

**Long-term caloric restriction increases UCP3 content
but decreases proton leak and reactive oxygen species production
in rat skeletal muscle mitochondria**

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Running Head: Proton Leak and ROS Production after Long-Term Calorie-Restriction

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ABSTRACT

Calorie restriction (CR) without malnutrition increases lifespan and delays the onset of a variety of diseases in a wide range of animal species. However, the mechanisms responsible for the retardation of aging with CR are poorly understood. We proposed that CR may act, in part, by inducing a hypometabolic state characterized by decreased reactive oxygen species (ROS) production and mitochondrial proton leak. Here we examine the effects of long-term CR on whole animal energetics as well as muscle mitochondrial energetics, ROS production and ROS damage. CR was initiated in male FBNF₁ rats at 6 months of age and continued for 12 or 18 months. Mean whole body oxygen consumption was 34.6% (P<0.01) and 35.6% (P<0.001) lower in CR rats than in controls after 12 and 18 months CR, respectively. Body mass-adjusted oxygen consumption was 11.1% and 29.5 % lower (both P<0.05) in the CR rats than in controls after 12 and 18 month CR. Muscle mitochondrial leak-dependent (state 4) respiration was decreased after 12 months compared to controls; however, following 18 months of CR there were slight but not statistically significant differences. Proton leak kinetics were affected by 12 months CR such that leak-dependent respiration was lower in CR mitochondria only at protonmotive force values exceeding 170mV. Mitochondrial H₂O₂ production and oxidative damage were decreased by CR at both time points and increased with age. Muscle UCP3 protein content increased with long-term CR, consistent with a role in protection from ROS, but inconsistent with the observed decrease or no change in proton leak.

Keywords: oxidative phosphorylation, oxidative stress, metabolic control analysis, aging

INTRODUCTION

Mitochondrial dysfunction is an important component of many of the pathologies associated with aging, such as type 2 diabetes mellitus, Alzheimer's disease, Parkinson's disease and some cancers (69,70). Indeed mitochondria have been implicated overall in the aging process although the mechanisms are not fully understood. One of the most widely accepted theories of aging, the oxidative stress theory, suggests that the aging process involves the accumulation of oxidative damage to mitochondria and other cellular components. Oxidative damage is induced by reactive oxygen species (ROS) produced primarily as a by-product of mitochondrial oxidative phosphorylation (24,65), a process that is responsible for ~85-90% of cellular oxygen consumption (1). As mitochondrial ROS can cause damage to mitochondrial DNA, proteins and membrane lipids, a self-perpetuating and destructive cycle can ensue in which increased ROS production leads to incremental damage and further ROS production. (25,28,51)

Calorie restriction (CR), without malnutrition, is a well known dietary intervention that consistently increases lifespan by delaying the aging process in a wide-variety of animal species (47,69,70). The mechanisms underlying aging retardation by CR are poorly understood. However, it has been suggested that they may involve a decrease in cellular oxygen consumption and ROS production (57). The role that hypometabolism may play in the actions of CR is unclear as several studies on whole-body, organ-specific and cellular oxygen consumption have yielded contradictory results (57). Other studies have demonstrated that CR mitigates age-associated increases in cellular ROS production and damage to cellular macromolecules in various tissues (2, 41,72).

Our laboratories have investigated the effects of CR on mitochondrial energetics and have focused on the role of mitochondrial proton leak (2, 27,36), a process that is responsible for 20-30% of resting cellular energy expenditure (8,60). The mechanism for mitochondrial proton leak itself is not well understood. However, the lipid composition of the mitochondrial inner membrane and the contribution of uncoupling proteins (UCPs) may be important, although recent literature suggests a diminished role of UCPs in proton leak (20,31,49,62,67). In many correlative studies proton leak has been associated with differences in thyroid hormone status, phylogeny, body size, membrane lipid composition, lipid peroxidation and aging (10,12,13,23,26,27,36,57,61).

Previous findings have demonstrated that proton leak is increased with age in intact hepatocytes (27). These findings were consistent with reports from other groups documenting aging-induced decreases in mitochondrial protonmotive force and increases in ROS production and damage in intact hepatocytes (23,61). In the post-mitotic tissue, skeletal muscle, we recently demonstrated that proton leak is altered with CR of short- and medium- term durations (2). Interestingly, short-term CR (2 wks and 2 months) resulted in a decrease in maximum proton leak-dependent (state 4) respiration concomitant with decreases in mitochondrial membrane potential. Following medium- term CR (6 months), maximum proton leak-dependent respiration decreased while mitochondrial membrane potential increased (2), indicating some intriguing differences in the response of muscle mitochondrial protonmotive force between short- and medium- term CR regimens. Mitochondrial H₂O₂ production was significantly decreased by CR of both short- and medium-term durations (2).

Therefore the aim of this study was to focus on the effects of long-term CR (12 months and 18 months), and to determine how the bioenergetic adaptations differed from those observed

after short- and medium term CR. The tissue of interest was again skeletal muscle as it is a post-mitotic tissue that is highly susceptible to the cumulative effects of oxidative stress (64). Assessments included whole body energy expenditure (by indirect calorimetry); characteristics of muscle mitochondrial oxidative phosphorylation; UCP3 content; H₂O₂ production, and ROS damage.

EXPERIMENTAL PROCEDURES

Treatment of animals. 4-mo-old male FBNF₁ rats were purchased from the National Institute on Aging (NIA) Aging Rodent Colony (Bethesda, MD). These rats are the F1 generation of the cross between Fisher 344 rats and Brown Norway rats. Rats were initially housed at the University of California (Davis) and allowed *ad libitum* intake of AIN-93M purified, defined diet (BioServ; Frenchtown, NJ). Mean food intakes were then measured and used to determine the amount of food to give the animals during the dietary intervention phase. At 6 months of age, rats were randomly assigned into control or CR treatment groups (n = 6-7 rats/ group). To prevent the development of obesity, control rats were restricted to 95% of *ad libitum* intake of AIN-93M purified defined diet, as previously described (2). The CR rats were given 60% of the energy intake of the *ad libitum* fed control rats (2). To avoid any nutrient deficiencies in CR rats, the carbohydrate component of the CR diet was decreased to increase the vitamin and mineral components. All animals were given free access to water, and were housed at 23°C with light 0700 to 1900. Approximately two months prior to the experimental endpoints the control and CR rats were shipped to the University of Ottawa. Upon arrival, rats were given a 3 week adaptation period in which food intake and body weights were measured daily. All studies were

conducted in accordance with the Canadian Council on Animal Care and the Institute of Laboratory Animal Resources (National Research Council, Washington, DC).

Indirect calorimetry. Whole body oxygen consumption and characteristics of fuel oxidation were assessed using an open-circuit indirect calorimeter (customized Oxymax system; Columbus Instruments, Columbus, OH). Two to three days prior to sacrifice, rats were placed in respiration chambers (11.7 liters / chamber). Data were recorded over a 24-h period with temperature maintained at 23°C, with light 0700 to 1900. The data set from each individual rat was then plotted and analyzed using the percent relative cumulative frequency (PCRF) approach as previously described (2,59). Briefly, the PCRF approach is used in the analysis of large data sets such as those collected by indirect calorimetry. The curves from each individual rat were compared statistically using the 50th percentile values and the curve slope (H values) (2,59).

Isolation of mitochondria from skeletal muscle. Animals were sacrificed after 12 months or 18 months of CR (they were thus 18 and 24 months of age, respectively). Hindlimb skeletal muscle mitochondria were isolated as previously described (2), using a modified method of Bhattacharya *et al* (4). In brief, freshly dissected muscle was placed in ice-cold isolation medium (10mM EDTA, 100mM Tris-HCl and 46mM KCl, pH 7.4), and visible connective tissue and fat were removed. Muscle was minced finely and placed in ice-cold isolation medium containing 0.5% (wt/vol) bovine serum albumin (BSA). The slurry was filtered through 100- μ m Nitex mesh and the filtrate was incubated in isolation medium containing 20% (wt/vol) Nagarse for 2 min with occasional stirring. This was then homogenized using an ice-cold glass/Teflon Potter-Elvehjem tissue grinder and fractionated by centrifugation at 484g (10 min, 4°C). The

supernatant was re-spun at 12,000 g (10 min, 4°C) twice. The final pellet was resuspended in ice-cold suspension buffer (120 mM KCl, 20 mM sucrose, 10 mM KH₂PO₄, 5.0 mM HEPES, 2.0 mM MgCl₂, 1.0 mM EDTA; pH 7.2 with KOH). A modified Lowry method was used to determine protein concentration with BSA as the standard.

Measurement of mitochondrial oxygen consumption. A Hansatech Clark-type oxygen electrode (Norfolk, UK) was used to measure mitochondrial oxygen consumption (0.5 mg/mL; 37°C). For assessments of complex II -driven respiration 5μM rotenone was used to block complex I. State 3 (maximum phosphorylating) respiration was determined in the presence of 10mM succinate, 0.65U/ml of hexokinase, and 100μM ADP/ATP. State 4 (non-phosphorylating, or maximal leak-dependent respiration) was determined following the addition of oligomycin (12μg/mg protein). Proton leak kinetics were assessed thereafter with incremental additions of malonate (0.3-10mM).

Measurement of mitochondrial protonmotive force. A methyltriphenylphosphonium (TPMP⁺)-sensitive electrode was used to assess mitochondrial protonmotive force (Δp). The electrode was constructed and used as previously described (2,34). All measurements of Δp were done in duplicate and simultaneous to oxygen consumption determinations. Nigericin (0.4ug/ml) was added to convert the pH component of Δp into mV units so that Δp could be measured in mV units.

Top-down metabolic control analysis. To determine if long-term CR altered the distribution of control within the oxidative phosphorylation system, we applied top-down metabolic control

analysis (2,8,26). Briefly, the oxidative phosphorylation system was studied as three blocks of reactions centered on the common intermediate, protonmotive force (Δp). The three blocks included reactions that produce Δp (substrate oxidation reactions) and those that use Δp (phosphorylation reactions and proton leak reactions). For each of the three subsystems, specific inhibitors were used to determine the kinetic response of changes in Δp and oxygen consumption. The kinetic response of proton leak to changes in Δp was measured using saturating amounts of oligomycin followed by incremental additions of malonate. The kinetic response of the substrate oxidation reactions were determined by titrating State 3 respiration with incremental additions of oligomycin. Finally, the kinetic response of the phosphorylation reactions was determined by titrating State 3 respiration with incremental amounts of malonate and corrections for leak-dependent oxygen consumption were made (8,26).

H₂O₂ production. Mitochondrial H₂O₂ production was determined using the p-hydroxyphenylacetate (PHPA) fluorometric assay (33). Freshly isolated mitochondria (0.25mg/ml) were incubated in 10mM potassium phosphate buffer (pH 7.4, containing 154mM KCL, 0.1mM EGTA, and 3mM MgCl₂), with 500ug PHPA, 4 U horseradish peroxidase, 5mM rotenone and 10mM succinate. H₂O₂ production was monitored over 10min using a miniFluorimeter (Hoefer, San Francisco, CA). Fluorescence readings were converted to H₂O₂ production rates using a standard curve.

Western blots of UCP3. Seventy-five micrograms of mitochondrial protein were loaded into each lane of a BioRad minigel system. As antibodies for the novel UCPs, including those for UCP3, often react with other mitochondrial proteins, we included both positive and negative

controls in all blots. Muscle mitochondrial protein from UCP3^{-/-} mice were used as the negative control and recombinant murine UCP3 (prepared in our laboratory) was used as the positive control. Molecular mass markers were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The primary antibody was purchased from Chemicon (UCP3, AB-3046; Tecmecula, CA) and was used at a 1:1000 dilution. The secondary antibody was a peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz, CA) and was used at a 1:500 dilution. For detection, blots were processed using enhanced chemiluminescence kits (Amersham Pharmacia; Baie d'Urfe, QC, Canada). Three Western blots were performed per mitochondrial sample from each of four rats in each group.

Determination of lipid peroxidation. Mitochondrial lipid peroxidation was determined through assaying thiobarbituric acid reactive substances (TBARS) (14). This method measures the quantity of malondialdehyde-thiobarbituric acid adducts (see Discussion). Briefly, sucrose (which interferes with the assay) was removed from the isolated mitochondria through centrifugation washes (12,000g, 3min, 4°C). Mitochondria were resuspended in 0.1M potassium phosphate buffer (pH 7.4). Samples were mixed with butylated hydroxytoluene (BHT) to prevent formation of artifactual peroxidized lipids during the assay. Absorbance was read at 535nm and a molar extinction coefficient of $1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ was used.

Statistical analysis. Group comparisons were made using a one-way ANOVA with Tukey's post hoc tests (Prism 4; Graph Pad, San Diego, CA). Statistical significance was defined as a P value <0.05. All results are presented as mean +/- standard error of the mean.

RESULTS

Body and organ weights. Body and organ weights are summarized in Table 1. Following 12 months of CR, there was a 35.7% decrease ($P<0.01$) in mean body weight between the CR and the age-matched control groups. There were decreases in all measured organ weights following 12 month of CR. Liver weight decreased to the greatest extent, with a 44.3% decrease ($P<0.01$). Thereafter, the greatest proportional decreases were in the kidneys and heart, which decreased by 26.9% ($P<0.01$) and 20.7% ($P<0.001$), respectively. As a crude indicator of adiposity we assessed epididymal adipose tissue (EWAT) weights. EWAT weight decreased 78.8% ($P<0.001$) following 12 months of CR.

Following 18 months of CR, animals had smaller livers, kidneys and hearts; decreases were 45.5% ($P<0.001$), 31.6% ($P<0.001$), and 29.1% ($P<0.001$), respectively. After 18 months of CR the decrease in EWAT weight was 75.6% ($P<0.001$), similar to that following 12 months of CR. There were no age-related changes in body weight, or in tissue weights between the control rats at the 12 and 18 months time points.

Whole body oxygen consumption and respiratory exchange ratios. Results were collected over 24-h time periods (Figures 1 and 2) and were plotted using a PRCF approach previously described (40,59). This approach allows quantitative comparisons of large sets of indirect calorimetry data, and involves sorting all data in ascending order, calculating their cumulative frequency, and expressing the frequencies in the form of percentile curves. The results collected over a 24 hour period were normally distributed, thus the 50th percentile values represent mean values. Mean whole body oxygen consumption was 34.6% ($P<0.01$) and 35.6% ($P<0.001$) lower

in CR rats than in respective controls at the 12 months and 18 months CR time points (Figure 1: A, C), and this was equivalent to the magnitude of CR between groups.

Reductions in total body oxygen consumption were also observed with CR when the data was normalized for body weight. Adjusted oxygen consumption (per gram body weight) (Figure 1: B, D) was 11.1% and 29.5% lower ($P<0.05$) in the CR rats than in controls at the 12 month or 18 month CR time points. The age-related change in total body oxygen consumption was not observed when the data were normalized for body weight. The equations describing the characteristics of PRCF curves (50th percentile value and the slopes of the curves) are provided for each curve in Figure 1.

The mean RER decreased in the CR rats compared to controls after 12 and 18 months of CR, respectively (Figure 2). We also observed an upward shift in RER values with age (18 vs 24 month old controls; ($P<0.05$)) consistent with an increase in carbohydrate oxidation with age.

H₂O₂ production. At the 12 month and 18 month CR time points, H₂O₂ production of muscle mitochondria of CR rats decreased 51% ($P<0.01$) and 49% ($P<0.01$), respectively, compared to controls (Figure 3). We also observed an age-related increase in the rate of H₂O₂ production; it was 18% higher ($P<0.01$) in the 24 month old compared to the 18 month old control rats.

Kinetics of proton leak, substrate oxidation, and phosphorylation reactions. The overall kinetics of proton leak in muscle mitochondria are summarized in Figure 4. The furthestmost point on the right of each curve represents state 4 respiration (maximal proton leak-dependent respiration). At the 12 month CR assessment point the CR rats had a 40% lower ($P<0.001$) maximal proton leak-dependent respiration compared to the control-fed rats. The kinetics of

proton leak were affected by 12 month CR such that leak-dependent respiration was lower in the CR mitochondria at Δp values exceeding 170mV, but not below this Δp value. After 18 months of CR, there were no differences ($P>0.05$) in maximal leak-dependent respiration rates between CR and control rats.

The overall kinetics of the substrate oxidation reactions for the 12 and 18 month CR time points are summarized in Figure 5. Figure 5 demonstrates that CR decreases the steady state oxygen consumption by changing the kinetic response of the Δp producers (substrate oxidation reactions). In other words, following CR, the amount of oxygen required to support substrate oxidation reactions at a given value of Δp is lower than in controls. State 3 oxygen consumption or slope of the line was not significantly ($P>0.05$) different between mitochondria from the 12 month CR rats and their control counterparts. Whereas, at 18 month CR we did observe a significant decrease in State 3 oxygen consumption compared to control rats ($P<0.001$). The overall kinetics (*i.e.*, the slopes of the lines) did not significantly change following 18 months CR. However, at both the 12 and 18 month CR time points, the amount of oxygen used to support the substrate oxidation reactions at any Δp value was lower in mitochondria of CR rats, compared to controls. Moreover, there were no age-related differences in State 3 oxygen consumption, but the slopes of the lines (representing the kinetics of the substrate oxidation reactions) significantly differed ($P<0.01$) with age.

Figure 6 summarizes the overall kinetics of the phosphorylation reactions. There were no differences ($P>0.05$) in kinetics between the CR and the control-fed rats at either the 12 or the 18 month assessment points. Moreover, there were no age-related differences in the overall kinetics of the phosphorylation reactions (*i.e.*, between control fed rats at the 12 and 18 month time points).

Metabolic control analysis. Tables 2 and 3 summarize the results of the metabolic control analyses, and include results of the flux control coefficients and concentration control coefficients for each of the three blocks of reactions in oxidative phosphorylation.

At 12 months, the only substantive shift in control by proton leak over the three branches of the oxidative phosphorylation system appears to be the increased control over phosphorylation reactions. The decreased control over proton leak reactions by substrate oxidation reactions and the increased control over phosphorylation flux by the phosphorylation reactions however also are substantial. At 18 months, shifts in the control by the substrate oxidation and the proton leak reactions over the flux through all blocks of reactions are similar. During state 4 respiration, there were no consistent CR-induced changes in the control of the oxidative phosphorylation system.

Concentration control coefficient results (bottom of Tables 2 and 3) demonstrate that Δp is primarily controlled by substrate oxidation reactions under state 3 conditions. CR did not result in any consistent changes in the distribution of control over Δp . Under state 4 conditions control is balanced between substrate oxidation and leak pathways and again there were no consistent changes in the distribution of control over Δp following 12 and 18 months of CR. In the control group, the increase in magnitude of the concentration control coefficients indicates that the protonmotive force becomes more sensitive to changes in substrate oxidation or phosphorylation system fluxes with age.

Lipid peroxidation. Following 12 months of CR there was a 46% decrease in mitochondrial lipid peroxidation, compared to controls ($P < 0.05$; Figure 7). 18 months of CR resulted in a 52%

decrease in peroxidation ($P<0.01$). Moreover, with regard to age-related changes in mitochondrial lipid damage, there was a 24.4% increase in lipid peroxidation in the 24 month old compared to the 18 month old control rats ($P<0.05$)

Western Blots. UCP3 protein content was increased in muscle mitochondria at both 12 and 18 month CR time points, compared to controls. A representative immunoblot is shown in Figure 8. UCP3 protein content was also consistently lower in the 24 month old control fed rats when compared to the 18 month control rats.

DISCUSSION

The objective of the current study was to determine the effects of long-term CR on mitochondrial proton leak, ROS production and UCP3 content in muscle. Although, several studies have investigated the effect of long-term CR on mitochondrial proton leak and ROS production, the above parameters have not as yet been assessed in parallel. Thus, the results presented herein are the first to provide direct evidence of the effects of long-term CR on these parameters. The findings of the current study are also informative in light of our previous findings on the effects of short-, and medium-term CR (2). CR has also been documented to have variable effects depending on the degree of restriction and tissue-specific effects. The current studies were conducted under the same conditions as those used to study the effects of short- and medium- term CR (2) and thus direct comparisons are valid.

Our previous studies demonstrated that short- and medium- term CR caused decreased maximal leak-dependent (State 4) oxygen consumption in rat muscle mitochondria (2), and as such the findings are overall consistent with the hypothesized role for decreased leak in the

induction of a CR-induced hypometabolic state and an extended lifespan. However, the findings indicated that the changes in leak kinetics were complex and dependent upon the duration of CR. In particular, CR of shorter duration led to significant reductions in both State 4 oxygen consumption and Δp , whereas following medium-term CR, State 4 oxygen consumption was decreased and Δp was increased. Results from metabolic control analyses corroborated these findings (*i.e.*, that control patterns respond differently to CR of short- and medium- term durations). In these same studies we also observed rapid (following 2 wk CR) and sustained (following 6 months) decreases in muscle mitochondrial H_2O_2 production due to CR. Thus results were indeed consistent with the notion that decreased oxygen consumption and ROS production are possible mechanisms behind the retardation of aging through CR. In the current study, we extended our analyses to the effects of long-term CR.

To test our hypothesis that the actions of CR are mediated through a reduction in oxygen consumption, we began by measuring whole body energy expenditure (Figure 1). Whole body oxygen consumption rates were lower in CR rats than in control rats at both the 12 months and 18 months CR time points. When the oxygen consumption data were normalized for body weight, similar effects of CR were observed, the only substantive difference was that normalization to body weight eliminated any age-related increase in oxygen consumption.

Our previous studies of short- and medium- term CR demonstrated that mass-adjusted oxygen consumption was not significantly affected by 2 weeks and 2 months of CR (2). It was not until after 6 months of CR that we observed a 40% decrease in mass-adjusted oxygen consumption ($p < 0.05$). Overall, our latest results are consistent with those of Dulloo and Girardier (19) and Gonzales-Pacheco *et al* (21) who studied the effects of 6 weeks of a 40% CR regimen on whole-body energy metabolism and demonstrated CR-induced decreases in mass-

adjusted oxygen consumption in the range of 14-18%. However, several other studies have demonstrated no changes in mass-adjusted oxygen consumption (44,45). Inconsistencies in the effects of CR on mass-adjusted oxygen consumption have also been observed in rhesus monkeys (5,37,38,56). Differences in the effects of CR upon whole body oxygen consumption may arise from differences in methods used to measure oxygen consumption or energy expenditure; differences in methods to normalize data; differences in the extent of the CR, and differences in the ages of the species studied, amongst other factors. Moreover, several assumptions are made when normalizing and analyzing indirect calorimetry data. As we have demonstrated here, the changes in the rates of tissue weight loss with CR vary from tissue to tissue, and the oxygen demands vary considerably from tissue to tissue (2,30,58). Our results show that the liver weight decreased to the greatest extent, followed by the kidney and heart. As expected EWAT weight (a crude indicator of adiposity) was also substantially reduced by CR. Degree of adiposity clearly also affects the interpretation of mass-adjusted oxygen consumption. Thus while indirect calorimetry is a useful tool in studies of the importance of hypometabolism in the actions of CR, the results must be interpreted with caution. The latter challenges associated with whole body studies highlight the importance of cellular and subcellular studies of the metabolic effects of CR.

There are many cellular pathways that contribute to resting energy expenditure including proton leak, $\text{Na}^+\text{K}^+\text{ATPase}$, and protein turnover (60). In this study we focused on mitochondrial proton leak because it is an important contributor to resting cellular energy expenditure (8,57,60). Proton leak is a process estimated to account for 20-30% of resting energy expenditure (9,60). Leak is also estimated to be responsible for up to 50% of the resting oxygen consumption of hind limb muscle in rats (60). However, the latter proportion has recently been

called into question based on ^{31}P magnetic resonance and optical spectroscopy approaches (17,42,43), which also include a number of assumptions, involving effects of ischemia, and metabolite compartmentalization. Further studies are required to assess the proportion of leak-dependent respiration in intact muscle cells and tissue, and are underway in our laboratory.

Ramsey *et al.*, (57) suggested that proton leak may be central to aging retardation by CR. Further it was proposed that CR induces a decrease in leak, followed by decreases in mitochondrial ROS production and oxidative damage. Evidence for this hypothesis accrues mainly from correlative data in studies of aging, and the negative correlations between proton leak and lifespan (54). Proton leak is also negatively correlated to mammalian body size (54), and with mitochondrial membrane linoleic acid composition (10,28). It is positively correlated with oxidative stress (65), with thyroid hormone status (26), and with mitochondrial membrane docosahexanoic acid composition (10,12).

To test the hypothesis proposed by Ramsey *et al.*, we measured proton leak in isolated mitochondria from long-term CR rats compared to control fed rats. We demonstrated that maximum leak-dependant respiration was 40% lower ($P < 0.001$) with 12 months of CR, compared to the control-fed rats at Δp values of over 170mV. This is consistent with the hypothesis, and with the results of short- and medium- term CR studies (2). After 18 months of CR, maximal leak-dependent respiration rates were slightly, but consistently lower, in the CR rats and their control counterparts. The observation of only a slight decrease in respiration was unexpected, given that previous findings from our laboratory have also demonstrated that maximal leak-dependent respiration was lower in skeletal muscle mitochondria from 33 month old rats following a 23 month 33% CR regimen, compared to age-matched controls (36). Differences in the results between these two studies may be related to the different strains of rat

involved, or to the different lengths of CR periods. Previous studies in our laboratory also demonstrated in isolated hepatocytes from 30 month old mice compared with 3 month old rats an age-related increase in proton leak and decrease in ATP production (27).

This is the first report on the effects of long-term CR on changes in the control of oxidative phosphorylation in skeletal muscle. Our metabolic control analysis demonstrated a switch toward a lesser control by proton leak over substrate oxidation and phosphorylation reactions under state 3 conditions following 12 months of CR. This is similar to our previous study of short-and medium- term CR demonstrated that the control of proton leak over the oxidative phosphorylation subsystems under state 3 conditions was decreased with CR. .

Consistent with our findings on the effects of short- and medium- term CR we found that long-term CR also decreased the rate of mitochondrial H_2O_2 production. Interestingly, we also observed an age-related increase in the rate of H_2O_2 production; it was 18% higher ($P<0.01$) in the 24 month old control rats compared to the 18 month old control rats. These results are consistent with the oxidative stress theory of aging (65) and with previous studies in heart (40% CR for 1 year) and *gastrocnemius* muscle (40% CR for 21 months), which demonstrated decreased H_2O_2 production (18,22). Studies in liver mitochondria have shown mixed results; long-term CR resulted in decreased H_2O_2 production whereas no difference in H_2O_2 production was observed following short- or medium- term CR (40% CR for 1 or 6 months) (58). These inconsistencies may be due to differences in tissue type (*i.e.*, mitotic, liver, *vs.* post mitotic, skeletal muscle and heart).

The findings presented here demonstrate not only increased ROS production with aging and its mitigation with CR, but also decreased mitochondrial lipid peroxidation with long-term (18 mo) CR. The limitations of the TBARS assay are that it is not specific for any one class of

peroxidation products and many compounds can interfere with the assay (6). However, this approach is thought to provide a good approximation of total malondialdehyde (MDA). Several other groups (52,55,72) have documented CR-induced decreases in mitochondrial lipid and protein oxidation in a variety of tissues. Zainal et al., (72) examined *vastus lateralis* muscle of 17-23 month old rhesus monkeys following 10 months of 30% CR. They found an age-related increase in protein carbonyls and HNE-modified proteins. They also observed an effect of CR oxidative damage was lower in muscle of CR monkeys compared to age-matched controls (72).

In vitro studies into the effects of uncoupling agents upon ROS production in isolated mitochondria have demonstrated that uncoupling can decrease ROS production (11,48). Moreover the recent studies of Speakman *et al* (66) have suggested that proton leak and augmented basal metabolism may protect from ROS production and aging. Speakman *et al* used three different normalization methods to test the associations between energy metabolism and longevity. They found that mice with high metabolism were more uncoupled, had greater energy metabolism, lived the longest and supported the ‘uncoupling to survive’ hypothesis (11,66). It is possible that the mice having high metabolic rates are more active, or that they have a greater preference for lipids as a fuel source, and that these factors relate to protection from aging. Regardless, the findings are incongruent with a large body of *in vivo* literature supporting an indirect relationship between proton leak and lifespan. Studies from our laboratory have demonstrated that proton leak is associated with aging (2,27,36). Whereas other studies have suggested that uncoupling is associated with a decrease in ROS production (11,48). Furthermore, UCPs have also been suggested to have a role in protection from ROS (20). The findings herein demonstrate that aging is associated with decreases in muscle mitochondrial UCP3, and that CR is associated with increased levels of UCP3. UCP3 gene expression in white

adipose tissue increased 2 fold after 9 months of long-term CR (29). These findings, in conjunction with our demonstrated age-induced increases, and the CR-induced decreases in H₂O₂ production, are thus consistent with a role for UCP3 in the protection from oxidative stress and aging. Following long-term CR others have documented increased UCP3 mRNA in heart (39), and these findings are consistent with our observed changes in UCP3 protein.

UCP3 has been suggested as a putative regulator of proton leak. Our results have demonstrated that short- and medium- term CR (2) and long-term CR (this paper) result in significant increases in UCP3 protein levels, but no corresponding increases in proton leak. In fact, our findings overall demonstrate that CR prevents aging-induced increases in proton leak (27,36,57). Moreover previous reports from our laboratory and others have clearly shown that increased UCP3 content in muscle does not result in alterations in proton leak (3,15). Our results are thus consistent with the idea that proton leak is not a function of UCP3 level, but could be related more to the extent of oxidative damage to the membrane (57). Thus our results demonstrating increased UCP3 content are consistent with the suggested role of UCP3 in decreasing ROS production (68) and facilitating increased rates of fatty acid oxidation (31).

When interpreting the results the potential importance of altered physical activity levels should be considered. Others have examined the influence of exercise in relation to CR and age and found that the beneficial effects of exercise were more effective in combination with CR (35,46). Although we did not assess the physical activity patterns in this study, it is possible that CR resulted in increased activity and the synergistic effects of physical activity and CR played a role in our findings.

Recent studies have demonstrated that the beneficial effects of CR have involve increases in content and activity of the mammalian Sir2 (SIRT1) protein in rats and human cells (16,32). Specifically, CR has been shown to induce the expression of SIRT1, resulting in the deacylation of Ku70 (a DNA repair factor which sequesters the proapoptotic factor, BAX), and in turn, resulting in decreased stress-induced apoptotic cell death. In addition, it was proposed that SIRT1 induction improves cell survival by increasing cellular stress response and repair mechanisms (16). Upregulation of SIRT1 is a thus thought to be a key mechanism induced by CR to alter the balance between cellular death and survival (16).

Our findings therefore corroborate and significantly extend earlier results on the effects of short-and medium-term CR, and are consistent overall with the idea that the metabolic mechanisms at the muscle mitochondrial level that come into play following CR depend largely upon length of CR. Further studies into the mechanism of action of UCP3 and into the mechanisms underlying proton leak are warranted.

ACKNOWLEDGEMENTS

The work was supported by the **National Institute on Aging Grant RO1 AG-17902**. The authors thank Dr. Martin Gerrits for his valuable comments.

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FIGURE LEGENDS

Figure 1: Percent relative cumulative frequency (PRCF) plots of whole body (A and C) and mass-adjusted (B and D) oxygen consumption for control and CR rats (n=6rats/group). Panel A and B: 12 month study; Panel C and D: 18month study. For each panel, the top graph represents whole body oxygen consumption (*i.e.*, per rat) and curve on the bottom represents mass adjusted oxygen consumption (*i.e.*, per gram body weight). For each graph the dashed line represents data from CR rats and the solid line represents data from control rats.

Figure 2: Percent relative cumulative frequency (PRCF) plots of the respiratory exchange ratios for control and CR Rats (n=6rats/group). Panel A: 12 month study; Panel B: 18 month study. For each graph the dashed line represents data from CR rats and the solid line represents data from control rats.

Figure 3: The effect of CR on H₂O₂ production in muscle mitochondria. Oxidant production was measured using a fluorometric PHPA assay (33). The results shown are expressed as means \pm SEM of 6 rats in each group. CR results are depicted by solid bars and controls by open bars. Statistical significance was determined using one-way ANOVA with a Tukey's post-hoc test. * indicates a statistically significant difference at the level $p<0.05$ and *** $p<0.001$.

Figure 4: Overall kinetics of proton leak reactions in muscle mitochondria from control (■) and CR (▲)rats following 12 months (A) and 18 months (B) of CR. The furthestmost point on the right represents maximal leak-dependent (State 4) oxygen consumption and was determined by addition of saturating amounts of oligomycin. The kinetic response of the proton leak block was determined by incremental additions of malonate (0.33-1.0mM) to the mitochondria at State 4. Each point represents the mean \pm SEM of duplicate experiments with 6 rats in each group.

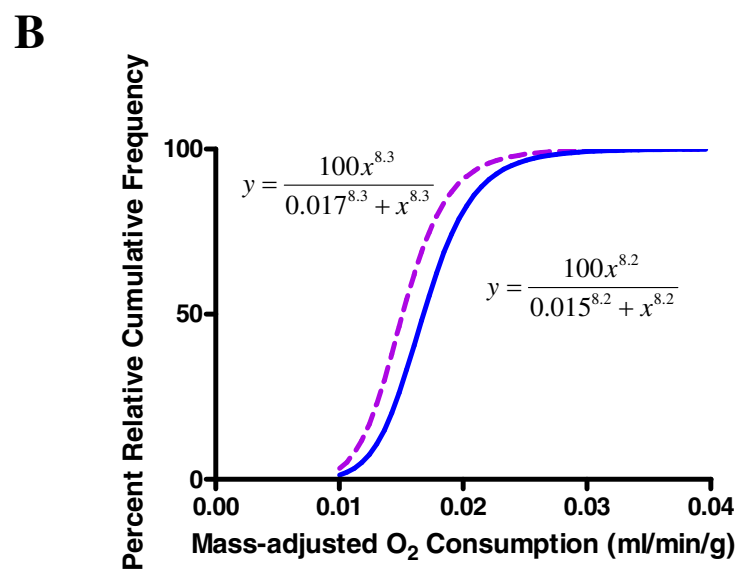
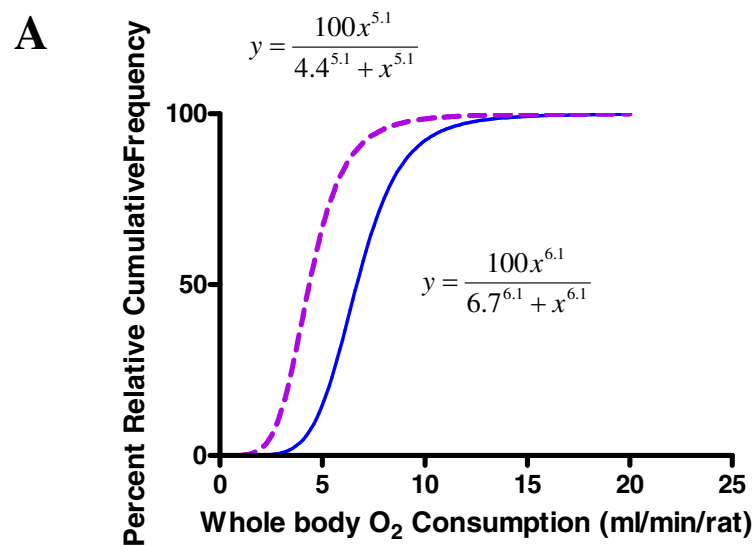
Figure 5: Overall kinetics of substrate oxidation reactions in muscle mitochondria of control (■) and CR (▲) rats following 12 months (A) and 18 months (B) of CR. The kinetic response of the substrate oxidation block was determined by titration of State 3 respiration with incremental additions of oligomycin. Each point represents the mean \pm SEM of duplicate experiments with 6 rats in each group.

Figure 6: Overall kinetics of phosphorylation reactions in muscle mitochondria of control (■) and CR (▲) rats following 12 months (A) and 18 months (B) of CR. The kinetic response of this block was determined by titration of State 3 respiration with incremental additions of malonate. Each point represents the mean \pm SEM of duplicate experiments with 6 rats in each group.

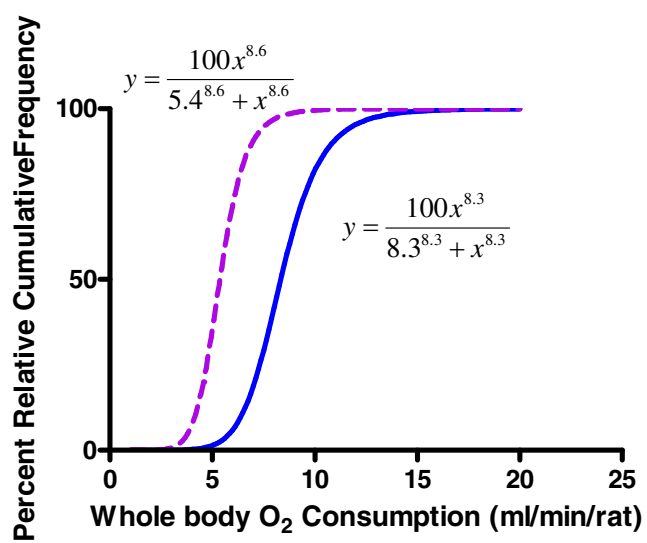
Figure 7: Lipid peroxidation content in muscle mitochondria. CR results are depicted by solid bars and controls by open bars. Statistical significance was determined using one-way ANOVA with a Tukey's post-hoc test. ** indicates a statistically significant difference at the level $p<0.01$.

Figure 8: Representative Western blot of muscle mitochondrial UCP3. Each lane was loaded with 75 ug of mitochondrial protein from 12 and 18 months CR rats and controls. Recombinant mouse UCP3 served as a positive control; it migrates at a molecular weight of 39 kDa, rather than 34 kDa, due to a 5 kDa fusion protein. Mitochondrial protein from UCP3 (-/-) mice served as a negative control.

Figure 1



C



D

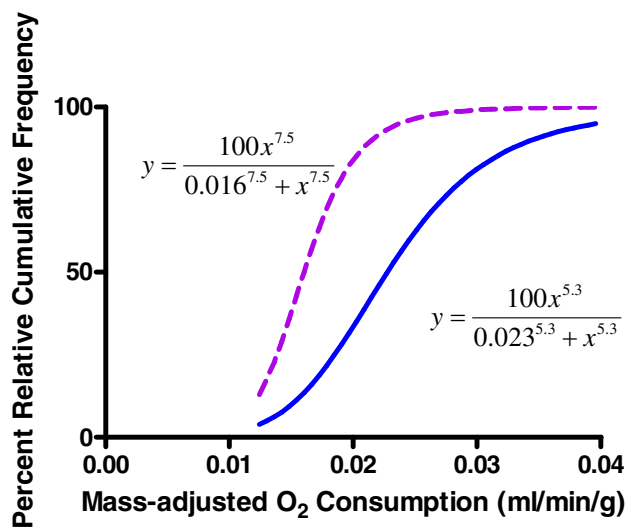
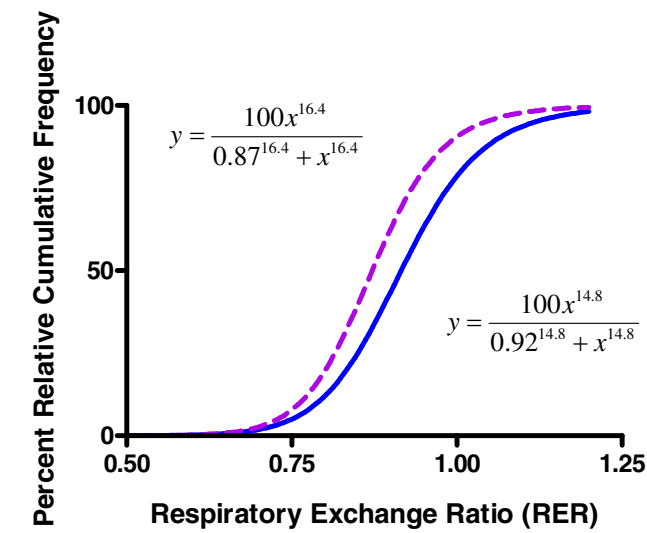


Figure 2

A



B

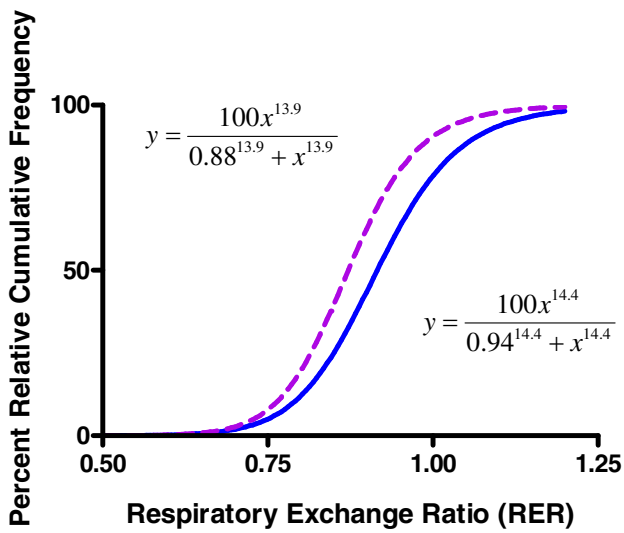


Figure 3

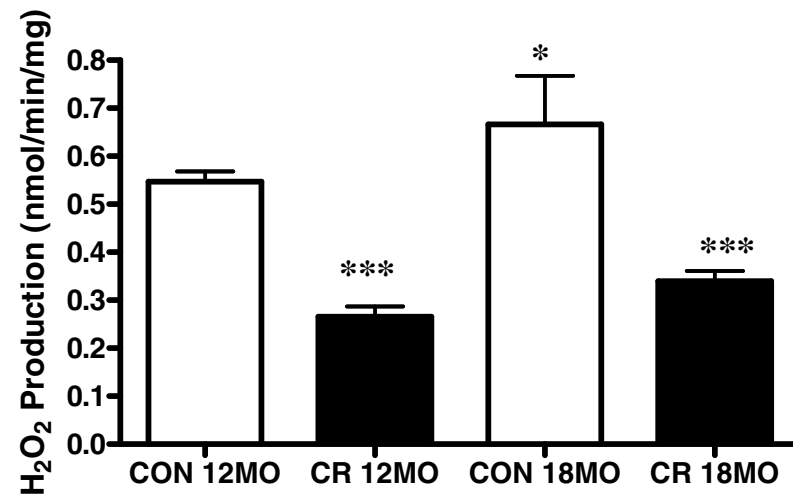
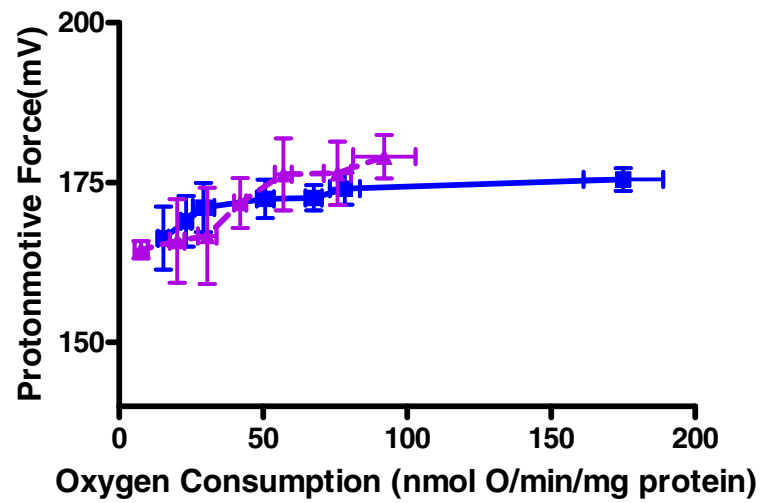


Figure 4

A



B

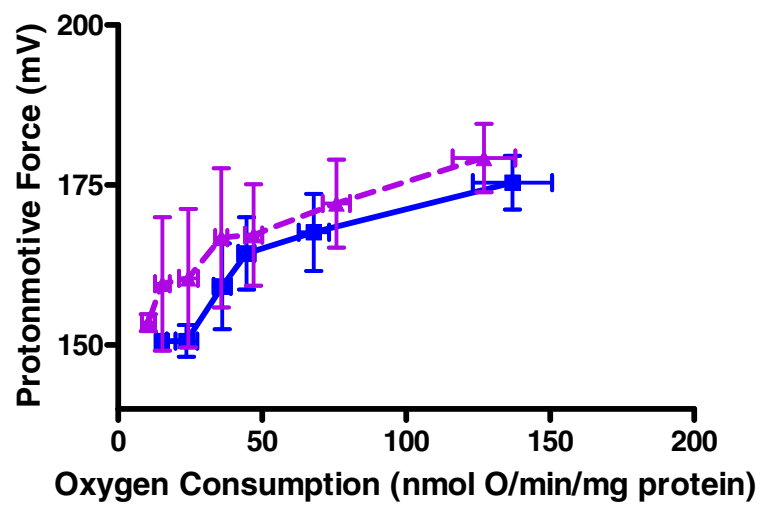
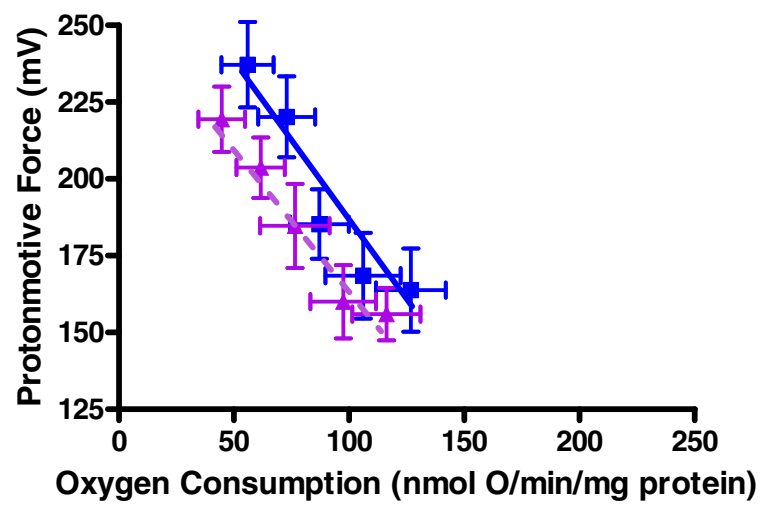


Figure 5

A



B

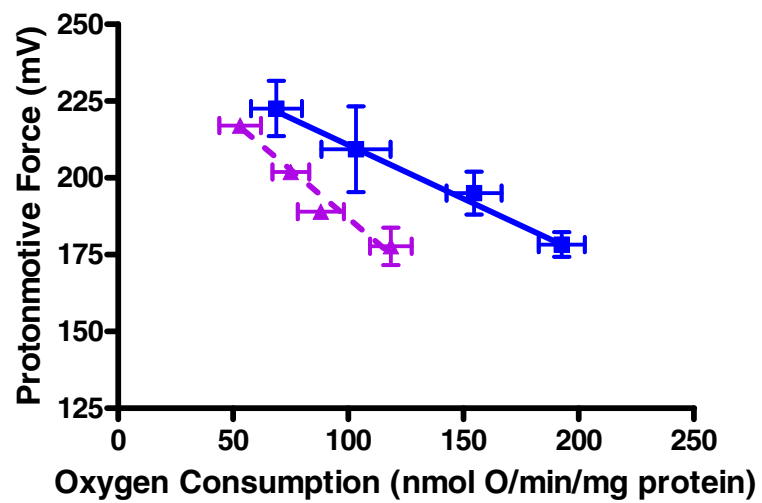
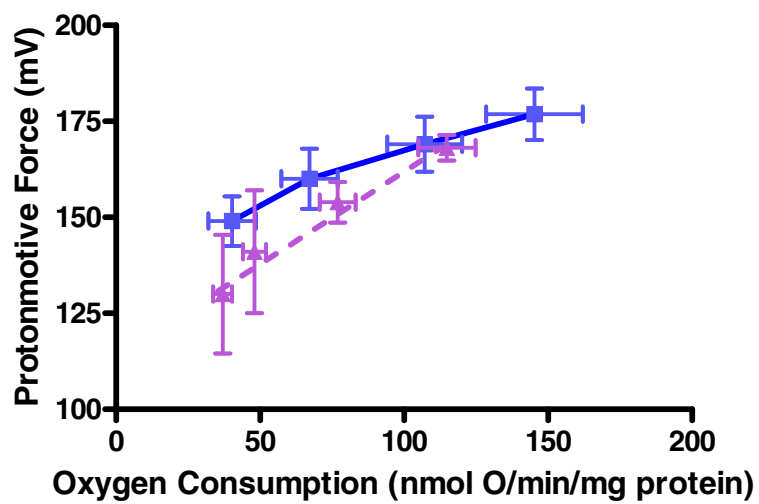


Figure 6

A



B

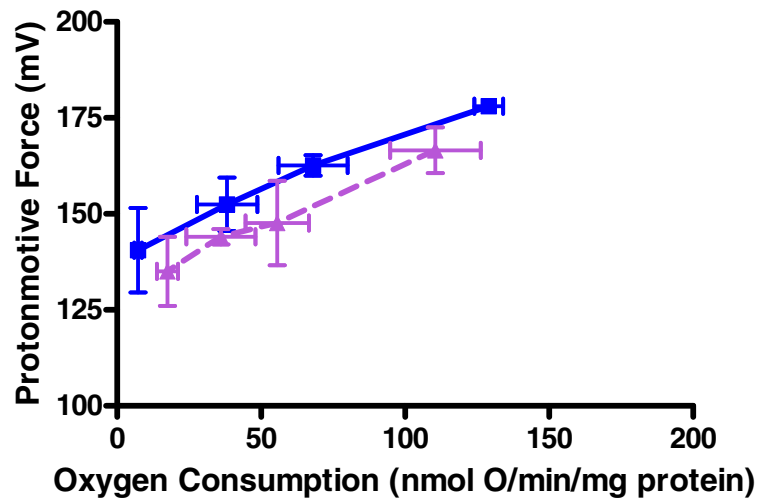


Figure 7

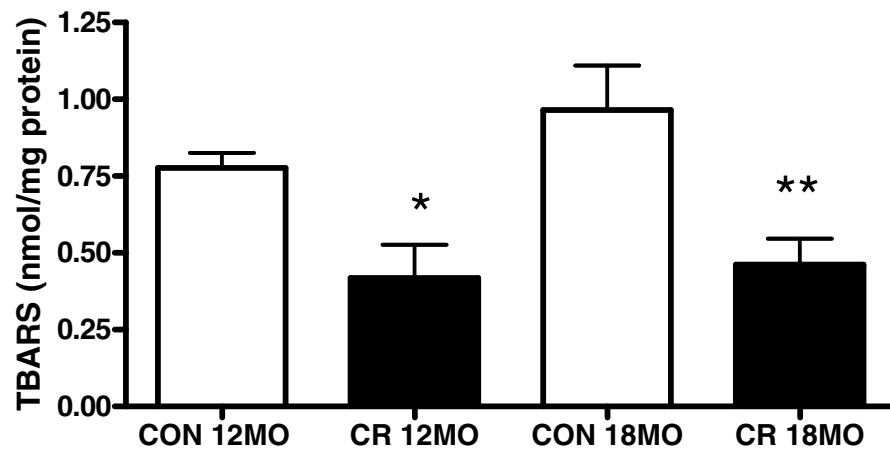


Figure 8

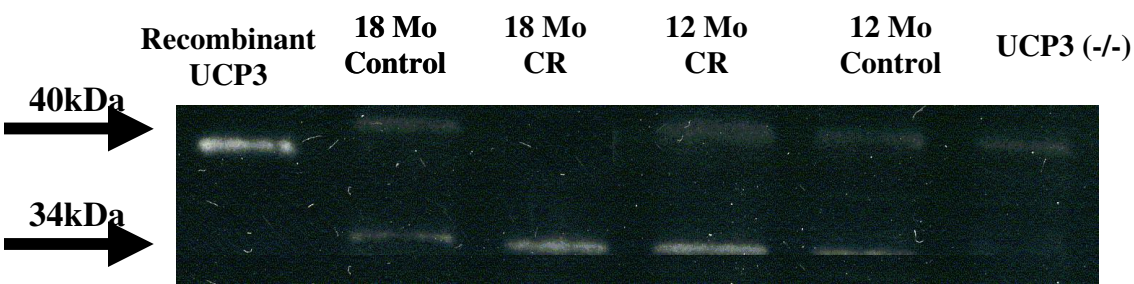


Table 1: Body and organ weights for control and 40% calorie restricted FBNF₁ rats.

Organ	12 Month CR			18 Month CR		
	Control	CR	% Change (CR vs.control)	Control	CR	% Change (CR vs.control)
Liver	14.2±0.25	7.91±0.19	44.3 ^{***}	15.1±1.95	8.23±0.56	45.5 ^{***}
Kidney	2.66±0.06	2.11±0.07	29.6 ^{***}	3.06±0.17	2.10±0.07	31.6 ^{***}
Heart	1.19±0.02	0.87±0.16	20.7 [*]	1.36±0.13	0.97±0.16	29.1 ^{***}
EWAT	11.3±1.02	2.39±0.21	78.8 ^{***}	10.8±1.7	2.63±0.64	75.6 ^{***}
Body Weight	483±10	320±8	35.7 ^{**}	515±16	315±3	38.8 ^{***}

Results are expressed as means ± SEM of 6 rats in each group. * indicates a statistically significant difference between the means of the paired columns at the level of $p<0.05$, ** at the level of $p<0.01$, and *** at the level of $p<0.001$. EWAT = epididymal white adipose tissue

Table 2: State 3 flux control coefficients for each of the three subsystems, and concentration coefficients over Δp in muscle mitochondria from 12 month and 18 month calorie restricted and control rats

	<u>12 Month CR</u>		<u>18 Month CR</u>	
	Control	CR	Control	CR
Flux Control Coefficients				
Substrate Oxidation Reactions				
C_S^{JS}	0.88	0.87	0.61	0.74
C_P^{JS}	0.07	0.01	0.06	0.04
C_L^{JS}	0.05	0.12	0.33	0.22
Phosphorylation Reactions				
C_S^{JP}	0.24	0.27	0.40	0.51
C_P^{JP}	0.81	0.98	0.99	0.95
C_L^{JP}	-0.05	-0.25	-0.39	-0.46
Proton Leak Reactions				
C_S^{JL}	0.87	0.72	0.65	0.39
C_P^{JL}	-0.16	-0.08	-0.36	-0.06
C_L^{JL}	0.29	0.32	0.71	0.67
Concentration Control Coefficients				
$C_S^{\Delta p}$	0.05	0.08	0.09	0.06
$C_P^{\Delta p}$	-0.03	-0.02	-0.08	-0.03
$C_L^{\Delta p}$	-0.02	-0.06	-0.01	-0.03

Abbreviations: Δp , protonmotive force; S, substrate oxidation; P, phosphorylation; L, proton leak; JS, flux through substrate oxidation reaction; JP, flux through phosphorylation reactions; JL, flux through proton leak reactions.

Table 3: State 4 flux control coefficients for each of the three subsystems, and concentration coefficients over Δp in muscle mitochondria from 12 month and 18 month calorie restricted and control rats.

	<u>12 Month CR</u>		<u>18 Month CR</u>	
	Control	CR	Control	CR
Flux Control Coefficients				
Substrate Oxidation Reactions				
C_S^{JS}	0.95	0.86	0.64	0.88
C_P^{JS}	-	-	-	-
C_L^{JS}	0.05	0.14	0.36	0.11
Phosphorylation Reactions				
C_S^{JP}	-	-	-	-
C_P^{JP}	-	-	-	-
C_L^{JP}	-	-	-	-
Proton Leak Reactions				
C_S^{JL}	0.69	0.71	0.65	0.46
C_P^{JL}	-	-	-	-
C_L^{JL}	0.33	0.29	0.35	0.54
Concentration Control Coefficients				
$C_S^{\Delta p}$	0.02	0.05	0.09	0.07
$C_P^{\Delta p}$	-	-	-	-
$C_L^{\Delta p}$	-0.02	-0.05	-0.09	-0.07

Abbreviations: Δp , protonmotive force; S, substrate oxidation; P, phosphorylation; L, proton leak; JS, flux through substrate oxidation reaction; JP, flux through phosphorylation reactions; JL, flux through proton leak reactions.