Journal of Gerontology: BIOLOGICAL SCIENCES
2001, Vol. 56A, No. 3, B116–B122

Effects of Caloric Restriction on Skeletal Muscle Mitochondrial Proton Leak in Aging Rats

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Long-term caloric restriction (CR) retards aging processes and increases maximum life span. We investigated the influence of CR on mitochondrial proton leaks in rat skeletal muscle. Because CR lowers oxidative damage to mitochondrial membrane lipids and proteins, we hypothesized that leak would be lower in mitochondria from old CR rats than in age-matched controls. Three groups ($n = 12$) were studied: 4-month-old "young" control rats (body weight: 404 g ± 7 SEM), 33-month-old CR rats (body weight: 262 g ± 3), and 33-month-old control rats (body weight: 446 g ± 5). CR rats received 67% of the energy intake of old control rats, with adequate intakes of all essential nutrients. Maximum leak-dependent O_2 consumption (State 4) was 23% lower in CR rats than in age-matched controls, whereas protonmotive force values were similar, supporting our hypothesis. The overall kinetics of leak were similar between the two groups of old rats; in the young, kinetics indicated higher protonmotive force values. The latter indication is consistent with aging-induced alterations in proton leak kinetics that are independent of dietary intervention. There was no influence of age or diet on serum T_4 level, whereas T_3 was lower in young than in old control rats. These results support and extend the oxidative stress hypothesis of aging.

An increase in oxidative stress has long been thought to be one of the causes of aging (1). The basic premise of the oxidative stress hypothesis of aging is that age-related loss of function is due to progressive and irreversible accumulation of molecular oxidative damage (2). Further, it is thought that longer life spans can be achieved by decreasing the level of oxidative damage incurred over a lifetime (2).

Recent studies in our laboratory have shown that there is an increased mitochondrial proton leak in hepatocytes of old versus young mice, as well as altered kinetics of adenosine triphosphate (ATP) turnover reactions and a modified distribution of metabolic control in oxidative phosphorylation reactions (3). Oxidative damage to mitochondrial membranes has been implicated in an augmented generation of reactive oxygen species (ROS), which, in turn, can lead to further membrane damage. The implications of such a vicious cycle of oxidative stress and damage have been described by in vitro studies such as those of Sohal and Sohal (4), who showed that mitochondrial H_2O_2 generation increases after exposure to 2,2-azobis(2-amino-propane)dihydrochloride.

Brookes and colleagues (5) have shown that sequential additions of 200 μM of peroxynitrite to rat brain mitochondria significantly stimulated proton leak. Importantly, the stimulation of proton leak was inhibited by Trolox, a vitamin E analog, thus supporting the involvement of lipid peroxidation in the mechanism of peroxynitrite cytotoxicity. Hagen and colleagues (6) reported that oxidant generation from the mitochondria of isolated rat hepatocytes was greater by old cells than by cells from young rats. Also, it has been shown that caloric restriction (CR) prevents the age-associated accumulation of oxidative damage to mouse skeletal muscle mitochondria (7).

These recent findings have led to our current investigation of the effects of CR on age-related changes in proton leak in rat skeletal muscle. Thyroid hormone status in these rats was also assessed, as thyroid hormones increase proton leak and maximum leak-dependent mitochondrial O_2 consumption (8–10). Restricting the caloric intake of mammals is a well-recognized means of extending maximum life span. Evidence indicates that CR may act, at least in part, by decreasing oxidative stress and increasing antioxidant defenses and repair mechanisms (2,11). With the use of this evidence and that of other studies (3,5–7), we hypothesize that the beneficial effects on aging of CR may be directly due to lowered rates of mitochondrial proton leak.

Oxidative phosphorylation is the overall process through which the oxidation of cellular energy substrates is coupled to ATP synthesis in mitochondria. The activity of the mitochondrial respiratory electron transport chain generates the protonmotive force (Δp) across the mitochondrial inner membrane. It is this electrochemical gradient that is used to drive protons back into the mitochondrial matrix. Protons are returned into the matrix via two separate pathways: ATP synthase and mitochondrial proton leak (Figure 1). ATP synthase catalyses the phosphorylation of adenosine diphos-

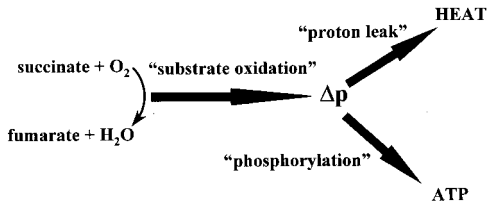

Figure 1. The oxidative phosphorylation system in mitochondria. The intermediate in the system, protonmotive force (Δp), is produced by the substrate oxidation branch of reactions; Δp is consumed by the proton leak and phosphorylating branches of reactions. The proton leak branch consists of the leak of protons and any cation cycles across the mitochondrial inner membrane. The phosphorylation branch consists of the synthesis of ATP (adenosine triphosphate) by means of ATP synthase and ATP consumption.

phate (ADP) (transfer of energy) as protons flow down their gradient. Conversely, the proton leak pathways lead to decreased Δp without the synthesis of ATP, but with the production of heat. Energy is dissipated as the activity of the mitochondrial respiratory chain increases in response to decreased Δp. Proton leak pathways are thought to be modulated by thyroid hormones (8–10) and phylogeny (12), and they are inversely related to body mass in mammals (13).

CR has consistently been shown to increase maximum life span by 20–40%, with the magnitude of life-span extension showing an inverse relationship with energy intake (14). For an increased life span, however, it is crucial that animals on CR diets receive adequate amounts of protein, vitamins, and minerals. In this study, we aimed to examine mitochondrial proton leak in oxidative skeletal muscle tissue, because the greatest attenuation of oxidative damage caused by CR appears to occur in postmitotic tissues such as skeletal muscle, brain, and heart (2,15).

Recent reviews describe age-related changes that occur in mitochondria. Changes include oxidative damage to mtDNA, oxidation of mitochondrial proteins, decreased membrane fluidity, and oxidation of membrane lipids (2,16,17). However, in our previous study of age-related changes in the oxidative phosphorylation system in hepatocytes of mice, we found no significant effects of age on the overall kinetics of substrate oxidation reactions (3). Substrate oxidation reactions comprised reactions involved in, and following, the oxidation of glucose, lactate, pyruvate, and endogenous substrates; it thus includes components of the electron transport chain—many of which are encoded in mtDNA.

Our previous findings did nonetheless show that the overall kinetics of mitochondrial proton leak were altered such that leak-dependent oxygen consumption was higher in hepatocytes from old versus young mice (3). Membrane lipid damage is likely an important factor contributing to the increased proton leak with aging, but we cannot exclude other potential mechanisms. Over the life span of an animal, there is a natural decline in the amount of linoleic acid (18:2) and concomitant increases in the amounts of long-chain polyunsaturated fatty acids (22:4 and 22:5), resulting in an increased probability of membrane lipid peroxidation (18,19). Thus, although mechanistic aspects of mitochondrial proton leak are as yet unclear, there are several intriguing relationships among mitochondrial fatty acid composition, mitochondrial proton leak, and life span.

The findings of this investigation show that maximum leak-dependent O_2 consumption in skeletal muscle mitochondria from old rats is significantly lower in those subjected to CR than in control-fed rats. These results support and extend the oxidative stress hypothesis of aging.

Methods

Treatment of Animals

Male Wistar rats were studied at either 4 or 33 months of age. The 4-month-old young controls were obtained from Charles River (St. Constant, PQ) and studied 2–3 weeks after arrival in Ottawa. These rats were allowed free access to Purina 5000 Chow (PMI Feeds, St. Louis, MO). The 33-month-old rats (old control and CR rats) were obtained from the specific pathogen-free Shared Aging Rodent Facility at the Veterans Administration Geriatric Research, Education and Clinical Center (Madison, WI). These rats were initially purchased at 5 weeks of age from the Lobund Laboratories at Notre Dame University. Upon arrival in Madison they were given free access to Purina 5000 Chow.

At approximately 10 months of age, the latter group of rats were randomly divided into two groups receiving either a control or energy-restricted diet (Teklad 91349 control diet and Teklad 91351 CR diet; Harlan Teklad, Madison, WI). The diet compositions are summarized in Table 1. The amount of protein, minerals, and vitamins was elevated in the diet of CR animals in order to ensure that the rats were not deficient in these nutrients. The energy intakes were 43.6 kcal/day for the controls and 29.2 kcal/day for the CR rats. For waste to be minimized during feeding, the powdered diets were suspended in a 0.5% agar solution to produce a semisolid diet. The rats were caged individually at 23°C with light from 7 AM to 7 PM. All rats were allowed free access to water. All animals were cared for in accordance with the guidelines of the Canadian Council on Animal Care, the Institute of Laboratory Animal Resources (National Research Council, USA), and with "Guiding Principles for Research Involving Animals and Human Beings."

Table 1. Composition of the Diets for the Old Control and Calorie Restricted Groups

Ingredients	Control Diet (g/100 g Diet)	Restricted Diet (g/100 g Diet)
Casein	22.0	27.7
DL-methionine	0.3	0.4
Sucrose	27.0	23.5
Corn starch	27.0	23.5
Corn oil	13.5	13.5
Cellulose	5.0	4.9
Mineral mix, AIN-76	3.5	4.4
Calcium carbonate	0.3	0.4
Vitamin mix	1.0	1.3
Brewer's yeast	0.4	0.5

Note: The mineral mix is Teklad 170915 and the vitamin mix is Teklad 40060 (Harlan Teklad, Madison, WI).

Isolation of Hind-Limb Skeletal Muscle Mitochondria

Rats were anesthesized with intraperitoneal injections of 0.325 mg of sodium pentobarbital per gram of body weight prior to decapitation. Skeletal muscle mitochondria were isolated as described by Bhattacharya and colleagues (20). The isolated mitochondria were resuspended in a medium containing the following: 120 mM of KCl, 20 mM of sucrose, 20 mM of glucose, 10 mM of KH_2PO_4, 5 mM of N-2-Hydroxyethylpiperazine-N′-ethanesulphonic acid (HEPES), 2 mM of $MgCl_2$, 1 mM of (Ethylenedioxy) diethylenedinitrilotetracetic acid (EGTA), and 0.5% defatted bovine serum albumin (BSA), with a pH of 7.2 with potassium hydroxide (KOH). BSA was defatted by using the method of Chen (21). Mitochondrial protein concentration was determined by the Biuret reaction.

Measurement of Mitochondrial Oxygen Consumption

Mitochondrial oxygen consumption was measured by using a Clark type of oxygen electrode (Hansatech, Norfolk, UK) maintained at 37°C. Mitochondria (0.5 mg of protein/ml) were incubated in 1 mL of medium containing 120 mM of KCl, 20 mM of sucrose, 20 mM of glucose, 10 mM of KH_2PO_4, 5mM of HEPES, 2 mM of $MgCl_2$, 1 mM of EGTA, and 0.5% defatted BSA, with a pH of 7.2 with KOH, plus 5 μM of rotenone and 0.4 μg of nigericin. Rotenone was added to inhibit oxidation of endogenous reduced nicotinamide adenine dinucleotide (NADH)-linked substrates. Nigericin is added to convert the ΔpH component of the mitochondrial protonmotive force to potential; thus, our assessments of mitochondrial membrane potential, described below in the following paragraphs, reflect total protonmotive force. Respiration was initiated with 10 mM of succinate and 100 μM of ATP. State 4 oxygen consumption, defined as maximum nonphosphorylating respiration, was achieved by using saturating amounts of oligomycin (12 μg/mg of mitochondrial protein) to completely inhibit ATP synthase, and hence the phosphorylation branch of oxidative phosphorylation (see Figure 1). The kinetics of the leak (i.e., response of leak-dependent oxygen consumption to imposed changes in protonmotive force) were subsequently determined by progressively inhibiting the electron transport chain with malonate (an inhibitor of Complex II) to give final concentrations between 0.2 and 3.17 mM. All measurements were performed in duplicate.

Measurement of Mitochondrial Protonmotive Force (Δp)

Δp was determined by using a triphenylmethylphosphonium ($TPMP^+$)-sensitive electrode (22), which was inserted into the incubation chamber of the oxygen electrode as described by Brown and Brand (23). Mitochondrial matrix volume and $TPMP^+$ potential-independent binding correction factor (a_m) were also determined as described by Brown and Brand (23). Δp can be calculated by using a modified Nernst equation as follows: $\Delta p = 61.5 \times \log(\{[TPMP^+]_{mat} \times a_m\}/[TPMP^+]_{ext})$, where $[TPMP^+]_{mat}$ refers to the concentration of $TPMP^+$ in the mitochondrial matrix, $[TPMP^+]_{ext}$ refers to the concentration of $TPMP^+$ in the medium surrounding the mitochondria, and a_m refers to the binding correction factor used to correct for potential-independent binding of $TPMP^+$ to mitochondria.

All measurements of Δp were performed in duplicate and simultaneously to the above-described measurements of oxygen consumption.

Determination of Thyroid Hormone Status

Serum T_3 and T_4 assays were performed by using ^{125}I-Radio Immuno-Assay kits from INCSTAR corporation (Stillwater, MN).

Materials.—Oligomycin, valinomycin, succinate, malonate, ADP, and ATP were obtained from Sigma Chemicals (St. Louis, MI). TPMP·Br was obtained from Aldrich (Milwaukee, WI). All radioactive compounds were obtained from Mandel-Dupont NEN (Guelph, ON).

Statistical analysis.—Data were analyzed by using an analysis of variance (ANOVA) followed by Tukey's post hoc tests. All data are reported as mean ± standard error of the mean. A value of $p < .05$ was considered statistically significant.

RESULTS

Body and Tissue Weights

Mean body weight was, as expected, significantly lower in old CR than old control rats. Body weight was 41% lower and approximates the 33% CR. Weights of epididymal white adipose tissue (EWAT) depots were determined as an index of adiposity. Both groups of older rats had higher mean total weights of EWAT than did the young controls (Table 2). However, if EWAT weights are normalized to the mean body weights, there are no significant differences between the two groups of old rats.

Because of the importance of brown adipose tissue thermogenesis in rodents with respect to energy balance, the weights of interscapular brown adipose tissue (IBAT) depots were also measured. The mean IBAT weight was significantly lower in old CR rats than in both other groups (Table 2). For example, the mean IBAT weight of the old CR rats was only 21% that of age-matched controls. The mean IBAT weight was significantly greater in old control

Table 2. Body and Fat Pad Weights for Old Calorie Restricted, Old Control, and Young Control Groups

Group	Body Weight (g)	Total EWAT (g)	Total IBAT (g)
Old			
Calorie restricted ($n = 12$)	262.4 ± 3.1	0.55 ± 0.04	1.13 ± 0.17
Control ($n = 12$)	446.0 ± 4.7	0.94 ± 0.06	6.12 ± 0.16
Young control ($n = 12$)	404.3 ± 6.9	0.35 ± 0.03	4.94 ± 0.45

Notes: EWAT = epididymal white adipose tissue; IBAT = interscapular brown adipose tissue. Results are presented as mean ± *SE*. Within each column, results from each group of rats were significantly different from every other group, as determined by the Tukey multiple comparison test. Overall analysis of variance, $p < .0001$; for comparisons between groups, all comparisons yielded values of $p \leq .01$.

than in young control rats, and it probably reflects a greater proportion of triglyceride in the depots of old rats.

Mitochondrial Proton Leak

Maximum leak-dependent oxygen consumption (State 4) was achieved with the addition of a saturating concentration of oligomycin (12 μg/mg of mitochondrial protein). The furthest point on the right of each curve identifies maximum State 4 oxygen consumption. It was found that the maximum State 4 oxygen consumption rate was 23% lower ($p < .05$) in mitochondria from old CR rats (Figure 2A) than that from old control rats (Figure 2B). The actual mean values were 72.5 ± 6.4 ($n = 8$), 59.0 ± 3.3 ($n = 10$), and 66.8 ± 5.3 ($n = 9$) nmol of O_2 per milligram of mitochondrial protein per minute for old control, CR, and young control groups, respectively. These values are presented in Figure 3.

Figure 2 (parts A, B, and C) further depicts the overall kinetics of proton leak reactions. Overall leak kinetics were not markedly different between the two groups of older rats. However, certain observations in comparison of results from the old and the young are of interest. Overall leak kinetics over a range of oxygen consumption values show that Δp is higher in young than in old rats. This is particularly evident in the comparison between results of young controls and those from old CR rats. These results indicate that the oxygen used to balance the leak over a range of Δp is lower (i.e., the leak is lower) in mitochondria from young than from old control rats, regardless of dietary intervention. Again, this supports the hypothesis of age-related increases in mitochondrial proton leaks.

It should also be noted that there was a large variation in data from the mitochondria of old control rats (Figure 2B). This was not observed in the data of the mitochondria from old CR or young controls (Figures 2A, and 2C) and is surprising, as all mitochondria were isolated by using the same protocol.

Thyroid Hormone Levels

Thyroid hormone levels (Table 3) were assessed in serum collected at the time of sacrifice. Average T_3 levels were significantly higher ($p < .05$) in old control than in young control rats. However, average values for CR rats were not significantly different from either group. Average T_4 levels were not significantly different between groups.

Discussion

We hypothesized that CR lowers the accrual with aging of oxidative damage to the mitochondrial inner membrane, which, in turn has been proposed as a cause of age-associated increases in mitochondrial proton leak (3). Our previous study (3) showed that mitochondrial proton leak was significantly higher in hepatocytes of old mice compared with cells from young mice. The differences were relatively small; thus a study of leak in a postmitotic tissue seemed appropriate. Skeletal muscle was selected as the site of study because oxidative stress and mitochondrial dysfunction may contribute to losses in muscle mass and quality (24).

The age-associated increase in the amount of oxidative stress can be ascribed to three main factors: an increase in the rate of generation of reactive oxygen metabolites such as H_2O_2, $O_2^{\cdot -}$, and $^{\cdot}OH$; a decline in protective antioxidative capabilities; and a decline in the efficiency of repair and removal of damaged molecules. A hypothesis that requires further study is that increases in mitochondrial proton leak,

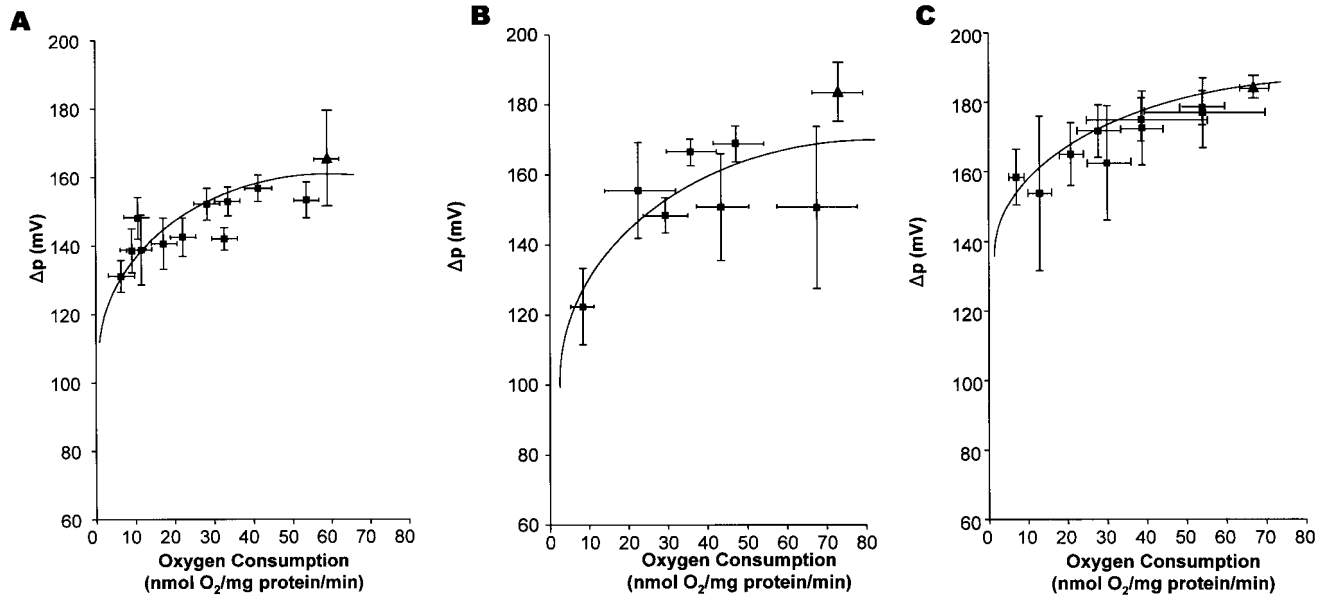

Figure 2. Relationship between protonmotive force (Δp) and oxygen consumption of hind-limb skeletal muscle mitochondrial proton leaks in **A**, old calorie restricted, **B**, old control, and **C**, young control rats. Maximum leak-dependent oxygen consumption (State 4) is the furthest point on the right of the graph (▲). All measurements were made in the presence of a saturating amount of oligomycin (12 μg/mg of mitochondrial protein). Overall kinetics of the proton leak (■) were determined by titrating Δp producers (i.e., substrate oxidation reactions) with increasing amounts of malonate (**A**: 0.2, 0.33, 0.53, 0.67, 0.86, 1.0, 1.19, 2.0, 2.18, 3.0, and 3.17 mM; **B**: 0.2, 0.53, 0.67, 0.86, 1.0, 1.19, and 3.17 mM; **C**: 0.2, 0.33, 0.53, 0.67, 0.86, 1.0, 1.19, 2.0, 2.18, and 3.0 mM). All points represent mean ± SEM of at least five (**A**), eight (**B**), and six (**C**) duplicate assays.

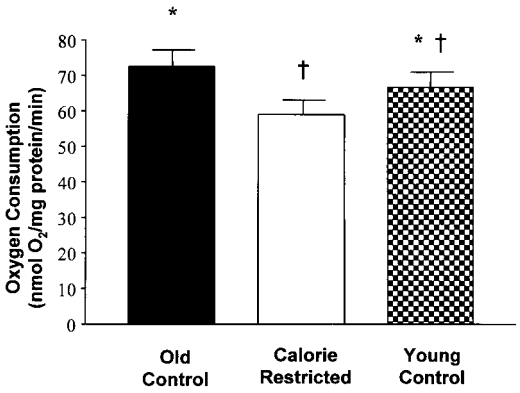

Figure 3. State 4 oxygen consumption of hind-limb skeletal muscle mitochondria from old control, calorie restricted, and young control rats. State 4 (maximum leak dependent) oxygen consumption was measured as that in the presence of a saturating amount of oligomycin (12 μg/mg of mitochondrial protein). Different symbols indicate significant differences ($p < .05$) in means by an analysis of variance with Tukey post hoc tests. Means were calculated by using $n = 8$ for old controls, $n = 10$ for calorie restricted rats, and $n = 9$ for young controls; each rat muscle preparation was assessed in duplicate.

may be both a consequence and a cause of oxidative stress, as an increased leak results in increased mitochondrial oxygen consumption and thus possibly subsequent oxidant production (2,3,5). This could then result in further damage to lipids and proteins in the mitochondrial and extramitochondrial environment.

Mitochondria constitute the greatest source of oxidants in the cell, as they account for 85% of cellular oxygen consumption and oxidants are continually being produced in the mitochondria as a by-product of substrate oxidation. Rats may consume up to 10^{12} molecules of O_2 per day and, because it is estimated that 2% of these oxygen molecules are not completely reduced to water, this results in the daily generation of 2×10^{10} molecules of H_2O_2 and $O_2^{\cdot -}$ (17). Mass-specific basal metabolic rate is inversely correlated with life span in mammals (2); thus, smaller mammals have higher mass-specific metabolic rates and shorter life spans than larger mammals. There is also, as mentioned earlier, the intriguing inverse correlation between mitochondrial proton leak and body mass in mammals (13).

In this investigation of mitochondrial proton leak in the skeletal muscle of CR, old control, and young rats, we observed that the maximum leak-dependent oxygen consumption was decreased by 23% in skeletal muscle mitochondria from old CR rats versus old control rats. The decrease in oxygen consumption at State 4 would lead to a decreased generation of free radical species at the level of the electron transport chain. It has been shown that mitochondria that are damaged by oxidative stress generate greater amounts of H_2O_2 than nonoxidized mitochondria (4). This leads to a vicious cycle wherein mitochondria that are damaged by oxidative stress become more "leaky" and would then generate more ROS and thus become further impaired. The fact that protonmotive force was higher in mitochondria from young rats than in CR and old control rats is to be noted. This is presumably an effect of aging that is independent of CR. Importantly, CR seems to exert its effect through changing mitochondrial oxygen consumption under State 4 conditions. This suggests that CR may act to increase life span in part by decreasing oxidative stress (2,25,26), and decreased proton leak-dependent oxygen consumption through CR may be one of the mechanisms that extends life span.

Table 3. Serum Thyroid Hormone Levels of Old Calorie Restricted, Old Control, and Young Control Groups

Group	(T_3) (nmol)	(T_4) (nmol)
Old		
Calorie restricted ($n = 12$)	1.07 ± 0.08	52.2 ± 5.0
Control ($n = 12$)	1.13 ± 0.07*	50.9 ± 2.1
Young control ($n = 12$)	0.83 ± 0.05	59.4 ± 4.8

Notes: T_3 = L-3,3′,5-triiodothyronine; T_4 = thyroxine. Results are presented as mean ± *SE*.

*Significant difference ($p < .05$) from young control rats.

Maintenance of normal mitochondrial membrane lipid composition is an important indication of the health of mitochondria. The majority of the age-related changes observed in mitochondrial membrane lipid composition occur in cardiolipin (27). Cardiolipin interacts with various mitochondrial membrane proteins, including ATP synthase and ADP–ATP translocator, and it plays an important role in maintaining their activity (27). Cardiolipin also appears to play a major role in controlling the permeability of the mitochondrial membrane to small molecules and in establishing proton gradients (27). CR in rodents has been shown to maintain the amount of 18:2 acyl side chains in the mitochondrial membrane and inhibits the oxidation of cardiolipin (18). These effects may minimize proton leak in CR animals and maintain mitochondrial inner membrane integrity. Thus, our observed lower state 4 oxygen consumption values in CR rats versus old control rats may be an indication of allayed mitochondrial membrane lipid oxidation in these rats.

Surprisingly, we did not find increased State 4 oxygen consumption in old versus young controls. This contradicts earlier findings from studies conducted in hepatocytes of mice (3) and rats (6). However, we were surprised in this study by the fairly large degree of variation in data within groups, and particularly within the old control group. This may be related to the fact that mitochondria from old rats are more fragile than those from young rats and are thus more easily damaged during isolation (28). Mitochondrial preparations from old control rats may thus have contained variable proportions of unhealthy mitochondria, leading to the observed variable results. Moreover, it is also possible that there are species differences in oxidant generation (29), which affect differences in proton leak between rats and mice. Had we a higher number of observations per group, it is likely that statistically significant observations would be obtained. The relative degree to which mitochondria are damaged during their isolation from old and young species is a subject that has received little attention, and more detailed studies (e.g., balance studies of marker enzymes) are needed.

As mentioned earlier, one factor known to affect mitochondrial proton leak is thyroid hormone status (8,9,30,31).

Although thyroxine (T_4) levels tend to remain constant with age in rodents, increases in L-3, 3′, 5-triiodothyronine (T_3) levels, such as we saw, have been observed (32). This has been attributed to defects in the ability of thyroid stimulating hormone to adequately regulate thyroid hormone levels (32). Herlihy and colleagues (33) found that CR in rats decreases 24-hour mean T_3 levels, but that T_4 levels are unchanged. However, we did not observe differences in serum T_3 between old CR and old control rats. It is interesting that the greatest values of leak-dependent oxygen consumption and of serum T_3 were observed for the old control group, because thyroid hormone levels have been positively correlated with mitochondrial proton leak (8,9,30,31).

The specific mechanism for the observed increased proton leak with age has not yet been identified, though, as outlined above, evidence suggests a mechanism involving membrane lipid damage. Another obvious possibility is through the modified activity of the recently identified uncoupling proteins (UCPs). UCPs are mitochondrial inner membrane proteins that may cause a proton leak. Four new UCPs have been cloned, and these include UCP2 (34,35), UCP3 (36, 37), UCP4 (38), and BMCP1 (39). Although their homologies to UCP1, and their ability to uncouple oxidative phosphorylation in a variety of in vitro expression systems, suggest that they might be uncoupling proteins, the mechanisms involved and their physiological functions are as yet unknown. UCP2 is expressed ubiquitously, with highest levels of expression in brown and white adipose tissues. UCP3 is expressed almost exclusively in skeletal muscle in humans, but also in the brown adipose tissue of rodents. UCP4 is expressed exclusively in the brain, whereas BMCP1 is expressed predominantly in the brain but is also expressed at lower levels (10- to 30-fold) in many other tissues. Thus, an increased UCP-mediated leak during aging is another possible mechanism for an increased proton leak. It is also important that one of the several putative functions for the novel UCPs is *decreased* production of ROS (40,41). The mechanism proposed is that UCPs allow increased electron flow during nonphosphorylating states, which in turn may decrease the relative reduction of redox centers in Complex I and of cytochrome b in Complex III, and thus decrease the probability of electron transfer to molecular oxygen.

The major finding from this study is that proton leak-dependent oxygen consumption in hind-limb skeletal muscle of rats is decreased in very old rats subjected to CR from 10 months of age. These results support previous findings, specifically those that demonstrate increased proton leak and oxidative stress with age (3,6). The recent results of Lass and colleagues (7) show that CR attenuates a 41% age-associated increase in superoxide production in submitochondrial particles isolated from mouse muscle, and these results are consistent with our findings. Although the mechanisms involved still require elucidation, decreased mitochondrial proton leak may be an important factor in the retardation of aging by CR.

Acknowledgments

This work was supported by grants from the Natural Science and Engineering Research Council (NSERC) of Canada (MEH) and the National Institutes of Health (P01 AG 11915; T32 AG 00213). This is publication number 01-02 from the Madison Veterans Administration Geriatric Research, Education and Clinical Center.

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References

1. Harman D. Aging: a theory based on free radical and radiation chemistry. *J Gerontol.* 1956;2:298–300.
2. Sohal RS, Weindruch R. Oxidative stress, caloric restriction and aging. *Science.* 1996;273:59–63.
3. Harper M-E, Monemdjou S, Ramsey JJ, Weindruch R. Age-related increase in mitochondrial proton leak and decrease in ATP turnover reactions in mouse hepatocytes. *Am J Physiol.* 1998;275:E197–E206.
4. Sohal RS, Sohal BH. Hydrogen peroxide release by mitochondria increases during aging. *Mech Ageing Dev.* 1991;57:187–202.
5. Brookes PS, Lands J, Clark J, Heales S. Peroxynitrite and brain mitochondria: evidence for increased proton leak. *J Neurochem.* 1998;70: 2195–2202.
6. Hagen TM, Yowe DL, Bartholomew JC, et al. Mitochondrial decay in hepatocytes from old rats: membrane potential declines, heterogeneity and oxidants increase. *Proc Natl Acad Sci USA.* 1997;94:3064–3069.
7. Lass A, Sohal BH, Weindruch R, Forster MJ, Sohal RS. Caloric restriction prevents age-associated accrual of oxidative damage to mouse skeletal muscle mitochondria. *Free Rad Biol Med.* 1998;25:1089–1097.
8. Hafner RP, Nobes CD, McGown AD, Brand MD. Altered relationship between protonmotive force and respiration rate in non-phosphorylating liver mitochondria isolated from rats of different thyroid hormone status. *Eur J Biochem.* 1988;178:511–518.
9. Hafner RP, Leake MJ, Brand MD. Hypothyroidism in rats decreases mitochondrial inner membrane cation permeability. *FEBS Lett.* 1989; 248:175–178.
10. Horrum MA, Tobin RA, Ecklund RE. Thyroid hormone effects of the proton permeability of rat liver mitochondria. *Mol Cell Endocrinol.* 1990;41:163–169.
11. Yu BP. Aging and oxidative stress: modulation by dietary restriction. *Free Rad Biol Med.* 1996;5:651–668.
12. Brand MD, Couture P, Else PL, Withers KW, Hulbert AJ. Evolution of energy metabolism. Proton permeability of the inner membrane of liver mitochondria is greater in a mammal than in a reptile. *Biochem J.* 1991;275:81–86.
13. Porter RK, Brand MD. Body mass dependence of H^+ leak in mitochondria and its relevance to metabolic rate. *Nature.* 1993;362:628–630.
14. Weindruch R, Walford RL. *The Retardation of Aging and Disease by Dietary Restriction.* Springfield, IL: Charles C Thomas; 1988.
15. Davies KJ. Oxidative stress: the paradox of aerobic life. *Biochem Soc Symp.* 1995;61:1–31.
16. Shigenaga M, Hagen T, Ames BN. Oxidative damage and mitochondrial decay in aging. *Proc Natl Acad Sci USA.* 1994;91:10771–10778.
17. Ames BN, Shigenaga M, Hagen T. Oxidants, antioxidants, and the degenerative diseases of aging. *Proc Natl Acad Sci USA.* 1993;90:7915–7922.
18. Laganiere S, Yu BP. Modulation of membrane phospholipid fatty acid composition by age and food restriction. *Gerontology.* 1993;39:7–18.
19. Yu BP, Suescun E, Yang S. Effect of age-related lipid peroxidation on membrane fluidity of phospholipase A2: modulation by dietary restriction. *Mech Ageing Dev.* 1992;65:17–33.
20. Bhattacharya SK, Thakar JH, Johnson PL, Shanklin DR. Isolation of skeletal muscle mitochondria from hamsters using an ionic medium containing ethylenediaminetetraacetic acid and nagarse. *Anal Biochem.* 1991;192:344–349.
21. Chen RF. Removal of fatty acids from serum albumin by charcoal treatment. *J Biol Chem.* 1967;242:173–181.
22. Kamo N, Muratsugu M, Hongoh R, Kobatake Y. Membrane potential of mitochondria measured with an electrode sensitive to tetraphenyl phosphonium and relationship between proton electrochemical potential in steady state. *J Memb Biol.* 1979;49:105–121.
23. Brown GC, Brand MD. Thermodynamic control of flux through mitochondrial cytochrome bc1 complex. *Biochem J.* 1985;225:399–405.

24. Weindruch R. Interventions based on the possibility that oxidative stress contributes to sarcopenia. *J Gerontol Biol Sci.* 1995;50A:B157–B161.
25. Yu BP, Yang R. Critical evaluation of the free radical theory of aging. A proposal for the oxidative stress hypothesis. *Ann NY Acad Sci.* 1996; 786:1–11.
26. Sohal RS, Ku HH, Agarwal S, Forester MJ, Lal H. Oxidative damage mitochondrial oxidant generation and antioxidant defenses during aging in response to food restriction in the mouse. *Mech Ageing Dev.* 1994;74:121–130.
27. Hoch F. Cardiolipins and biomembrane function. *Biochim Biophys Acta.* 1992;1113:71–133.
28. Wilson PD, Franks LM. The effect of age on mitochondrial ultrastructure and enzymes. *Adv Exp Med Biol.* 1975;53:171–183.
29. Sohal RS, Sevensson I, Brunk UT. Relationship between antioxidant defenses and longevity in different mammalian species. *Mech Ageing Dev.* 1990;53:209–215.
30. Harper M-E, Brand MD. Hyperthyroidism stimulates mitochondrial proton leak and ATP turnover in rat hepatocytes but does not change the overall kinetics of substrate oxidation reactions. *Can J Physiol Pharmacol.* 1994;72:899–908.
31. Harper M-E, Brand MD. The quantitative contributions of mitochondrial proton leak and ATP turnover reactions to the changed respiration rate of hepatocytes from rats of different thyroid status. *J Biol Chem.* 1993;268:14850–14860.
32. Hotta H, Ooka H, Sato A. Changes in basal secretion rates of thyroxine and 3,3′,5-triiodothyroxine from the thyroid gland during aging of the rat. *Jpn J Physiol.* 1991;41:75–84.
33. Herlihy JT, Stacy C, Bertrand HA. Long-term food restriction depresses serum thyroid hormone concentrations in the rat. *Mech Ageing Dev.* 1990;53:9–16.
34. Fleury C, Neverova M, Collins S, et al. Uncoupling protein 2: a novel gene linked to obesity and hyperinsulinemia. *Nature Genet.* 1997;15: 269–272.
35. Gimeno RE, Dembski M, Weng X, et al. Cloning and characterization of and uncoupling protein homolog: a potential mediator of human thermogenesis. *Diabetes.* 1997;46:900–906.
36. Boss O, Samec S, Paoloni-Giacobino A, et al. Uncoupling protein 3: a new member of the mitochondrial carrier family with tissue specific expression. *FEBS Lett.* 1997;408:39–42.
37. Vidal-Puig A, Solanes G, Grujic D, Flier JS, Lowell BB. UCP3: an uncoupling protein homologue expressed preferentially and abundantly in skeletal muscle and brown adipose tissue. *Biochem Biophys Res Comm.* 1997;235:79–82.
38. Mao W, Yu XX, Zhong A, et al. UCP4, a novel brain-specific mitochondrial protein that reduces membrane potential in mammalian cells. *FEBS Lett.* 1999;443:326–330.
39. Sanchis D, Fleury C, Chomiki N, et al. BMCP1, a novel mitochondrial carrier with high expression in the central nervous system of humans and rodents, and respiration uncoupling activity in recombinant yeast. *J Biol Chem.* 1998;273:34611–34615.
40. Negre-Salvayre A, Hirtz C, Carrera G, et al. A role for uncoupling protein-2 as a regulator of mitochondrial hydrogen peroxide generation. *FASEB J.* 1997;11:809–815.
41. Boss O, Muzzin P, Giacobino J-P. The uncoupling proteins, a review. *Eur J Endocrinol.* 1998;139:1–9.

Received February 14, 2000
Accepted October 3, 2000
Decision Editor: John A. Faulkner, PhD