloric restriction, consistent with the decline observed in 8-oxodG. These results support the idea that caloric restriction may reduce the aging rate at least in part by decreasing the rate of mitochondrial ROS production and thus inhibiting reactive oxidant-induced mtDNA damage.

**Perspectives**

This study indicates that age-related alterations in energy metabolism are tissue specific and that caloric restriction is unable to attenuate these effects in subsarcolemmal mitochondria of the heart and the gastrocnemius muscle, a predominately fast fiber type. An overview of the changes in ATP content, rate of ATP production, and H2O2 production in isolated mitochondria of gastrocnemius and heart muscle with aging and caloric restriction is shown in Table 3. We suggest that the decline in ATP content and ATP production in skeletal muscle may result from an accumulation of mtDNA damage with age, which interferes with respiratory chain ATP production. Consequently, this accrual of damage may lead to a fiber-specific decline in skeletal muscle activity with age and may be a factor involved in the increased incidence of sarcopenia in skeletal muscle (1, 13, 50). Mitochondrial functional measurements on different fiber types and muscle contractile property analysis need to be performed simultaneously to get a more complete picture of the effects

**Table 3. Overview of changes in ATP content, rate of ATP production, and H2O2 production in isolated mitochondria of gastrocnemius and heart muscle with aging and caloric restriction**

<table>
<thead>
<tr>
<th></th>
<th>Aging</th>
<th>Caloric Restriction</th>
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<tr>
<td>Gastrocnemius muscle</td>
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<tr>
<td>ATP content</td>
<td>↓</td>
<td>→</td>
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<tr>
<td>ATP production</td>
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</tr>
<tr>
<td>H2O2 production</td>
<td>→</td>
<td>→</td>
</tr>
<tr>
<td>Gastrocnemius mass</td>
<td>↓</td>
<td>→</td>
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<tr>
<td>Heart</td>
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<td>ATP content</td>
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<td>ATP production</td>
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<tr>
<td>H2O2 production</td>
<td>→</td>
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<td>Heart mass</td>
<td>→</td>
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↑, Increase; ↓, decrease; →, no change. 12AD, 26AD, and 26CR male Fischer 344 rats were obtained from the National Institute of Aging colony (Harlan Sprague Dawley, Indianapolis, IN).
of sarcopenia. In contrast, the aged heart will require the continued availability of energy throughout its life, and this may explain why ATP content and rate of production did not decline with age in heart mitochondria. It has been shown that in the majority of healthy older adults, cardiac output is maintained throughout life (37). The amount of ATP produced within the mitochondria is directly proportional to the ADP available, so any change in ADP concentration should directly affect the ATP concentration (40, 46, 49). Hence, it is supportive that ATP content and rate of production varied in unison, since production and consumption are regulated by ATP-to-ADP ratio. Compared to skeletal muscle, heart mitochondria exhibit an increased amount of mtDNA mutations, which are thought to interfere with respiratory chain complexes and that changes are tissue specific (1) adaptations in DNA repair and/or turnover, 2) reduction in stress hormones, 3) reduction in glycoxidative stress, and 4) alterations in gene expression that increase resistance to stress.

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