Long-term calorie restriction reduces proton leak and hydrogen peroxide production in liver mitochondria

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1Department of Molecular Biosciences, School of Veterinary Medicine, University of California, Davis, California; 2Department of Biochemistry, Microbiology, and Immunology, Faculty of Medicine, University of Ottawa, Ottawa, Ontario, Canada; and 3Department of Medicine, University of Wisconsin Medical School and Veterans Administration Geriatric Research, Education and Clinical Center, Madison, Wisconsin

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Hagopian, Kevork, Mary-Ellen Harper, Jesmon J. Ram, Stephen J. Humble, Richard Weindruch, and Jon J. Ramsey. Long-term calorie restriction reduces proton leak and hydrogen peroxide production in liver mitochondria. Am J Physiol Endocrinol Metab 288: E674–E684, 2005. First published November 23, 2004; doi: 10.1152/ajpendo.00382.2004.—Calorie restriction (CR) without malnutrition increases maximal life span in diverse species. It has been proposed that reduction in energy expenditure and reactive oxygen species (ROS) production could be a mechanism for life span extension with CR. As a step toward testing this theory, mitochondrial proton leak, H2O2 production, and markers of oxidative stress were measured in liver from FBNF1 rats fed control or 40% CR diets for 12 or 18 mo. CR was initiated at 6 mo of age. Proton leak kinetics curves, generated from simultaneous measures of oxygen consumption and membrane potential, indicated a decrease in proton leak after 18 mo of CR, while only a trend toward a proton leak decrease was observed after 12 mo. Significant shifts in phosphorylation and substrate oxidation curves also occurred with CR; however, these changes occurred in concert with the proton leak changes. Metabolic control analysis indicated no difference in the overall pattern of control of the oxidative phosphorylation system between control and CR animals. At 12 mo, no significant differences were observed between groups for H2O2 production or markers of oxidative stress. However, at 18 mo, protein carbonyl content was lower in CR animals, as was H2O2 production when mitochondria were respiring on either succinate or pyruvate plus malate in the presence of rotenone. These results indicate that long-term CR lowers mitochondrial proton leak and H2O2 production, and this is consistent with the idea that CR may act by decreasing energy expenditure and ROS production.

CALORIE RESTRICTION (CR) without malnutrition is the only intervention that has consistently been shown to increase maximum life span in laboratory rodents (60). In addition to increasing life span in rodents, CR also delays the onset of diabetes, hypertension, and many types of cancer as well as other age-related physiological and pathological conditions (60). This shows that CR not only extends life span but also retards the rate of biological aging. The mechanism(s) responsible for the actions of CR, however, is still unknown.

Two theories of aging, the rate of living theory (42) and the free radical theory (24), have been proposed as possible mechanisms for the retardation of aging and diseases by CR (56). However, both of these theories may contain the same mechanism, since mitochondria are central to both energy metabolism and reactive oxygen species (ROS) production. Because both processes contain a common mitochondrial link, it is reasonable to assume that alterations in energy expenditure (defined here as any process that converts food or stored energy to heat and work) could influence ROS production. Thus both theories may be linked by common reduction in mitochondrial ROS production. Although studies have clearly indicated a reduction in mitochondrial ROS production with CR (4, 17, 38, 56), the relationship of CR to mitochondrial energy metabolism is less clear. Whole animal studies have been used to conclude that CR either decreases (15) or does not alter energy metabolism (39); however, measures of cellular energy-expending processes are needed to truly determine the effect of CR on energy metabolism. Mitochondrial proton leak (defined here as proton movement across the mitochondrial inner membrane, independent of ATP formation) was chosen as the focus for this study, because proton leak is both a major contributor to resting energy expenditure and has been proposed to play a role in regulation of ROS production (50, 53). Also, proton leak appears to increase with age (27), and this could be due to stimulation of proton leak by oxidative damage to the mitochondrial inner membrane (10).

Proton leak is a process that uncouples oxidative phosphorylation by allowing protons to bypass H+-ATPase and passively cross the mitochondrial inner membrane. Mitochondrial proton leak is responsible for −20–30% of resting oxygen consumption/energy expenditure (49, 50). Although proton leak is often called a futile cycle, it is likely that a process that consumes such a large amount of energy exists to serve some function. Suggested functions of proton leak include heat production, protection from ROS generation, and regulation of carbon fluxes (49). Much interest has focused lately on the role proton leak may play in regulating ROS production. This link stems from studies in isolated mitochondria that have shown a decrease in H2O2 production after the addition of an uncoupler (5). It has been clearly demonstrated that uncouplers decrease ROS production when added to isolated mitochondria (53). However, it is possible that the effect of uncouplers on ROS production in isolated mitochondria is different from the effects of long-term in vivo uncoupling on ROS production. Addition of uncouplers to isolated mitochondria does not allow for any genetic response to uncoupling and does not allow for...
mitochondrial adaptation to sustained alterations in proton leak. We have previously shown that both proton leak and H$_2$O$_2$ production were decreased in skeletal muscle of rats after 6 mo of CR (4). Similarly, hypothyroidism decreases both proton leak (25) and H$_2$O$_2$ production (59). In contrast to uncoupler studies in isolated mitochondria, proton leak studies at the whole animal level suggest that long-term proton leak changes in vivo are positively correlated with mitochondrial ROS production.

Therefore, we propose that long-term CR lowers mitochondrial proton leak and that this reduction will be associated with a reduction in mitochondrial ROS production. To test this hypothesis, we measured proton leak kinetics and H$_2$O$_2$ production in liver mitochondria isolated from rats maintained on a 40% CR diet for a period of 12 and 18 mo. Top-down metabolic control analysis was also completed on the mitochondria to better determine the effect of CR on the regulation of oxidative phosphorylation. Markers of protein and lipid oxidative damage, as well as organ weights, were also measured.

**METHODS**

**Animals and diets.** Male FBN$_1$ rats were purchased from the National Institute on Aging (NIA) Aging Rodent Colony (Bethesda, MD) and were used for all phases of this study. At 6 mo of age, the rats were randomly assigned to either control or CR groups. The control group was fed an AIN-93M purified diet at 95% of average ad libitum food intake, whereas the CR group was fed a modified AIN-93M diet (Research Diets, New Brunswick, NJ) at 60% of ad libitum intake, as previously described (47). The corn starch (carbohydrate) component of the restricted diet was decreased, resulting in an increase in the percentage of protein, fat, minerals, and vitamins in the restricted diet, thus achieving equal absolute daily intakes of essential nutrients between control and CR groups. The energy intakes were 62 kcal/day for the control and 38 kcal/day for the restricted rats. All animals were fed between 8 and 9 AM each day. The rats were caged individually, maintained on a 12:12-h light-dark cycle at 23°C, and allowed free access to water during all stages of the study. At the 12-mo CR time point (18 mo of age), five control and six CR animals were used for the study, whereas at the 18-mo CR time point (24 mo of age), seven control and seven CR animals were used. All animal protocols were approved by the University of California-Davis Internal Animal Care and Use Committee and are in accordance with the guidelines of the Institute of Laboratory Animal Resources (National Research Council).

**Chemicals.** All chemicals were purchased from Sigma (St. Louis, MO) except for hexokinase and ATP, which were from Roche (Indianapolis, IN), and Percoll, which was from Amersham (Piscataway, NJ). Bio-Rad protein assay kits were from Bio-Rad Laboratories (Hercules, CA), and bicinchoninic acid (BCA) protein assay kits and BSA standards were from Pierce (Rockford, IL).

**Mitochondria isolation and Percoll gradient fractionation.** All animals were food deprived overnight before euthanasia. Rats were killed by decapitation, and livers were removed rapidly, weighed, and placed into ice-cold isolation medium (250 mM sucrose, 10 mM Tris·HCl, 1 mM EGTA, 0.1% defatted BSA, pH 7.4). The livers were finely chopped and rinsed in the isolation medium to remove excess blood, and mitochondria were isolated with the use of previously described methods (13). Briefly, liver (10% wt/vol in isolation medium) was transferred to an ice-cold glass-Teflon homogenizer and homogenized with six strokes of the pestle. The homogenate was centrifuged at 1,000 g for 5 min at 4°C, and the resulting supernatant was decanted into clean centrifuge tubes and centrifuged at 12,000 g for 10 min (4°C). The supernatant was discarded, and the pellet was gently resuspended in isolation medium using a cooled glass rod and centrifuged again at 12,000 g. The pellet was then resuspended in isolation medium without BSA and centrifuged at 12,000 g, and the resulting mitochondrial pellet was suspended in isolation medium without BSA and stored on ice.

For H$_2$O$_2$ production assays, a portion of the isolated mitochondria was further fractionated on a 30% (vol/vol) Percoll gradient in 225 mM mannitol, 1 mM EGTA, and 25 mM HEPES buffer, pH 7.4, and centrifuged at 95,000 g for 30 min at 4°C in a Beckman L8-80M ultracentrifuge, using a 60-Ti Beckman rotor (52). Centrifugation resulted in the mitochondria separating into a band near the lower part of the gradient. This band was removed and washed twice with washing buffer (2 mM HEPES buffer containing 220 mM mannitol, 70 mM sucrose, and 0.5 mM EGTA, pH 7.4) to remove the Percoll and centrifuged after each wash at 6,400 g for 10 min at 4°C. The resulting mitochondria were washed twice with 150 mM KCl and centrifuged as above, and the final pellet was resuspended in washing buffer. This step is essential for the removal of catalase, which comes not only from tissue homogenization but also from the lysis of red blood cells (47).

**Measurement of organ weights.** Internal organs (liver, brain, heart, kidneys, lungs, stomach, small intestine, and large intestine) were removed immediately after death, cleaned of fat or connective tissue, and weighed. Ingesta or feces were removed from stomach and intestines before weighing.

**Mitochondrial oxygen consumption.** Mitochondrial oxygen consumption was measured as previously described (47). A Clark-type oxygen electrode (Hansatech, Norfolk, UK) with an incubation chamber maintained at 37°C was used for oxygen consumption measurements. All measurements were completed in duplicate using mitochondria (1.0 mg/ml) in incubation medium (100 mM KCl, 20 mM succinate, 20 mM glucose, 10 mM KH$_2$PO$_4$, 5 mM HEPES, 2 mM MgCl$_2$, 1 mM EGTA, pH 7.2) with 5 μM rotenone and 0.4 μg nigericin. Nigericin, in the presence of KCl, is used to convert the ΔpH component of the protonotive force to units of mitochondrial membrane potential (mV). State 3 respiration was defined as the oxygen consumption rate in the presence of 10 mM succinate, 1.50 units/ml hexokinase, and 200 μM ADP. State 4 oxygen consumption was defined as the oxygen consumption rate in the presence of 10 mM succinate and the ATP synthase inhibitor, oligomycin (8 μg/ml mitochondrial protein).

**Measurement of mitochondrial membrane potential.** Membrane potential was measured with a triphenylmethylnaphthosphonium (TPMP$^+$)-sensitive electrode (World Precision Instruments) as previously described (6). Before the start of each measurement, a TPMP$^+$ standard curve was generated in the presence of sample through successive additions of five TPMP$^+$ aliquots of equal concentrations until a final 5 mM TPMP$^+$ concentration was achieved. Mitochondrial membrane potential (Δφ$_{m}$) was determined by comparing TPMP$^+$ measurements after the addition of succinate with the TPMP$^+$ standard curve generated in that sample before the addition of succinate. At the end of runs, 0.1 μM FCCP was added to the sample to dissipate Δφ$_{m}$ and determine any drift in the TPMP$^+$ electrode. Δφ$_{m}$ was calculated using the modified Nernst equation

\[
\Delta \phi_m = 61.5 \times \log([\text{TPMP}^+_{\text{int}}] \times a_{\text{m}}/[\text{TPMP}^+_{\text{ext}}])
\]

where [TPMP$^+_{\text{int}}$] is the concentration of TPMP$^+$ in the mitochondrial matrix, and [TPMP$^+_{\text{ext}}$] is the concentration of TPMP$^+$ outside the mitochondria. The factor $a_{\text{m}}$ is the mitochondrial TPMP$^+$ binding correction, and the value used for liver was 0.4 (51). Membrane potentials and oxygen consumption were measured simultaneously in all of the samples.

**Top-down metabolic control analysis and proton leak kinetics.** Metabolic control analysis, as developed by Kacer and Burns (31) and Heinrich and Rapoport (28), is an approach that allows the control exerted by specific enzyme reactions over flux through a metabolic pathway to be quantified. Traditionally, this approach has determined

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the control structure of metabolic pathways by successively manipulating the activities of individual enzymes and determining the effect of these manipulations on flux through the pathway. In contrast to this traditional approach, the top-down approach determines the control exerted by blocks of reactions, rather than individual enzymes, over both the flux through the metabolic pathway and the concentration of pathway metabolites. The theory and application of top-down metabolic control analysis are the subject of several reviews (7, 26, 46).

Briefly, this approach involves dividing the metabolic pathway of interest into two or three blocks of reactions around a common intermediate. In our case, the mitochondrial oxidative phosphorylation system is divided into substrate oxidation, proton leak, and phosphorylation components that are linked by \( \Delta \psi_{in} \) (the common intermediate). To determine control over oxidative phosphorylation, the \( \Delta \psi_{in} \) is manipulated by titrating the individual blocks of reactions with either inhibitors or activators. From this information, elasticity coefficients may be calculated that mathematically represent the dependence of flux (through each of the 3 blocks of reactions) on membrane potential. In other words, elasticity coefficients represent the change in flux for each block of reactions induced by a change in \( \Delta \psi_{in} \). Thus a large elasticity coefficient indicates that flux through a specific block of reactions is very sensitive to alterations in membrane potential, whereas a small elasticity coefficient indicates that flux through the block of reactions is relatively insensitive to alterations in membrane potential. Flux control coefficients and concentration control coefficients are then calculated for the blocks of reactions from elasticity coefficients and respiration rates, using published equations (22). Flux control coefficients describing control over a particular component (i.e., proton leak, substrate oxidation, or phosphorylation) add up to one, with a value of one indicating complete control, a value of zero indicating no control, and a value of 0.5 indicating 50% control. In contrast, concentration control coefficients quantify the control exerted by the individual components over the common intermediate (\( \Delta \psi_{in} \)) and add up to zero. For concentration control coefficients, the components exerting the greatest control will have the largest absolute values.

The kinetic response of the proton leak to \( \Delta \psi_{in} \) was determined by titrating the electron transport chain with malonate (0.3–10 mM) in the presence of oligomycin (8 \( \mu \)g/mg protein). The kinetic response of the substrate oxidation pathway to \( \Delta \psi_{in} \) was determined by titration with hexokinase (0.25–1.50 U/ml) in the presence of 200 mM ATP. The kinetic response of the phosphorylation system to \( \Delta \psi_{in} \) was determined by titration with malonate (0.3–10 mM) in the presence of 200 mM ADP and 1.50 U/ml hexokinase. Because this measurement contains components of both phosphorylation and proton leak, a correction was subsequently made for the proton leak-related oxygen consumption at each membrane potential.

Mitochondrial \( \text{H}_{2}\text{O}_{2} \) production. The rate of \( \text{H}_{2}\text{O}_{2} \) generation by intact mitochondria (0.50–0.60 mg total protein in 3 ml of assay) was determined fluorimetrically (29) in an assay medium (10 mM potassium phosphate buffer, pH 7.4, containing 154 mM KCl, 0.1 mM EGTA, 3 mM MgCl\(_2\)) in the presence of 4 U of horseradish peroxidase, 500 \( \mu \)g/assay \( p \)-hydroxyphenylacetic acid, and either 10 mM succinate or 10 mM pyruvate-5 mM malate as substrates. Briefly, increased fluorescence (\( \lambda_{exc} = 320 \text{ nm, } \lambda_{em} = 400 \text{ nm} \)) at 37°C was monitored by a Perkin-Elmer LS-55 luminescence spectrometer equipped with a water peltier heating system and a magnetic stirring sample compartment. Levels of \( \text{H}_{2}\text{O}_{2} \) were expressed as picomoles \( \text{H}_{2}\text{O}_{2} \) per minute per milligram protein. Rates were determined by converting fluorescence readings, using a standard curve generated over a range of \( \text{H}_{2}\text{O}_{2} \) concentrations.

Markers of oxidative stress. Mitochondrial protein carbonyls and thiobarbituric acid-reactive substances (TBARS) were measured as markers of protein and lipid oxidative stress, respectively. Protein carbonyls were determined by derivatization with 2,4-dinitrophenylhydrazine, according to Levine et al. (36), as described previously (47). Carboxyl content was measured spectrophotometrically, using a Perkin-Elmer Lambda-25 spectrophotometer set at 366 nm, with a molar extinction coefficient of 22,000 M\(^{-1}\) cm\(^{-1}\). Results were expressed as nanomoles carbonyl per milligram protein.

Lipid peroxidation levels or TBARS were determined as malondialdehyde-thiobarbituric acid adds according to Buege and Aust (3), as previously described (47). Samples were treated with butylated hydroxytoluene (0.07 mM final concentration) to avoid generation and overestimation of peroxidation developed during the heating step (21). Absorbance was read spectrophotometrically in a Perkin-Elmer Lambda-25 spectrophotometer set at 535 nm. Concentrations were determined by using a molar extinction coefficient of 1.56 \( \times \) 10\(^5\) M\(^{-1}\) cm\(^{-1}\) and expressed as nanomoles TBARS per milligram protein.

Protein assays. In all samples, protein concentrations were determined by use of a Bio-Rad protein assay kit. However, protein concentrations for the protein carbonyl assays were determined with a BCA protein assay kit, since the presence of high guanidine-HCl in samples interfered with the Bio-Rad assay system. BSA was used to generate standard curves for both protein assays. In the case of the BCA assay, the standard curve was generated in the presence of guanidine-HCl at concentrations similar to those found in the samples. No differences in mitochondrial protein yield (mg protein/g wet wt of liver) were observed between control and CR rats.

Statistical analysis. Comparisons between control and CR groups were completed using Student’s t-tests, and comparisons within a treatment group were completed using paired Student’s t-tests, with \( P \leq 0.05 \) considered significant and \( 0.05 < P \leq 0.1 \) considered a trend. All statistical analyses were completed using JMP software (SAS Institute, Cary, NC). Results are presented as means ± SD or SE (as indicated in legends to tables and figures).

RESULTS

Organ and body weights. Organ and body weight results are summarized in Table 1. As expected, body weight was significantly lower (\( P < 0.05 \)) in CR compared with control rats at

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<th>Table 1. Organ weights in control and CR rats</th>
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<td>Liver</td>
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<td>Large intestine</td>
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<td>Body wt</td>
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|                                | 18 Mo   |     |         |
|---------------------------------------------|
| Liver                           | 14.21 (SD 0.68) | 7.17 (SD 0.57)* | −50 |
| Heart                          | 1.29 (SD 0.08)  | 0.93 (SD 0.11) | NS |
| Brain                          | 2.18 (SD 0.21) | 2.03 (SD 0.06)* | NS |
| Kidneys                        | 2.91 (SD 0.17) | 2.09 (SD 0.06)* | −28 |
| Lungs                          | 1.70 (SD 0.20) | 1.79 (SD 0.19) | NS |
| Stomach                        | 1.67 (SD 0.12) | 1.53 (SD 0.08) | NS |
| Small intestine                | 1.72 (SD 0.31) | 1.58 (SD 0.17) | NS |
| Large intestine                | 1.11 (SD 0.23) | 1.32 (SD 0.22) | NS |
| Body wt                        | 577.4 (SD 29.0) | 344.6 (SD 8.6)* | −40 |

Values are presented as means (SD). CR, calorie restriction; NS, not significant (\( P > 0.05 \)). *\( P < 0.05 \), CR different from control at same assessment period.
both the 12- and 18-mo assessments. The 35 and 40% differences in body weight between the CR and control rats at the 12- and 18-mo assessments, respectively, are similar in magnitude to the levels of CR used in this study. Interestingly, the only organ that showed a weight change of a magnitude similar to or greater than that in CR rats was liver, which showed a 40 and 50% difference $(P < 0.05)$ between control and CR groups at 12 and 18 mo of CR, respectively. Heart and kidneys were the only other organs that showed a weight difference $(P < 0.05)$ between control and CR groups. Heart showed a weight reduction of 24–28% in CR rats, and kidney weights were reduced 22–28% by CR. All other organs weights were not significantly different between the two treatment groups.

Proton leak, substrate oxidation, and phosphorylation systems kinetics. Mitochondrial proton leak, substrate oxidation, and phosphorylation kinetics are shown in Fig. 1 (12-mo assessment) and Fig. 2 (18-mo assessment). At 12 mo of CR, maximum leak-dependent respiration (the furthest point to the right in the kinetics curve) showed a trend $(P = 0.10)$ toward being lower in the CR compared with the control group, whereas membrane potentials were not different between groups at this maximum leak-dependent respiration point (Fig. 1C). However, membrane potential values in other parts of the curve (Fig. 1C) were higher $(P < 0.05)$ in CR vs. control rats when compared at similar oxygen consumption rates. This shift in the proton leak kinetics curve toward higher membrane potential values in the CR group suggests a modest decrease in proton leak with CR. Similar to the proton leak curve, the phosphorylation (Fig. 1B) and substrate oxidation (Fig. 1A) plots showed slight differences between the two diet groups. The 12-mo respiratory chain kinetics plot was shifted by CR in a manner such that the CR animals showed a trend toward higher membrane potentials at similar respiratory rates compared with controls (Fig. 1B). In the 12-mo respiratory chain kinetics plot, membrane potential values were similar between control and CR groups; however, respiration rates were lower $(P < 0.05)$ in the CR group compared with control animals (Fig. 1A).

Proton leak, phosphorylation, and respiratory chain kinetics plots were very different between control and CR groups at 18 mo of CR. Maximum leak-dependent respiration showed a trend toward being lower in the CR vs. control group $(P = 0.08)$, whereas membrane potential was higher $(P < 0.05)$ in the CR vs. control group at this point (Fig. 2C). Overall, proton leak kinetics curve showed a shift with CR such that the CR animals consistently had higher membrane potentials than the control animals over a range of oxygen consumption rates. These results show a lower proton leak rate in the CR group compared with the control group at 18 mo of CR. Similar to the proton leak results, CR animals consistently had higher $(P < 0.05)$ membrane potentials over a range of respiration rates in both phosphorylation (Fig. 2B) and substrate oxidation (Fig. 2A) kinetics plots. Respiratory control ratios were also significantly different $(P < 0.05)$ between control $(3.37 \pm 0.13)$ and CR $(3.73 \pm 0.07)$ groups at the 18-mo assessment. This difference in the respiratory control ratio is consistent with the conclusion that CR decreases liver mitochondrial proton leak.

Mitochondrial H2O2 production. H2O2 production was measured in intact mitochondria isolated from control and CR rats at 12 and 18 mo of CR. At the 12-mo assessment point (Fig. 3), no differences $(P > 0.05)$ were observed between control and CR groups when the mitochondria were respiring on either succinate or pyruvate plus malate. Also, no differences $(P > 0.05)$ were observed between groups when inhibitors (antimycin A or rotenone) were used with either substrate.

Higher levels of H2O2 were produced by both control and CR mitochondria when respiring on succinate vs. pyruvate-malate $(P < 0.05)$. When antimycin A was included in the assay, the highest $(P < 0.05)$ levels of H2O2 production were detected with both substrates and in both control and CR mitochondria. However, in the presence of rotenone, significantly $(P < 0.05)$ lower levels of H2O2 were detected when
succinate was the substrate, in both controls and CR, when compared with substrate alone, whereas significantly higher levels of H2O2 production were detected when pyruvate-malate was the substrate (P < 0.05) when compared with substrate alone.

At the 18-mo assessment, mitochondria from the CR group respiring on succinate alone showed a decreased H2O2 production (P < 0.05) compared with the control group (Fig. 4). When mitochondria were respiring on pyruvate-malate, CR mitochondria showed decreased H2O2 production (P < 0.05) when compared with controls after the addition of rotenone.

No significant changes were observed between the control and CR groups when mitochondria were respiring on either pyruvate plus malate alone or after the addition of antimycin A.

Markers of oxidative stress. Protein carbonyls and lipid peroxidation values for 12 and 18 mo are shown in Figs. 5 and 6, respectively. For protein carbonyls, no differences were observed between the control and CR groups at 12 mo (Fig. 5). Substrate oxidation kinetics were completed with 200 μM ATP and 10 mM succinate and titrated with hexokinase (0.25–1.50 U/ml). Phosphorylation kinetics were completed with 200 μM ADP, 1.5 U/ml hexokinase, and 10 mM succinate and titrated with malonate (0.3–10.0 mM). Proton leak kinetics were completed with 10 mM succinate and oligomycin (8 μg/mg mitochondrial protein). All measurements were completed as described in METHODS. Values are presented as means ± SE.
whereas a significant increase was observed in the control vs. CR groups at the 18-mo assessment (Fig. 6). However, lipid peroxidation was not different between the two groups at either the 12- or 18-mo assessments.

**Top-down metabolic control analysis.** Top-down metabolic control analysis was completed on mitochondria at both the 12- and the 18-mo assessments to determine possible changes in the regulation of oxidative phosphorylation with CR. Flux control coefficients over subsystem fluxes and concentration control coefficients over mitochondrial membrane potential ($\Delta\psi_m$) are summarized in Table 2. (state 3 conditions) and Table 3 (state 4 conditions). Under state 3 conditions, CR did not alter the control of oxidative phosphorylation at either 12 or 18 mo of CR. At both assessment points and in both treatment groups, the phosphorylation system exerted the greatest control over substrate oxidation flux. For both control and CR groups, the remaining control of substrate oxidation flux was equally divided between the substrate oxidation and proton leak subsystems at the 12-mo assessment, whereas greater control was exerted by substrate oxidation than proton leak at the 18-mo assessment.

The phosphorylation subsystem exerted the greatest control over its own flux in both groups and at both time points, and the substrate oxidation system also exerted a considerable positive control over phosphorylation flux in both control and

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<th>12 Mo Control</th>
<th>12 Mo CR</th>
<th>18 Mo Control</th>
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<td>Substrate oxidation</td>
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<tr>
<td>$C^S_s$</td>
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<tr>
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**Table 2. State 3 flux control coefficients over subsystem fluxes and concentration control coefficients over $\Delta\psi_m$ in liver mitochondria**
by the 18-mo assessment, these processes in the CR vs. control mitochondria. However, substrate oxidation and proton leak flux such that proton leak flux were primarily controlled by the proton leak system. At the 12-mo assessment, there was a shift in control of the CR pattern to the same in both control and CR groups at both the 12- and 18-mo assessments.

Under state 3 conditions, proton leak flux was controlled almost exclusively by the proton leak system itself. This control pattern was the same in both control and CR groups at both the 12- and 18-mo assessments.

Under state 4 conditions, substrate oxidation and proton leak flux were primarily controlled by the proton leak system. At the 12-mo assessment, there was a shift in control of state 4 in phospholipid oxidation and proton leak flux such that proton leak exerted less control and substrate oxidation more control over these processes in the CR vs. control mitochondria. However, by the 18-mo assessment, state 4 flux control coefficients were identical in the control and CR groups.

Concentration control coefficients over ΔΨm in liver mitochondria from control and restricted animals after 12 or 18 mo of CR. See legend to Table 2 for explanation of terms.

CR animals. Proton leak exerted a smaller negative control over flux through the phosphorylation system that was of a similar magnitude in both the control and CR groups.

Under state 3 conditions, proton leak flux was controlled almost exclusively by the proton leak system itself. This control pattern was the same in both control and CR groups at both the 12- and 18-mo assessments.

Under state 4 conditions, substrate oxidation and proton leak flux were primarily controlled by the proton leak system. At the 12-mo assessment, there was a shift in control of state 4 in phospholipid oxidation and proton leak flux such that proton leak exerted less control and substrate oxidation more control over these processes in the CR vs. control mitochondria. However, by the 18-mo assessment, state 4 flux control coefficients were identical in the control and CR groups.

Concentration control coefficients over ΔΨm in liver mitochondria from control and restricted animals after 12 or 18 mo of CR. See legend to Table 2 for explanation of terms.

CR is the only intervention that has consistently been shown to increase maximum life span in laboratory rodents (60). Life span extension with CR is due to a reduction in energy intake and not a reduction in the consumption of a specific nutrient (38). Because energy metabolism is central to the actions of CR, it is attractive to propose that reduction in energy expenditure may be a mechanism contributing to life span extension with CR. However, whole animal studies reporting no difference in mass-adjusted energy expenditure between control and CR rats (39) have been used to refute the idea that a reduction in energy expenditure may be a mechanism contributing to the actions of CR. The data normalization procedures used in whole animal studies, however, may mask important shifts in energy metabolism that occur at the cellular level. This may occur because current methods for normalizing energy expenditure for body size differences often assume that the composition of lean mass will be similar between CR and control animals. Increasing evidence indicates that this assumption is false.

To better understand body composition changes with CR that could contribute to alterations in energy expenditure, organ weights were measured in both control and CR animals. Our results support the conclusion that CR does not result in a uniform change in organ weights. In our study, only body and liver weights showed a reduction in the CR group similar to the magnitude of the CR, with the size of liver weight reduction actually slightly exceeding the level of CR at 18 mo (Table 1). In contrast, other organs either showed no change in weight (e.g., brain, lungs) between control and CR rats or a decrease in weight (e.g., heart, kidneys) that did not reach the magnitude of the restriction. Similarly, previous studies have also shown that long-term CR does not result in uniform changes in the weights of internal organs (20, 23, 60), and this nonuniform change in organ mass with CR could have important implications for drawing conclusions about cellular energy expenditure from whole animal data. Different tissues or organs do not have uniform rates of energy expenditure, and internal organs contribute >50% of resting energy expenditure despite the fact that they represent <10% of total body weight (48). Taken together, our study and those previously published (20, 23, 60) indicate that internal organs represent a greater portion of body weight in CR compared with control animals. These body composition alterations complicate whole animal comparisons between control and CR groups, and, therefore, measurements of cellular energy-consuming processes are required to determine the role alterations in energy expenditure may play in the actions of CR.

To better understand the effect of CR on cellular energy expenditure, it is important to measure the activity of major cellular energy-consuming pathways. Mitochondrial proton leak is a process of particular interest, because it is responsible for 20–30% of resting energy expenditure (49, 50) and may play an important role in regulating ROS production (49, 53). In contrast, other organs either showed no change in weight (e.g., brain, lungs) between control and CR rats or a decrease in weight (e.g., heart, kidneys) that did not reach the magnitude of the restriction. Similarly, previous studies have also shown that long-term CR does not result in uniform changes in the weights of internal organs (20, 23, 60), and this nonuniform change in organ mass with CR could have important implications for drawing conclusions about cellular energy expenditure from whole animal data. Different tissues or organs do not have uniform rates of energy expenditure, and internal organs contribute >50% of resting energy expenditure despite the fact that they represent <10% of total body weight (48). Taken together, our study and those previously published (20, 23, 60) indicate that internal organs represent a greater portion of body weight in CR compared with control animals. These body composition alterations complicate whole animal comparisons between control and CR groups, and, therefore, measurements of cellular energy-consuming processes are required to determine the role alterations in energy expenditure may play in the actions of CR.

Table 3. State 4 flux control coefficients over subsystem fluxes and concentration control coefficients over ΔΨm in liver mitochondria

<table>
<thead>
<tr>
<th>Flux Type</th>
<th>12 Mo</th>
<th>18 Mo</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>CR</td>
</tr>
<tr>
<td>Substrate oxidation flux</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C^4S</td>
<td>0.17</td>
<td>0.28</td>
</tr>
<tr>
<td>C^3S</td>
<td>0.72</td>
<td>0.77</td>
</tr>
<tr>
<td>Prophosphorylation flux</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C^4P</td>
<td>0.17</td>
<td>0.28</td>
</tr>
<tr>
<td>C^3P</td>
<td>0.83</td>
<td>0.72</td>
</tr>
<tr>
<td>Proton leak flux</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C^4L</td>
<td>0.17</td>
<td>0.28</td>
</tr>
<tr>
<td>C^3L</td>
<td>0.83</td>
<td>0.72</td>
</tr>
<tr>
<td>Concentration control coefficients over ΔΨm</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C^4m</td>
<td>0.04</td>
<td>0.05</td>
</tr>
<tr>
<td>C^3m</td>
<td>-0.04</td>
<td>-0.05</td>
</tr>
</tbody>
</table>
Clearly, the duration of CR and/or age and tissue type play a major role in determining proton leak response to CR.

In contrast to our findings, a recent study by Lambert and Merry (35) reported that proton leak is increased with CR in rat liver mitochondria. The differences in results between our study and that of Lambert and Merry could be related to differences in the experimental design of the two studies. Lambert and Merry used Brown Norway rats that were started on CR at 60 days of age, and CR was maintained through weight clamping rather than a set level of CR. Thus rat strain, age of CR initiation, and protocol for maintaining CR all differed from our study, and it is possible that this contributed to the proton leak differences between studies. If proton leak is truly increased under some CR conditions, future studies will be needed to determine why the animal would divert scarce energy resources to increasing the activity of a major energy-consuming pathway.

The mechanism(s) responsible for the development of changes in liver mitochondrial proton leak with long-term CR are not known. Although uncoupling proteins (UCPs) have been proposed to play a role in regulating proton leak (1), liver parenchymal cells do not contain UCPs, and, therefore, changes in proton leak in liver are not likely due to altered expression of known UCPs (40). However, it is possible that prolonged increases in liver β-oxidation may induce UCP2 expression (19). Alternatively, adenine nucleotide translocase (11) or a newly identified mitochondrial carrier protein (57) could potentially contribute to mitochondrial proton leak in liver. Membrane oxidative damage has also been shown to increase proton leak (10). Furthermore, mitochondrial proton leak in hepatocytes is increased with aging, a process characterized by increased tissue oxidative damage (27). However, differences in proton leak between control and CR animals appeared to be emerging at the 12-mo assessment. These results suggest that changes in proton leak may precede the development of oxidative damage to the mitochondria; however, it is possible that oxidative damage at least partially contributes to the proton leak differences observed between groups at the 18-mo assessment. Because oxidative damage appears to increase proton leak (10), and proton leak appears to be associated with increased oxidative damage, these precesses may interact to create a “vicious cycle” of increasing ROS production and oxidative damage. Membrane fatty acid composition and surface area have also been proposed to play a major role in regulating the rate of proton leak (8, 9, 45), and it is likely that changes in either of these parameters could contribute to the observed alterations in proton leak with CR. Although little is known about the effect of CR on mitochondrial membrane surface area, several studies have shown that CR changes mitochondrial membrane fatty acid composition (33, 34, 47). These studies (33, 34) have also shown that changes in membrane composition occur in both control and CR animals throughout the life span. Thus the delayed changes in proton leak that were observed in this study could be due to a diet and age interaction affecting membrane composition.

In addition to measures of proton leak kinetics, mitochondrial \( \text{H}_2\text{O}_2 \) production was also measured at both assessment points. At the 12-mo assessment, no significant differences were observed between the control and CR groups (Fig. 3). At the 18-mo assessment (Fig. 4), however, a significant lowering of \( \text{H}_2\text{O}_2 \) production was observed in liver mitochondria from CR animals. When mitochondria were respiring on pyruvate plus malate, differences \((P < 0.05)\) in the \( \text{H}_2\text{O}_2 \) production between control and CR groups were only observed in the presence of rotenone. Complex I is the only respiratory chain complex on the substrate side of this inhibitor, and, therefore, the difference in \( \text{H}_2\text{O}_2 \) production between control and CR groups respiring on pyruvate plus malate in the presence of rotenone is due entirely to complex I. Succinate, a complex II-linked substrate, differs from pyruvate plus malate in that electrons from complex II may be directed to complex III (via CoQ) or undergo a backflow to complex I. The finding that rotenone decreases \( \text{H}_2\text{O}_2 \) production in CR and control mitochondria respiring on succinate clearly implicates complex I as a site for ROS generation through electron backflow from complex II. This mechanism has been reported previously (2, 12). The differences between control and CR \( \text{H}_2\text{O}_2 \) production, when mitochondria were respiring on succinate alone, were abolished in the presence of rotenone, indicating that the difference between the two groups was due to ROS generation at complex I. Furthermore, it appears that CR has little effect on ROS production at complex III, since differences between groups were not observed when mitochondria were respiring on pyruvate plus malate alone or in the presence of antimycin A. This conclusion is also supported by the fact that no differences between groups in \( \text{H}_2\text{O}_2 \) production were observed in mitochondria respiring on succinate in the presence of rotenone.

Our finding that differences in liver ROS production between control and CR groups do not occur until at least late middle age is in agreement with several studies indicating that differences in markers of liver oxidative damage only occur between control and CR animals later in life. Studies measuring 8-hydroxy-2′-deoxyguanosine as a marker of DNA oxidative damage have reported that differences emerge between control and CR animals at either 23 mo of age in mice (54) or 30 mo of age in rats (32). Studies of lipid peroxidation in liver have also reported differences between control and CR rats only after the animals reached 30 mo of age (18). Similarly, a study measuring protein carbonyl content in liver from rats reported that significant age-related increases in protein oxidative damage did not occur until the animals were 24 mo old (58). These oxidative damage measures support our conclusion that differences in liver \( \text{H}_2\text{O}_2 \) production do not occur between control and CR animals until later in the life span.

In addition to oxidative stress measures, we are aware of only one study (37) that has measured liver \( \text{H}_2\text{O}_2 \) production in rats maintained on long-term CR. Similar to our study, the above-mentioned study reported a decrease in liver \( \text{H}_2\text{O}_2 \) production with CR. However, the work of López-Torres et al. (37) differed from our results in that a reduction in \( \text{H}_2\text{O}_2 \) production was observed when mitochondria were respiring on pyruvate plus malate alone, whereas no differences in \( \text{H}_2\text{O}_2 \) production were observed with succinate alone. The reason for the difference between the two studies is not clear, although it could be related to the age at which CR was initiated (6 mo for our study vs. 12 mo for the López-Torres et al. study).

In addition to measures of proton leak kinetics, substrate oxidation and phosphorylation system kinetics were also measured in control and CR groups. Data from the measures of proton leak, substrate oxidation, and phosphorylation kinetics were analyzed together for top-down metabolic control analy-
leak is associated with a reduction in mitochondrial H$_2$O$_2$ with long-term CR, a finding consistent with the idea that energy metabolism is altered in CR vs. control animals at either time point. The reason for these shifts is not entirely clear, although there is some evidence for age-related changes in components of both substrate oxidation and phosphorylation pathways. The phosphorylation system, as studied under our conditions, consists primarily of H$^+$-ATPase (complex V) and transport proteins (adenine nucleotide translocase and phosphate transporter), whereas the substrate oxidation system in our study consists of the electron transport chain (ETC) (excluding complex I). A few studies have indicated age-related impairments in the ETC (14), H$^+$-ATPase (41), and adenine nucleotide translocase (61); however, these studies were not completed in rat liver. Nevertheless, it is possible that the changes observed in the substrate oxidation and phosphorylation kinetics curves between control and restricted animals reflect a resistance in age-related impairments to mitochondrial membrane components. However, it is important to note that our results clearly show that CR does not preferentially alter or protect one specific component of the mitochondrial oxidative phosphorylation system, since regulation of oxidative phosphorylation was not altered by CR despite the fact that significant changes in kinetics curves for each of the subsystems were observed between diet groups. Also, our results did not show the age-related alterations in liver oxidative phosphorylation that have been previously reported when comparing 3- vs. 30-mo-old mice (27), and thus our rats may not have reached an age where age-related disregulation of oxidative phosphorylation occurs. The lack of a CR-induced alteration in the regulation of liver oxidative phosphorylation at 6 mo of CR (47) or at the later time points described in this study contrasts with the rapid and sustained alterations in control of oxidative phosphorylation seen in skeletal muscle with CR (4). This further demonstrates the tissue-specific alterations in energy metabolism that occur with CR and indicates that broad conclusions about energy metabolism alterations with CR should not be made from single tissue or time point measurements.

Our results show that mitochondrial proton leak is decreased with long-term CR, a finding consistent with the idea of reduced energy expenditure, and that this reduction in proton leak is associated with a reduction in mitochondrial H$_2$O$_2$ production. However, this finding appears to contradict the common notion that mitochondrial uncoupling and UCPs function to inhibit ROS production (30, 53). This conclusion, that uncoupling protects against ROS production, stems initially from work showing that addition of uncoupling agents to isolated mitochondria decreases ROS formation (5). The mechanism for this decrease in ROS production is an increased flux through the respiratory chain that prevents the chain from remaining in a reduced state. Alternatively, proton leak may also decrease ROS formation by lowering Δ$\psi_{\text{m}}$ (53). However, the relationship between proton leak and ROS production appears to be more complex than these generalities. In the case of CR, an increase in proton leak could be one method to decrease ROS production; however, this would be energetically costly, and there is no clear evolutionary reason why a CR animal would benefit from shifting scarce energy resources toward a reduction in ROS generation. However, in this study we have shown that long-term CR induces a reduction in both proton leak and ROS production in liver. We have previously reported a similar positive correlation between proton leak and ROS production in skeletal muscle with CR, although the time course for skeletal muscle changes was different from those observed in liver (4). These results suggest that, under long-term physiological conditions, a reduction in proton leak is associated with a reduction in ROS production. In support of this conclusion, long-lived animal species tend to have low rates of H$_2$O$_2$ production (43, 55) and low proton leak rates (44). Similarly, hypothyroidism is associated with a decrease in both proton leak (25) and H$_2$O$_2$ production (59). Proton leak has been proposed to serve many functions, and it is possible that alterations in ROS production are not a primary function of proton leak but instead occur secondarily to other functions of this process. Possibly, the central function of proton leak may involve regulating flux through the ETC under resting conditions. A sustained change in proton leak will thus be associated with an alteration in ETC flux, and it seems unlikely that such a change would be met without any mitochondrial adaptations. It is possible that mitochondria adapt to a sustained reduction in proton leak with a decrease in ETC and/or decreased membrane surface area. There is evidence that CR decreases ETC chain activity in skeletal muscle mitochondria (14), and this may reflect a downregulation in mitochondrial ETC content. A downregulation of mitochondrial ETC content secondary to CR-induced reductions in proton leak (or other energy-expending processes) could result in a reduction in ROS production by decreasing available sites for ROS production. However, this is speculation, and additional studies will be needed to determine whether a reduction in proton leak is a critical mechanism leading to a reduction in ROS production and whether long-term changes in proton leak modulate ROS production by inducing alterations in mitochondrial ETC content.

The results of this study indicate that long-term CR is associated with both a decrease in liver mitochondrial proton leak and a reduction in mitochondrial H$_2$O$_2$ production. These changes are consistent with the idea that energy metabolism is decreased with CR. Furthermore, these results contradict the current opinion that a primary function of proton leak is to reduce ROS production and instead suggest that a reduction in proton leak may be a mechanism for reducing ROS production.

GRANTS

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LONG-TERM CALORIE RESTRICTION AND MITOCHONDRIAL PROTON LEAK

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