Published in final edited form as:

Exp Gerontol. 2008 December ; 43(12): 1069–1076. doi:10.1016/j.exger.2008.09.010.

Effect of aging, caloric restriction, and uncoupling protein 3 (UCP3) on mitochondrial proton leak in mice

Danny K. Asami¹, Roger B. McDonald¹, Kevork Hagopian², Barbara A. Horwitz³, David Warman³, Aileen Hsiao¹, Craig Warden⁴, and Jon J. Ramsey²

¹ Department of Nutrition, University of California, Davis, Davis, California

² Department of Molecular Biosciences, University of California, Davis, Davis, California

³ Section of Neurobiology, Physiology, and Behavior, University of California, Davis, Davis, California

⁴ Department of Pediatrics, University of California, Davis, Davis, California

Abstract

Mitochondrial proton leak may modulate reactive oxygen species (ROS) production and play a role in aging. The purpose of this study was to determine proton leak across the life span in skeletal mitochondria from calorie-restricted and UCP2/3 overexpressing mice. Proton leak in isolated mitochondria and markers of oxidative stress in whole tissue were measured in female C57BL/6J mice fed ad libitum (WT-Control) or a 30% calorie-restricted (WT-CR) diet, and in mice overexpressing UCP2 and UCP3 (Positive-TG), their non-overexpressing littermates (Negative-TG) and UCP3 knockout mice (UCP3KO). Proton leak in WT-CR mice was lower than that of control mice at 8 and 26 months of age. The Positive-TG mice had greater proton leak than the Negative-TG and UCP3KO mice at 8 months of age, but this difference disappeared by 19 and 26 months. Lipid peroxidation was generally lower in WT-CR vs. WT-Control mice and UCP3KO mice had greater concentrations of T-BARS (thiobarbituric acid reactive substances, a measure of lipid peroxidation) than did Positive-TG and Negative-TG. The results of this study indicate that sustained increases in muscle mitochondrial proton leak are not responsible for alterations in life span with calorie restriction or UCP3 overexpression in mice. However, UCP3 may contribute to the actions of CR through mechanisms distinct from increasing basal proton leak.

Keywords

aging; knockout mice; lipid peroxidation; protein carbonyls

INTRODUCTION

Mitochondrial proton leak may be responsible for as much as 20–30% of resting cellular energy expenditure (Brand et al., 1994; Rolfe et al., 1994). The function of proton leak is not entirely known, although it is thought that this process plays a role in regulating mitochondrial reactive oxygen species (ROS) production (Brand, 1997; Skulachev, 1998). In 1956, Denham Harman

Corresponding Author: Roger McDonald, Ph.D. Department of Nutrition, University of California, Davis, CA 95616, (530) 219-5941 (office), (530) 752-8966 (FAX), rbmcdonald@ucdavis.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

postulated that aging was the result of the deleterious interactions of free radicals with cell constituents (Harman, 1956), and this free radical theory of aging continues to be among the most popular theories to explain aging. Therefore, it is possible that mitochondrial proton leak plays a role in aging by modulating ROS production, and it has been proposed that mitochondrial uncoupling may lead to an increase in life span (Brand, 2000; Speakman et al., 2002). However, the role mitochondrial proton leak plays in aging is still the subject of debate.

One strategy for investigating the possible role proton leak plays in aging is to induce a sustained increase in mitochondrial proton leak, and determine the impact on life span or other age-related parameters (see McDonald et al., this issue). For this to work, it is important to demonstrate first that the mechanism used to increase mitochondrial proton leak truly produces a sustained increase across a major portion of the life span. It is now generally considered that proton leak consists of a basal component which is unregulated and an inducible, regulated component (Brookes, 2005). The mechanisms responsible for either component of proton leak are not entirely known, although it is thought that uncoupling proteins may play a central role in the regulation of inducible proton leak (Affourtit et al., 2007; Brookes, 2005). It is generally considered that the physiological role of UCP3 involves only the inducible proton leak, although it has been shown that basal proton leak is also increased in skeletal muscle mitochondria from mice overexpressing UCP3 (Cadenas et al., 2002; Son et al., 2004). It has been suggested that the increase in basal proton leak in these mice is due to artifactual uncoupling since mitochondria from these animals are not inhibited by GDP (Cadenas et al., 2002). While these mice may not adequately reflect the physiological function of UCP3, they may provide a good model for investigating the effect of sustained increases in basal proton leak. Previous studies with UCP3-TG mice, however, have used only young adult animals, and it is not known if uncoupling observed in these animals would continue through later life. Therefore, it is important to measure proton leak across the life span in UCP3-TG mice to determine if they provide a model for sustained increases in basal proton leak.

Another strategy for investigating the influence of proton leak on aging is to determine changes in proton leak following initiation of an intervention that is known to extend maximum life span. Calorie restriction without malnutrition extends both mean and maximum life span, delays the onset and lowers the frequency of age-related diseases, and retards the age-related decline in several physiological functions (Ramsey et al., 2000). The mechanism responsible for life span extension with CR is not known, although it has been proposed that either an increase (Merry 2002) or decrease (Ramsey et al., 2000) in mitochondrial proton leak may contribute to the actions of CR. Studies investigating the influence of CR on rat liver mitochondrial proton leak, however, have produced conflicting results (Hagopian et al., 2005; Lambert and Merry, 2004; Lambert and Merry, 2005) and studies in rat skeletal muscle have reported that CR induces sustained decreases in proton leak (Bevilacqua et al., 2004; Bevilacqua et al., 2005). Additional studies are clearly needed to determine the influence of CR on proton leak, and it is also necessary to determine if the results reported in rats are also observed in other species. The mouse is one of the most common animal models used for aging research, and it is important to determine the influence of CR on mitochondrial proton leak in these animals.

The purpose of this study was to investigate the affect of CR and UCP overexpression on age-related alterations in skeletal muscle mitochondrial proton leak. We compared the leak kinetics of mice overexpressing UCP2 and UCP3 with their non-overexpressing littermates and UCP3 knockout mice. Proton leak was also compared between wild-type (WT) mice on 30% CR diet and ad libitum fed controls.

MATERIALS AND METHODS

Animals and Animal Care

Genetically altered and CR mice were obtained, housed and maintained identically to that previously described (McDonald et al., this issue). All experiments were approved by the University of California Institutional Animal Care and Use Committee.

Experiment I: Mitochondrial Proton Leak—Mitochondrial isolations and proton leak assessments were performed on groups of Postvie-TG, Negative-TG, UCP3KO, WT-Control, and WT-CR mice at 8, 19, and 26 months of age ($n = 8$ mice/group/age) as well as at 30-months for WT-CR mice ($n = 8$).

Mitochondrial isolation: Following a 10–12 hour fast, mice were killed by cervical dislocation. Subsequent steps were performed on ice at 4°C unless stated otherwise. Using the methods of Cadenas et al. (Cadenas et al., 2002) with slight modification, the skeletal muscle from both hind limbs was removed rapidly, cleaned of fat and connective tissue, weighed, and placed in ice-cold isolation medium containing 100 mM KCl, 50 mM Tris-HCl, 2 mM EGTA, pH 7.4. Immediately following, the muscle was placed on a chilled Teflon cutting surface, minced with a razor blade, and rinsed twice with ice-cold isolation medium. The tissue was stirred for 5 min in isolation medium plus 1 mM ATP, 5 mM MgCl₂, 0.5% (w/v) defatted BSA, and 10 units of protease/g of tissue (Sigma protease type VIII), pH 7.4, and then homogenized using a Polytron tissue homogenizer with two, 10-second bursts at 1,500 rpm. The homogenate was centrifuged at 500 x *g* for 10 min at 4°C, and the resulting supernatant was filtered through a 250 μm Nitex nylon monofilament mesh (Sefar America Inc., Kansas City, MO) and centrifuged at 10,000 *g* for 10 min. The supernatant was discarded, and the mitochondrial pellet was resuspended in isolation medium and centrifuged at 500 x *g* for 10 min. The supernatant was again filtered through 250 μm Nitex fabric and centrifuged at 10,000 *g* for 10 min and the resulting pellet was resuspended in isolation medium and centrifuged at 3,800 x *g* for 10 min. The mitochondrial pellet was resuspended in isolation medium and protein concentration determined by the Bradford method using a Bio-Rad assay kit, with BSA as the standard.

Measurement of mitochondrial oxygen consumption: Mitochondrial oxygen consumption measurements were completed as described previously (Ramsey et al., 2004) with slight modification. Briefly, oxygen consumption was measured using a Clark-type oxygen electrode (Hansatech, Norfolk, UK), with the incubation chamber maintained at 37°C and calibrated with air-saturated incubation medium (120 mM KCl, 5 mM KH₂PO₄, 5 mM HEPES, 2 mM MgCl₂, 1 mM EGTA, and 0.3% (w/v) defatted BSA, pH 7.4). Mitochondria (0.25 mg/ml protein) were incubated in incubation medium containing 5 μM rotenone and 5 mM succinate. Respiratory control ratios (RCR) were calculated as the state 3 respiration rate in the presence of 200 μM ADP divided by the state 4 rate after ADP phosphorylation. The mean RCR for each animal group ranged from 3.21 to 3.45. These RCR values are similar to previously reported values with succinate as a substrate (Cadenas et al., 2002). All respiration results are expressed per milligram of mitochondrial protein.

Measurement of mitochondrial membrane potential: Membrane potential was measured with a methyltriphenylphosphonium (TPMP⁺)-sensitive electrode simultaneously with all oxygen consumption measures described above using previously published methods (Brand, 1995). Mitochondrial membrane potential ($\Delta\Psi_m$) was calculated using the modified Nernst equation:

$$\Delta\Psi_m = 61.5 \times \log \left\{ \frac{[\text{TPMP}^+_{\text{mat}}] \times a_m}{[\text{TPMP}^+_{\text{ext}}]} \right\}$$

where $[TPMP^{+}_{mat}]$ is the concentration of $TPMP^{+}$ in the mitochondrial matrix and $[TPMP^{+}_{ext}]$ is the concentration of $TPMP^{+}$ outside the mitochondria. The factor a_m is the mitochondrial $TPMP^{+}$ binding correction, and the value used for skeletal muscle was 0.4 (Rolfe et al., 1994).

Mitochondria (0.25 mg/ml protein) were incubated in incubation medium containing 5 μ M rotenone, 0.4 μ g nigericin/mg mitochondrial protein, and 8 μ g oligomycin/mg mitochondrial protein. Rotenone was used to block complex I and ensure that energy flux through the electron transport chain was controlled through $FADH_2$ -linked substrates such as succinate. Nigericin, in the presence of KCl, was used to convert the ΔpH component of the protonmotive force to units of mitochondrial membrane potential (mV) and oligomycin was used to inhibit ATP synthase. The electrode was calibrated with sequential addition of 1 μ M $TPMP^{+}$, up to 5 μ M and followed by the addition of 5 mM succinate to start the reaction. Respiration and membrane potential were progressively inhibited through successive steady states by additions of malonate up to 10 mM. At the end of each trial, 0.2 μ M FCCP was added to dissipate the membrane potential and release all $TPMP^{+}$ back to the medium, allowing correction for any small electrode drift.

Proton leak kinetics: The kinetic response of the proton leak to $\Delta\Psi_m$ was determined by titrating the electron transport chain with malonate (0.16–1.25 mM), an inhibitor of complex II in the chain, in the presence of oligomycin (8 μ g/mg protein). Mitochondrial membrane potential and oxygen consumption during state 4 respiration were measured simultaneously using a methyltriphenylphosphonium-sensitive electrode and a Clark-type oxygen electrode. Proton leak kinetics were visualized by plotting membrane potential against oxygen consumption.

Experiments II: Oxidative Stress—Oxidative stress measurements in the supernatant fraction containing mitochondrial proteins isolated from of lower limb muscles were performed on groups of Positive-TG, Negative-TG, UCP3KO, WT-Control, and WT- CR mice at 8, 19, and 26 months of age ($n = 8$ –10 mice/group/age).

Tissue preparation: Following a 12–14 hour fast, mice were killed by decapitation. The left and right lower hind limb muscles (gastrocnemius, soleus, and plantaris), were removed, weighed, and snap frozen in liquid nitrogen. The liquid nitrogen frozen tissue was stored at -80°C for 48 hours prior to homogenization.

Frozen lower hind limb muscle were place into either a phosphate buffered saline (PBS: 10mM NaK, 150mM NaCL, pH 7.2) for the thiobarbituric acid reactive substances (T-BARS) or radioimmuno precipitation assay buffer (RIPA: 0.5M Tris-HCl, pH 7.4, 1.5M NaCl, 2.5% deoxycholic acid, 10% NP-40, 10mM EDTA) for the protein carbonyl assay. Ten microliters of Protease Inhibitor Cocktail (Sigma, St. Louis, MO) was added per ml of homogenization buffer to prevent protein degradation. Tissues were homogenized using a Kontes glass tissue grinder. Homogenates were aliquoted into 1.5 ml microcentrifuge tubes and stored at -80°C until protein carbonyl assays were preformed. Measurement of T-BARS was performed on fresh homogenate. Prior to assaying for specific marker of oxidative stress, homogenates were centrifuged at 5000 x g for 5 min. Supernatant was carefully decanted from the remaining pellet and immediately used in experimental analysis.

Pilot studies were conducted to determine the reliability of our isolation, storage, and homogenation procedures to accurately measure markers of oxidative stress. Protein carbonyls were stable for 3 weeks of storage at -80°C . However, the concentration of T-BARS began to increase in tissue homogenates after 48 hours of storage.

Lipid peroxidation and protein determination: The amount of lipid peroxidation in tissue was determined by measurement of T-BARS (Buege and Aust, 1988). Briefly, supernatant from tissue homogenates were mixed with 4% BHT, 5% SDS, 0.1 N HCl, 10% PTA, and 0.7% TBA. Butylated hydroxytoluene (0.01%) was added to the assay mixture to prevent autoxidation of the sample, and the mixture was heated at 95°C for 90 minutes. After cooling, the mixture was centrifuged at 1000 x g for 20 minutes, and the absorbance of the organic phase was measured at 535 nm. The concentration T-BARS was determined by standard curve and was expressed as nM of malonaldehyde per mg protein. Protein was determined by the methods of Lowry using the DC Protein Assay kit (Bio-Rad laboratories, Hercules, Ca)

Protein carbonyls: Measurement of whole tissue protein carbonyls was based a modification of the enzyme-linked immunosorbent assay (ELISA) procedures of Winterbourn and Buss (Winterbourn and Buss, 1999). Briefly, tissue homogenates were centrifuged at 12,000 x g for 10 min, the supernatant discarded and the pellet reconstituted with PBS. Aliquots of the reconstituted pellet were incubated with 2,4-dinitrophenylhydrazine (DNPH) at 4°C for 16–24 hours. Then, the samples were incubated for 1 hour at 37°C with anti-DHPH antibody followed by 1 hour incubation at 21°C in the dark with streptavidin-biotinylated horseradish peroxidase. Protein carbonyls, expressed as nM/mg protein, in tissue samples were determined from a standard curve generated from reduced serum albumin containing various concentration of carbonyls.

Statistical analysis: Analysis of variance (ANOVA) was used to evaluate the effects of age, feeding state, and animal type. Data were analyzed using two-way ANOVA to test for a significant effect of animal group and age. When the two-way interaction was significant ($P < 0.05$), differences between groups or ages were assessed by a post-hoc one-way ANOVA. Differences were considered significant at $P < 0.05$. All values presented in the figures and tables are means $\pm$ SE.

Although the transgenic and knockout mice were bred on a C57BL/6J background, our lifespan studies have shown differences in body weight between the transgenics and the C57BL/6J mice from Jackson Laboratory at specific ages (McDonald et al., this issue). Thus, to make certain that the WT-CR, Positive TG and UCP3KO were compared to their appropriate controls our statistical design evaluated possible differences in proton leak and ROS within two groupings. One group consisted of the Positive-TG, Negative-TG, and UCP3KO mice, while the other group included the C57BL/6J CR and ad libitum animals. The two groups were analyzed independent of each other.

RESULTS

Experiment I: Mitochondrial Proton Leak

Whole hind limb skeletal muscle weights—There was a significant main effect of age on skeletal muscle weights in the transgenic/knockout group that reflected a general decrease with age (Table 1). Post-hoc analysis indicated that differences in muscle weight reached significance only between 19 and 26 months of age for Positive-TG, Negative-TG and UCP3KO. There was no significant main effect of genotype on muscle weight at any of the ages examined, although some individual difference existed.

There was a significant interaction between age and dietary regime in the C57BL/6J mice. The interaction reflected the age-related decrease in muscle weight of the WT-Control group versus the maintenance of muscle weight in the WT-CR mice through 19 months of age.

Mitochondrial proton leak kinetics—The overall kinetics of proton leak in skeletal muscle mitochondria are summarized in Figures 1–3. The furthestmost point on the right of

each curve represents state 4 respiration (maximal proton leak-dependent respiration) and successive data points along the curve signify discrete and measured additions of malonate, a competitive inhibitor of complex II. Shifts of the plotted leak kinetics curve either rightward (increase in mitochondrial oxygen consumption), downward (decrease in membrane potential), or a combination of both responses, are indicative of increased mitochondrial proton leak.

At identical inhibitor (malonate) concentrations, there was no significant difference in mitochondrial membrane potential between Positive-TG and Negative-TG mice. However, at defined protonmotive force (Δp) values exceeding 140 mV, mitochondrial leak-dependent respiration was greater ($P < 0.05$) for Positive-TG mice than their Negative-TG littermates at 8 months of age (Fig. 1A). This resulted in a shift in the proton leak kinetics curve indicative of increased mitochondrial proton leak in the Positive-TG mice. At 19 and 26 months of age (Fig. 1B, 1C), there were no significant differences ($P > 0.05$) in oxygen consumption between the groups, resulting in a convergence of the proton leak kinetics curves and no differences in proton leak between the groups at these later assessment ages. This convergence of the leak kinetics curves was primarily due to an age-related decrease in proton leak in Positive-TG mice.

Mitochondrial proton leak was greater in the Positive-TG vs. UCP3KO mice at 8 and 19 months of age (Fig. 1A, 1B). At the 26-month assessment, there was no significant difference ($P > 0.05$) in proton leak between the groups (Fig. 1C). However, the proton leak kinetics curve does suggest a trend whereby the UCP3KO mice are becoming leakier than the Positive-TG mice. This was due to both an age-related decrease in proton leak in the Positive-TG group and an increase in proton leak in the oldest age group of UCP3KO mice.

The results in Figure 2A show that at 8 months of age, the WT-CR animals exhibited a higher membrane potential and lower leak-dependent respiration rate compared to their age-matched WT-Controls ($P < 0.05$), indicating decreased proton leak. However, no proton leak difference was observed between control and WT-CR groups at 19 months of age (Fig. 2B). At 26 months of age, significant differences ($P < 0.05$) in both membrane potential and respiration reemerged between WT-Control and WT-CR groups (Fig. 2C). This resulted in a biphasic change in proton leak, such that lower proton leak was observed in the WT-CR group at both 8 and 26 months of age.

Although there was no significant difference ($P > 0.05$) in proton leak between the 8-, 19-, and 26-month-old WT-CR mice, proton leak was greater ($P < 0.05$) in WT-CR mice at 30 months of age (Fig. 3) when compared to the other age groups. We did not have a sufficient number of ad libitum wild-type mice survive to 30 months of age to provide controls for the 30 month old WT-CR mice. The CR mice in Figure 3 are compared to their age-matched WT-controls in figure 2. At 8 months, WT-controls exhibited significantly ($P < 0.05$) lower membrane potentials and higher rate of respiration than at 19 months of age (Fig. 2). This resulted in decreased proton leak at 19 months of age, and this was solely responsible for the convergence of leak kinetics curves between control and WT-CR groups at this assessment point. Proton leak increased at 26 months such that the leak kinetics curve at this age was intermediate to the curves at 8 and 19 months.

Experiments II: Oxidative Stress

T-BARS- C57BL/6J Mice—There was a significant main effect of diet on T-BARS concentration in the lower-hind limb muscle of the C57BL/6J mice that reflected the generally lower mean values in the CR vs. Control groups (Fig. 4). Lower hind limb muscle isolated from 19- and 26-month WT-Control mice had significantly greater T-BARS than did the 8-month-old mice. Conversely, 26-month-old WT-CR mice had significantly lower T-BARS than did 8- and 19-

month-old mice. T-BARS in hind limb muscle of the 26-month-old Control vs. the 26-month WT-CR mice were significantly greater.

T-BARS- Genetically Altered Mice—There was a significant main effect of genotype on T-BARS in the lower-hind limb muscle of genetically altered mice that reflected the greater values in the UCP3KO vs. Positive-TG and Negative-TG mice (Figure 4). T-BARSs were significantly greater at all ages in the UCP3KO mice compared to age-matched values in the Positive-TG and Negative-TG groups.

Protein Carbonyls - C57BL/6J Mice—There was a significant main effect of diet on protein carbonyls in the lower hind limb muscle of C57BL/6J mice that reflect lower values in the WT-CR vs. WT-Control (Figure 4). Individual differences (WT-CR vs. WT-Control) reach significance in the hind limb muscle between the 8- and 26-month mice.

Protein Carbonyls- Genetically Altered Mice—There were no significant main effects of age or genotype on protein carbonyl production in hind limb muscle or genetically altered mice (Figure 4). The generally lower values of protein carbonyls production in the UCP3KO vs. Positive-TG and Negative-TG resulted in a significant main effect of genotype.

DISCUSSION

The purpose of this study was to investigate the effect of CR and UCP overexpression on age-related alterations in skeletal muscle mitochondrial proton leak in mice. The leak of protons across the mitochondrial inner membrane is responsible for 20–30% of resting cellular energy expenditure (Brand, 2000; Rolfe and Brown, 1997). Despite this considerable energy cost, the primary function of mitochondrial proton leak is still the subject of debate. However, it has been known for over 30 years that the addition of uncoupling agents to isolated mitochondria decreases ROS production (Boveris and Chance, 1973). Therefore, it has been suggested that a major role of proton leak may be to control the rate of aging by modulating ROS production (Brand, 2000). The finding by Speakman et al. (Speakman et al., 2004) of increased proton leak in skeletal muscle isolated from long-lived mice supports this suggestion. The mechanisms responsible for protein leak are not entirely known, although there has been considerable interest in the possible role UCP3 may play in regulating skeletal muscle protein leak. UCP3 is primarily expressed in skeletal muscle, and prior research has demonstrated that proton leak is increased in muscle mitochondria from transgenic mice overexpressing UCP3 (Cadenas et al., 2002; Son et al., 2004). However, previous studies in transgenic mice overexpressing UCP3 have been conducted in young animals, and it remains to be seen if uncoupling observed in early life would continue into late life. Therefore, we measured the effect of UCP3 expression on proton leak in skeletal muscle mitochondria isolated from 8-, 19-, and 26-month old mice that overexpress or lack a target gene for UCP3. The goals of this portion of the study were twofold: first, to determine if chronic elevations in UCP3 result in changes in basal proton leak; second, to determine if elevations in UCP3 lead to uniform alterations in muscle proton leak across the life span.

As expected, a significant increase in skeletal muscle mitochondrial proton leak was observed in the UCP3 overexpressors (UCP-TG) compared to non-TG littermates at 8 months of age. Also, an increase in proton leak was observed in the UCP-TG compared to UCP3 KO mice through 18 months of age. These results are consistent with previously published studies which indicate increased skeletal muscle proton leak in young UCP-TG mice when compared to WT control mice (Cadenas et al., 2002; Son et al., 2004; Horvath et al., 2003) or UCP3KO mice (Cadenas et al., 2002). However, the results of our study clearly indicate that the initial increases in proton leak observed in the UCP-TG mice disappear with advancing age. This is due primarily to an age-related decrease in proton leak in the UCP-TG group, although the

mechanism for this decrease is not entirely clear. It has been proposed that proton leak in UCP-TG mice is due to artifactual uncoupling since mitochondria from these animals are not activated by superoxide or inhibited by GDP (Cadenas et al., 2002). While this may limit the usefulness of this mouse model for investigating the physiological function of UCP3, it does not present a problem if the primary goal of the study is to simply induce sustained mitochondrial uncoupling. In our case, the primary goal was to determine if UCP3 overexpression induces a sustained alteration in basal proton leak across the life span, regardless of whether this occurs as an artifact of overexpression or as a normal physiological function of the protein. If uncoupling in UCP-TG is due to an artifact of UCP3 overexpression, it is important to note that this artifactual uncoupling disappears with time despite the fact that UCP3 mRNA and protein levels remain high (McDonald et al., this issue). Therefore, the mechanism for proton leak in UCP-TG mice is clearly due to a factor(s) that is sensitive to aging.

Possible factors that could contribute to an age-related decline in proton leak in the UCP-TG include a decrease in the insertion of UCP3 protein in the mitochondrial membrane or decreases in factors that stimulate the protein. Our results indicate that there is a decrease in mitochondrial UCP3 protein at 26 months of age, despite the fact that UCP3 mRNA expression remains high (McDonald et al., this issue). Thus, it is likely that this decrease in mitochondrial UCP3 protein is the major contributor to the age-related decline in proton leak in the UCP-TG mice. It has also been reported that UCP3 is stimulated by fatty acids, reactive alkenals, and superoxide (reviewed in: (Brand and Esteves, 2005; Esteves et al., 2006)). The purpose of the present study was to investigate the influence of UCP3 overexpression on basal proton leak and not to investigate the influence of proposed UCP3 stimulators. However, it is possible that age-related differences in stimulators could have influenced the results and future studies will need to investigate the influence of aging on possible stimulators/inhibitors of UCP3 to further determine mechanisms for the age-related decrease in proton leak in the UCP-TG mice. It is particularly important to note that the present study was completed in isolated mitochondria, and therefore, the influence on proton leak of mitochondrial interactions with various cellular compounds could not be determined. Thus, factors which may normally play a role in regulating UCP activity could have been missing from our mitochondrial preparations. Nonetheless, the results of the current study clearly indicate that UCP-TG mice do not provide an adequate model for sustained increases in basal proton leak across the life span.

It is interesting to note that lipid peroxidation was increased in UCP3 KO compared to positive-TG and negative-TG mice, while no differences in protein carbonyls were observed between these groups of mice. The mechanism for this difference is not entirely clear, although it has been proposed that UCP3 may function as a transporter of fatty acid anions or fatty acid peroxides out of the mitochondrial matrix (Schrauwen, et al., 2006). Such a function could prevent the accumulation of lipid peroxides in the mitochondria and this may help explain why only increased oxidative damage to lipids was observed in the UCP3KO mice.

CR, without malnutrition, is known to increase mean and maximum life span (Ramsey et al., 2000), and if alterations in basal proton leak play a role in aging it is expected that changes in proton leak would be observed with CR. Also, CR provides a physiological model of sustained increases in skeletal muscle UCP3 protein levels (Bevilacqua et al., 2004; Bevilacqua et al., 2005). However, the results of our study showed that CR did not increase basal mitochondrial proton leak in skeletal muscle. In fact, CR decreased proton leak at the 8 and 26-month assessments. This reduction in basal proton leak in the WT-CR mice occurred despite a significant increase, as compared to ad libitum fed mice, in UCP3 expression and protein concentration (McDonald et al., this issue). These results are consistent with previous research with rats investigating the effect of CR on mitochondrial proton leak in skeletal muscle (Bevilacqua et al., 2004; Bevilacqua et al., 2005). In these studies, it had been reported that

CR produces an increase in skeletal muscle UCP3 expression through 25 months of age. Despite this increase in UCP3 expression, a decrease in mitochondrial proton leak was observed at 6 and 12 months of CR while this difference disappeared by 18 months of CR (Bevilacqua et al., 2004; Bevilacqua et al., 2005). Our results indicate that skeletal muscle mitochondria from mice and rats show similar proton leak responses to CR.

In addition to elevating UCP3 expression, CR may be inducing other changes which could account for the observed decrease in proton leak. For example, it has been suggested that mitochondrial proton conductance is dependent upon the concentration of adenine nucleotide translocase (ANT) (Brand and Esteves, 2005). However, to our knowledge no studies have investigated the effect of long-term CR on levels of ANT protein in skeletal muscle. Changes in proton leak could also be the result of membrane permeability changes due to altered fatty acid composition of the membrane phospholipids (Brand et al., 1994). Previous studies report a positive correlation between polyunsaturated fatty acids (e.g., C22:6n-3) and proton leak and a negative correlation between linoleic acid (C18:2n-6) and proton leak (Brand et al., 1994; Porter et al., 1996; Brookes et al., 1998). Additionally, long-term CR results in increased mitochondrial linoleic acid content and decreased docosahexanoic acid (C22:6n-3) (Hulbert, 2005; Laganieri and Yu, 1993). Based on these correlative results, it may be expected that the membrane fatty acid changes would contribute to a decrease in proton leak.

The decrease in proton leak and increased UCP3 concentration in muscle of the CR animals was associated with decreased levels of oxidative stress as compared to those observed in the wild type control. Conversely, increased lipid peroxidation observed in muscle isolated from UCP3KO vs. the Negative-TG and Positive-TG mice across the life span was associated with a decreased proton leak. Based on these results, we conclude that basal proton leak does not consistently correlate with measures of oxidative stress in the mouse models studied. These results also do not support the idea that UCP3 protects against oxidative stress by increasing basal proton leak, however, we can not rule out the possibility that UCP3 could induce a regulated proton leak that could be stimulated under stress conditions. It has been reported that superoxide activates uncoupling proteins (Echtay et al., 2002), and it is possible that short-term stimulation of proton leak may be helpful in protecting against spikes in ROS production. Also, it has been reported that UCP3 may decrease ROS production without uncoupling mitochondria (MacLellan et al., 2005), although it is not clear what mechanism is responsible for this action of UCP3.

Regardless of the mechanisms controlling mitochondrial proton leak, it is important to know how this process is altered with aging. Two questions regarding aging and mitochondrial proton leak still need to be answered. First, does aging cause an increase in mitochondrial proton leak? Second, does an age-related increase in proton leak contribute to loss of physiological function? Our study was intended to provide an initial step towards addressing the first question. Mitochondrial proton leak is stimulated by ROS production and oxidative damage to the mitochondrial membrane (Brookes, 2005; Brookes et al., 1998; Brookes, 1998), presumably via lipid peroxidation (Brookes et al., 1998). It has been repeatedly demonstrated that aging is associated with an increase in ROS production and oxidative stress (Sohal and Weindruch, 1996), and therefore an increase in proton leak may be expected with aging. In support of this conclusion, it has been reported that mitochondrial proton leak is increased in skeletal muscle from 33-month-old compared to 4-month-old rats (Lal et al., 2001). Similarly, it has been reported that proton leak is greater in hepatocytes from 30-month-old compared to 3-month-old mice (Harper et al., 1998). However, in our study, the alterations to mitochondrial proton leak kinetics associated with aging were not consistent across all five animal groups. Both WT control and UCP3KO mice demonstrated an increase in proton leak from 19 to 26 months of age. However, mitochondrial proton leak decreased progressively in UCP-TG mice but was unchanged in their non-TG littermates between the 8- and 26-month assessment points. Across

this same age range, our CR group also showed no significant alterations in proton leak kinetics. However, between the 26- and 30-month assessments, CR mice had the largest increase in mitochondrial proton leak for all groups. The results from the oldest age groups of CR, UCP3KO, and WT controls are consistent with an increase in proton leak with advanced age. However, our results are not consistent with the idea of a progressive age-related increase in proton leak, but instead suggest that increases in proton leak may occur within a relatively brief time span at advanced age. UCP-TG and non-TG mice at 26 months may not have yet reached this physiological threshold and thus increases in proton leak were not observed.. To improve our understanding of the physiological changes associated with aging, future studies should involve reexamination of the alterations to proton leak kinetics over more frequent assessment points to the end of the life span. Once this has been determined it may be possible to establish if age-related changes in proton leak contribute to loss of physiological function.

It is important to note that an age-related decrease in hindlimb muscle weight was observed in all groups of mice. Previous studies have indicated an increase in mitochondrial dysfunction and oxidative damage with age-related loss of muscle mass (Bua et al., 2002; Dirks et al., 2006), and we were interested in determining if increases in proton leak were consistently associated with decreases in muscle weight. In our study, increases in mitochondrial proton leak did not occur in all groups at time points when significant decreases in skeletal muscle weight occurred and this indicates alterations in proton leak do not consistently occur in concert with age-related loss of muscle mass.

The increase in UCP3 protein levels with CR that has been reported in mice (McDonald et al., this issue) and rats (Bevilacqua et al., 2004; Bevilacqua et al., 2005) is interesting. While our studies clearly indicate that elevated muscle UCP3 does not increase basal proton leak, we can not rule out the possibility that these increases in UCP3 levels play an important role in the animal's adaptation to chronic CR. The function of UCP3 is still the subject of debate and it is possible that these proteins perform an important function distinct from uncoupling. Recently, it has been reported that UCP3 may function primarily as a Ca^{2+} transporter (Trenker et al., 2007). Clearly, such a function could play an important role in the metabolic adaptation to sustained CR, since Ca^{2+} influences insulin secretion (Graier et al., 2008) and the activities of key enzymes involved in substrate oxidation (Hansford and Zorov, 1998; McCormack, et al., 1990). Also, it has been proposed that UCP3 plays a role in fatty acid metabolism (Schrauwen, et al., 2006; Jezek et al., 2004) and such a function may be extremely important for the adaptation to CR.

In conclusions, CR or altered expression of UCP3 attributed to gene manipulation does not produce sustained increases in basal proton conductance across the inner membrane of skeletal muscle mitochondria. In fact, CR produces a decrease in muscle proton leak. Nonetheless, the significantly greater skeletal muscle UCP3 in CR vs. WT mice throughout the life span suggest that UCP3 may contribute to the actions of CR through mechanisms distinct from increasing basal proton leak.

Acknowledgements

The authors wish to thank Keya Walker for her technical assistance in the completion of this research. This work was supported by NIA grant AG19984.

References

- Affourtit C, Crichton PG, Parker N, Brand MD. Novel uncoupling proteins. Novartis Found Symp 2007;287:70–80. 80–91. [PubMed: 18074632]

- Bevilacqua L, Ramsey JJ, Hagopian K, Weindruch R, Harper ME. Effects of short- and medium-term calorie restriction on muscle mitochondrial proton leak and reactive oxygen species production. *Am J Physiol Endocrinol Metab* 2004;286:E852–861. [PubMed: 14736705]
- Bevilacqua L, Ramsey JJ, Hagopian K, Weindruch R, Harper ME. Long-term caloric restriction increases UCP3 content but decreases proton leak and reactive oxygen species production in rat skeletal muscle mitochondria. *Am J Physiol Endocrinol Metab* 2005;289:E429–438. [PubMed: 15886224]
- Boveris A, Chance B. The mitochondrial generation of hydrogen peroxide. General properties and effect of hyperbaric oxygen. *Biochem J* 1973;134:707–716. [PubMed: 4749271]
- Brand, MD. The measurement of mitochondrial protonmotive force. In: Brown, GC.; Cooper, CE., editors. *Bioenergetics: A Practical Approach*. Oxford University Press; Oxford, UK: 1995. p. 39-62.
- Brand MD. Regulation analysis of energy metabolism. *J Exp Biol* 1997;200:193–202. [PubMed: 9050227]
- Brand MD. Uncoupling to survive? The role of mitochondrial inefficiency in ageing. *Exp Gerontol* 2000;35:811–820.
- Brand MD, Chien LF, Ainscow EK, Rolfe DF, Porter RK. The causes and functions of mitochondrial proton leak. *Biochem Biophys Acta* 1994;1187:132–139. [PubMed: 8075107]
- Brand MD, Esteves TC. Physiological functions of the mitochondrial uncoupling proteins UCP2 and UCP3. *Cell Metab* 2005;2:85–93. [PubMed: 16098826]
- Brookes PS. Mitochondrial proton leak and superoxide generation: an hypothesis. *Biochem Soc Trans* 1998;26:S331. [PubMed: 10047845]
- Brookes PS. Mitochondrial H(+) leak and ROS generation: an odd couple. *Free Radic Biol Med* 2005;38:12–23. [PubMed: 15589367]
- Brookes PS, Buckingham JA, Tenreiro AM, Hulbert AJ, Brand MD. The proton permeability of the inner membrane of liver mitochondria from ectothermic and endothermic vertebrates and from obese rats: correlations with standard metabolic rate and phospholipid fatty acid composition. *Comp Biochem Physiol B: Biochem Molec Bio* 1998;119:325–334. [PubMed: 9629666]
- Bua EA, McKiernan SH, Wanagat J, McKenzie D, Aiken JM. Mitochondrial abnormalities are more frequent in muscles undergoing sarcopenia. *J Appl Physiol* 2002;92:2617–2624. [PubMed: 12015381]
- Buege JA, Aust SD. Microsomal lipid peroxidation. *Meth Enzymol* 1988;52:302–310. [PubMed: 672633]
- Cadenas S, Echtay KS, Harper JA, Jekabsons MB, Buckingham JA, Grau E, Abuin A, Chapman H, Clapham JC, Brand MD. The basal proton conductance of skeletal muscle mitochondria from transgenic mice overexpressing or lacking uncoupling protein-3. *J Biol Chem* 2002;277:2773–2778. [PubMed: 11707458]
- Dirks AJ, Hofer T, Marzetti E, Pahor M, Leeuwenburgh C. Mitochondrial DNA mutations, energy metabolism and apoptosis in aging muscle. *Ageing Res Rev* 2006;5:179–195. [PubMed: 16647308]
- Echtay KS, Roussel D, St-Pierre J, Jekabsons MB, Cadenas S, Stuart JA, Harper JA, Roebuck SJ, Morrison A, Pickering S, Clapham JC, Brand MD. Superoxide activates mitochondrial uncoupling proteins. *Nature* 2002;415:96–99. [PubMed: 11780125]
- Esteves TC, Parker N, Brand MD. Synergy of fatty acid and reactive alkenal activation of proton conductance through uncoupling protein 1 in mitochondria. *Biochem J* 2006;395:619–628. [PubMed: 16451125]
- Graier WF, Trenker M, Malli R. Mitochondrial Ca²⁺, the secret behind the function of uncoupling proteins 2 and 3? *Cell Calcium* 2008;44:36–50. [PubMed: 18282596]
- Hagopian K, Harper ME, Ram JJ, Humble SJ, Weindruch R, Ramsey JJ. Long-term calorie restriction reduces proton leak and hydrogen peroxide production in liver mitochondria. *Am J Physiol Endocrinol Metab* 2005;288:E674–684. [PubMed: 15562252]
- Hansford RG, Zorov D. Role of mitochondrial calcium transport in the control of substrate oxidation. *Mol Cell Biochem* 1998;184:359–369. [PubMed: 9746330]
- Harman D. Aging: a theory based on free radical and radiation chemistry. *J Gerontol* 1956;11:298–300. [PubMed: 13332224]
- Harper ME, 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:E197–206. [PubMed: 9688619]

- Horvath TL, Diano S, Miyamoto S, Barry S, Gatti S, Alberati D, Livak F, Lombardi A, Moreno M, Goglia F, Mor G, Hamilton J, Kachinskas D, Horwitz B, Warden CH. Uncoupling proteins-2 and 3 influence obesity and inflammation in transgenic mice. *Int J Obes Relat Metab Disord* 2003;27:433–442. [PubMed: 12664076]
- Hulbert AJ. On the importance of fatty acid composition of membranes for aging. *Journal of Theoretical Biology* 2005;234:277–288. [PubMed: 15757684]
- Jezek P, Záčková M, Růžicka M, Skobisová E, Jabůrek M. Mitochondrial uncoupling proteins--facts and fantasies. *Physiol Res* 2004;53(Suppl 1):S199–211. [PubMed: 15119950]
- Laganier S, Yu BP. Modulation of membrane phospholipid fatty acid composition by age and food restriction. *Gerontology* 1993;39:7–18. [PubMed: 8440492]
- Lal SB, Ramsey JJ, Monemdjou S, Weindruch R, Harper ME. Effects of caloric restriction on skeletal muscle mitochondrial proton leak in aging rats. *J Gerontol A Biol Sci Med Sci* 2001;56:B116–122. [PubMed: 11253148]
- Lambert AJ, Merry BJ. Effect of caloric restriction on mitochondrial reactive oxygen species production and bioenergetics: reversal by insulin. *Am J Physiol Regul Integr Comp Physiol* 2004;286:R71–79. [PubMed: 12969875]
- Lambert AJ, Merry BJ. Lack of effect of caloric restriction on bioenergetics and reactive oxygen species production in intact rat hepatocytes. *J Gerontol A Biol Sci Med Sci* 2005;60:175–180. [PubMed: 15814858]
- MacLellan JD, Gerrits MF, Gowing A, Smith PJ, Wheeler MB, Harper ME. Physiological increases in uncoupling protein 3 augment fatty acid oxidation and decrease reactive oxygen species production without uncoupling respiration in muscle cells. *Diabetes* 2005;54:2343–2350. [PubMed: 16046300]
- McCormack JG, Halestrap AP, Denton RM. Role of calcium ions in regulation of mammalian intramitochondrial metabolism. *Physiol Rev* 1990;70:391–425. [PubMed: 2157230]
- Merry BJ. Molecular mechanisms linking calorie restriction and longevity. *Int J Biochem Cell Biol* 2002;34:1340–1354. [PubMed: 12200030]
- Porter RK, Hulbert AJ, Brand MD. Allometry of mitochondrial proton leak: influence of membrane surface area and fatty acid composition. *Am J Physiol Regul Integr Com Physiol* 1996;271:R1550–1560.
- Ramsey JJ, Hagopian K, Kenny TM, Koomson EK, Bevilacqua L, Weindruch R, Harper ME. Proton leak and hydrogen peroxide production in liver mitochondria from energy-restricted rats. *Am J Physiol Endocrinol Metab* 2004;286:E31–40. [PubMed: 14662512]
- Ramsey JJ, Harper ME, Weindruch R. Restriction of energy intake, energy expenditure, and aging. *Free Radic Biol Med* 2000;29:946–968. [PubMed: 11084284]
- Rolfe DF, Brown GC. Cellular energy utilization and molecular origin of standard metabolic rate in mammals. *Physiol Rev* 1997;77:731–758. [PubMed: 9234964]
- Rolfe DF, Hulbert AJ, Brand MD. Characteristics of mitochondrial proton leak and control of oxidative phosphorylation in the major oxygen-consuming tissues of the rat. *Biochem Biophys Acta* 1994;1188:405–416. [PubMed: 7803454]
- Schrauwen P, Hoeks J, Hesselink MK. Putative function and physiological relevance of the mitochondrial uncoupling protein-3: involvement in fatty acid metabolism? *Prog Lipid Res* 2006;45:17–41.
- Skulachev VP. Uncoupling: new approaches to an old problem of bioenergetics. *Biochem Biophys Acta* 1998;1363:100–124. [PubMed: 9507078]
- Sohal RS, Weindruch R. Oxidative stress, caloric restriction, and aging. *Science* 1996;273:59–63. [PubMed: 8658196]
- Son C, Hosoda K, Ishihara K, Bevilacqua L, Masuzaki H, Fushiki T, Harper M-E, Nakao K. Reduction of diet-induced obesity in transgenic mice overexpressing uncoupling protein 3 in skeletal muscle. *Diabetologia* 2004;47:47–54. [PubMed: 14673524]
- Speakman JR, Selman C, McLaren JS, Harper EJ. Living fast, dying when? The link between aging and energetics. *J Nutr* 2002;132:1583S–1597S. [PubMed: 12042467]
- Speakman JR, Talbot DA, Selman C, Snart S, McLaren JS, Redman P, Krol E, Jackson DM, Johnson MS, Brand MD. Uncoupled and surviving: individual mice with high metabolism have greater mitochondrial uncoupling and live longer. *Aging Cell* 2004;3:87–95. [PubMed: 15153176]

- Trenker M, Malli R, Fertschai I, Levak-Frank S, Graier WF. Uncoupling proteins 2 and 3 are fundamental for mitochondrial Ca^{2+} uniport. *Nat Cell Biol* 2007;9:445–452. [PubMed: 17351641]
- Winterbourn CC, Buss IH. Protein carbonyl measurement by enzyme-linked immunosorbent assay. *Meth Enzymol* 1999;300:106–111. [PubMed: 9919514]

Figure 1.

Proton leak kinetics in muscle mitochondria isolated from mice that overexpress UCP2/3 (Positive-TG), the non-overexpressing littermates of these mice (Negative-TG) and mice that do not have the gene for UCP3 (UCP3KO) at 8- (A), 19- (B), and 26- (C) months of age. The furthestmost point on the *right* represents maximal leak-dependent oxygen consumption and was determined by addition of saturating amounts of oligomycin. Kinetic responses of proton leak block were determined by incremental additions of malonate (0.16–1.25 mM) to the mitochondria at State 4. Each point represents the mean $\pm$ SE; $n = 8$ mice/group.

Figure 2.

Proton leak kinetics in muscle mitochondria isolated from mice fed ad-libitum (WT-Control) or calorie restricted to 70% of ad-libitum fed mice (WT-CR) at 8- (A), 19- (B), and 26- (C) months of age. The furthestmost point on the *right* represents maximal leak-dependent oxygen consumption and determined by addition of saturating amounts of oligomycin. Kinetic response of proton leak block was determined by incremental additions of malonate (0.16–1.25 mM) to the mitochondria at State 4. Each point represents the mean $\pm$ SE; $n = 8$ mice/group.

Figure 3.

Proton leak kinetics in muscle mitochondria isolated from mice that have been calorie restricted to 70% of ad-libitum fed mice at 8-, 19-, 26- and 30-months of age. The furthestmost point on the *right* represents maximal leak-dependent oxygen consumption and determined by addition of saturating amounts of oligomycin. Kinetic response of proton leak block was determined by incremental additions of malonate (0.16–1.25 mM) to the mitochondria at State 4. Each point represents the mean $\pm$ SE; $n = 8$ mice/group.

Figure 4.

T-BARSs, and protein carbonyl concentration in lower hind limb muscle of gene-manipulated mice, ad-libitum fed and calorie restricted C57BL/6J mice. Within a strain (C57BL/6J or Genetically Altered) and group (WT-Control or WT-CR; Positive-TG, Negative-TG, or UCP3KO), bars sharing or not having an upper case letter do not differ significantly (i.e., age differences). Within a strain (C57BL/6J or Genetically Altered) and age, bars sharing or not having a lower case letter do not differ significantly (i.e., group difference). MDA, malonaldehyde. Positive-TG, overexpression of UCP2/3; Negative-TG, non-overexpression littermates of Positive-TG; UCP3KO, UCP3 knockout mice; WT-Control, ad libitum fed C57BL/6J; WT-CR, calorie restricted C57BL/6J.

Table 1
Whole hind limb skeletal muscle weight (g) of female ad libitum-fed and calorie-restricted mice¹.

Age	Positive-TG	Negative-TG	UCP3KO	WT Control	WT-CR
8 mo	1.98 ± 0.06 ^A	2.06 ± 0.08 ^A	2.10 ± 0.05 ^A	1.99 ± 0.06 ^{Aa}	1.69 ± 0.04 ^{Ab}
19 mo	1.81 ± 0.04 ^A	1.90 ± 0.06 ^A	1.92 ± 0.05 ^A	1.71 ± 0.09 ^B	1.68 ± 0.07 ^A
26 mo	1.47 ± 0.07 ^{Bab}	1.32 ± 0.09 ^{Bb}	1.56 ± 0.07 ^{Ba}	1.66 ± 0.09 ^B	1.53 ± 0.05 ^B
30 mo	n/a	n/a	n/a	n/a	1.33 ± 0.09 ^C

Values are mean ± S.E.

¹Life span studies and various physiological measurements suggest that the UCP transgenic mice may constitute a different strain than the wild type mice. Thus, our statistical design evaluated possible differences in proton leak within two groupings. The two groups are separated by the vertical line and were analyzed independently of each other. Within a column and group, values sharing an upper case letter superscript do not differ significantly ($P > 0.05$). Within a row and a group, values sharing lower case letter superscripts do not differ significantly ($P > 0.05$)