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

Danny K. Asami [a], Roger B. McDonald [a,*], Kevork Hagopian [b], Barbara A. Horwitz [c], David Warman [c], Aileen Hsiao [a], Craig Warden [d], Jon J. Ramsey [b]

[a] Department of Nutrition, University of California, 1 Shields Avenue, Davis, CA 95616, USA
[b] Department of Molecular Biosciences, University of California, Davis, CA, USA
[c] Section of Neurobiology, Physiology, and Behavior, University of California, Davis, CA, USA
[d] Department of Pediatrics, University of California, Davis, CA, USA

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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.

1. 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 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 the 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

* Corresponding author. Tel.: +1 530 219 5941; fax: +1 530 752 8966.
E-mail address: rbmcdonald@ucdavis.edu (R.B. McDonald).

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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, 2005) and studies in rat skeletal muscle have reported that CR induces sustained decreases in proton leak (Bevilacqua et al., 2004, 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.

2. 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.

2.1. Experiment I: Mitochondrial proton leak

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

2.1.1. Mitochondrial isolation

Following a 10–12 h 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. (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 $MgCl_2$, 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-s bursts at 1500 rpm. The homogenate was centrifuged at 500 *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 *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 3800*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.

2.1.2. 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 KH_2PO_4, 5 mM HEPES, 2 mM $MgCl_2$, 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.

2.1.3. 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:

$$\Delta\Psi_m = 61.5 \times \log([TPMP^+_{mat}] \times a_m)/[TPMP^+_{ext}]$$

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 μM rotenone, 0.4 μg nigericin/mg mitochondrial protein, and 8 μg 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 μM $TPMP^+$, up to 5 μM 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 μM FCCP was added to dissipate the membrane potential and release all $TPMP^+$ back to the medium, allowing correction for any small electrode drift.

2.1.4. 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 μg/mg protein). Mitochondrial membrane poten-