Effect of caloric restriction on mitochondrial reactive oxygen species production and bioenergetics: reversal by insulin

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Lambert, A. J., and B. J. Merry. Effect of caloric restriction on mitochondrial reactive oxygen species production and bioenergetics: reversal by insulin. Am J Physiol Regul Integr Comp Physiol 286: R71–R79, 2004. First published September 11, 2003; 10.1152/ajpregu.00341.2003.—To gain insight into the antiaging mechanisms of caloric restriction (CR), mitochondria from liver tissue of male Brown Norway rats were used to study the effects of CR and insulin on mitochondrial reactive oxygen species production and bioenergetics. As assessed by hydrogen peroxide measurement, CR resulted in a decrease in the production rate of reactive oxygen species. This decrease was attributed to a decrease in protonmotive force in mitochondria from the CR animals. The decrease in protonmotive force resulted from an increase in proton leak activity and a concomitant decrease in substrate oxidation activity. Each of these effects of CR was reversed by subjecting CR animals to 2 wk of insulin treatment. To achieve continuous and stable insulin delivery, animals were placed under temporary halothane anesthesia and miniosmotic pumps were implanted subcutaneously. To gain further insight into how CR and insulin exerted its effects on mitochondrial bioenergetics, the effects of CR and insulin were quantified using modular metabolic control analysis. This analysis revealed that the effects of CR were transmitted through different reaction branches of the bioenergetic system, and insulin reversed the effects of CR by acting through the same branches. These results provide a plausible mechanism by which mitochondrial reactive oxygen species production is lowered by CR and a complete description of the effects of CR on mitochondrial bioenergetics. They also indicate that these changes may be due to lowered insulin concentrations and altered insulin signaling in the CR animal.

control analysis; aging

Biological aging in mammals is characterized by a progressive decline in cellular function that leads to age-related pathology and death. To date, the most effective way to slow down this deleterious process in laboratory rodents is caloric restriction (CR). By restriction of calories throughout life span, the mean, maximum, and 10th decile of survival in rats and mice is increased. The effect of CR feeding on survival is directly proportional to the intensity of CR and its duration (39). In addition to the positive effects on survivorship, the onset and incidence of age-related disease are retarded (46). CR also ameliorates the age-related decline in a wide variety of processes such as DNA repair and protein turnover (8, 33, 44). As yet the underlying mechanism by which CR brings about these beneficial changes remains to be resolved. An attenuation of the rate of accrual of tissue oxidative damage by decreased generation of oxidants and free radicals provides a plausible explanation of the effect of CR on aging (40).

It has been proposed that mitochondria play a pivotal role in the aging process, for these organelles are considered to be the main producers of reactive oxygen species (ROS) and their DNA is particularly susceptible to free radical damage (9, 45). Mitochondria are also central to bioenergetics, as their main function is to use the electrochemical energy in the protonmotive force Δp (which is generated by proton pumping coupled to electron transport) to synthesize ATP from ADP. Whether CR brings about retarded aging via changes in mitochondrial metabolism remains uncertain, but it is a provocative idea given the reduced rate of ROS generation in mitochondria isolated from tissues of CR animals (17, 36, 43). To date, a complete description of the effects of CR on mitochondrial bioenergetics has not been reported nor has the mechanism underlying the reduced ROS generation from these organelles under CR feeding been identified.

Metabolic control analysis is an approach used to describe and quantify control in complex systems, and it encompasses control analysis, elasticity analysis, and regulation analysis (24). Control analysis describes and quantifies the distribution of control within a system. Elasticity analysis describes the kinetic responses of reactions to changes in levels of intermediates and is used to identify sites of action of effectors within a system. Regulation analysis quantifies the response of a system to an external effector such as an added inhibitor or hormone. Complex systems can be simplified by grouping related processes and intermediates into reaction blocks or modules, known as the “top-down” or “modular” approach of metabolic control analysis (7). This method has been applied to gain understanding of control and regulation in a wide variety of systems, particularly energy metabolism in mitochondria and whole cells. Examples of the approach include analysis of control of energy metabolism in isolated rat liver mitochondria (18); identification of sites of action of thyroid hormones on mitochondrial energy metabolism in isolated rat liver mitochondria (20); analysis of the effects of cadmium on oxidative phosphorylation in isolated potato tuber mitochondria (27–29); characterization of oxidative phosphorylation in pig skeletal muscle mitochondria (35); analysis of calcium regulation of oxidative phosphorylation in rat skeletal muscle mitochondria (26); analysis of ATP turnover, glycolysis, and oxidative phosphorylation in rat hepatocytes (1, 3); analysis of the effects of epinephrine and glucagon on rat hepatocyte metabolism (2); analysis of the effects of aging on mitochondrial energy metabolism in mouse hepatocytes (21); analysis of signal transduction in lymphocytes (31); and analysis of DNA microarray expression data (11).

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We have applied metabolic control analysis to obtain a complete description of the control and regulation of oxidative metabolism in isolated mitochondria in response to CR, and we have used this description to explain the effects of CR on mitochondrial ROS production. Furthermore, CR is known to decrease plasma concentrations of insulin (25), and mice lacking the insulin receptor in adipose tissue, or heterozygous insulin-like growth factor receptor knockout mice, have extended longevity (5, 22). It thus appears that insulin and insulin-like signaling may have a central role in the mammalian aging process. We therefore subjected CR animals to 2 wk of insulin replacement to investigate whether the effects of CR on ROS production and energy metabolism are under insulin control.

MATERIALS AND METHODS

Animals and feeding. All animal husbandry and procedures involved were in accordance with the United Kingdom Animals (Scientific Procedures) Act 1986. Male Brown Norway rats were purchased from Harlan UK and maintained under barrier conditions on a 12:12-h light/dark cycle (0800–2000) at 22 ± 1°C. CR rats were housed singly, and the intake of the pellet diet was limited such that body weights were maintained at 55% of the age-matched fully fed rats. Caloric restriction was initiated at 60 days of age. The restricted diet was preweighed and supplied between 1030 and 1100. CR rats consumed most of their food within ~5 h, effectively making them daytime meal eaters. However, ad libitum-fed rats tend to feed during the dark cycle. Therefore, the fully fed control rats in this study (also housed singly) were given free access to food between 1000 and 1500 to synchronize them to the same feed/fast cycle as the rats on CR. The ages of the animals used in this study were 6.4 ± 0.1 and 18.4 ± 0.1 mo (means ± SE). Hormone treatments and surgery. Solutions of insulin were prepared by dissolving in isotonic saline made pH <2 with acetic acid and making a final concentration of 27 μM with isotonic saline containing 5% (vol/vol) rat serum. Miniosmotic pumps (200–μl/H9262) were filled with 200 μl of the insulin solution and primed in isotonic saline at 37°C for at least 1 h before surgery. The pumps were implanted subcutaneously between the scapulae while the rats were under anesthesia. Rats were initially anesthetized with 1.5% isoflurane and given analgesic (0.05 mg buprenorphin hydrochloride injected per animal). They were then maintained for the operation (duration ~5 min/animal) on 1% isoflurane and 50:50 oxygen-nitrous oxide. Surgery was performed between 0900 and 1100 before the animals were fed. A group of sham-treated animals (n = 10) was prepared using osmotic pumps containing saline without insulin. Blood samples were collected (before feeding) at various intervals post surgery over the treatment period of 2 wk and plasma insulin concentrations were determined by ELISA.

Metabolic control analysis—theory. Consider a simple system: A × B. This system consists of three system variables: an intermediate X (which has a magnitude or concentration); a reaction, group of reactions, or process A (which has a rate or flux, Jx) producing X; and a reaction, group of reactions, or process B (which also has a rate or flux, Jy) consuming X. If a change is made to the activity of the X-producers A (such as addition of an inhibitor) and it is found that the activity of the X-consumers B changes, then process A is said to have control over process B. For example a flux control coefficient of CA = 1 would mean that process A has complete control over the flux through process B. A concentration control coefficient of Cx = 0 would mean that process B has no control over the concentration of the intermediate X. Flux control coefficients sum to 1 and concentration control coefficients sum to 0. The kinetic responses of A and B to changes in X are termed elasticities and can be used to calculate the control coefficients; elasticities allow the sites of action (which reaction, group of reactions, or process in the system) of an effector to be identified. Regulation analysis allows the responses to the effector to be quantified. Consider an external hormone H that is added to the system described above. An integrated response coefficient IRH = 0.5 would indicate that the effect of the hormone on the concentration of X is to increase it by 50%. The power of this approach is that the responses can be partitioned so that the particular routes by which a response is affected can be quantified. For example partial integrated response coefficients δX/H = −0.1 and δX/H = 0.6 would mean that in response to H, process A increases X by 10% and because A is a producer, this is by decreasing the production of X. Acting through B, H increases X by 60%, and because B is a consumer, this is by decreasing the consumption of X. The overall result is an increase in the concentration of X by 50%, quantified by summing the partial integrated response coefficients (−0.1 + 0.6 = 0.5). In this way, the effects of H on the rate of flux through A and B, and the routes by which these responses are affected, can be calculated.

Metabolic control analysis—method. Under phosphorylating (“state III”) conditions, the mitochondrial bioenergetic system was defined as a tripartite network consisting of one intermediate Δp and three subsystems (branches or modules): the Δp producer (substrate oxidation by the electron transport chain, S) and two Δp consumers (the leak flux across the inner mitochondrial membrane, L, and phosphorylation of ADP to ATP, P) as described (18) and shown in Fig. 1. Under nonphosphorylating conditions (“state IV”), the system becomes a two-branch system of substrate oxidation and proton leak, because the phosphorylation branch is not in effect.

The first step in metabolic control analysis of mitochondria is elasticity analysis; this involves comparing the kinetic responses of the subsystems to Δp to see which subsystem(s) have been affected under the conditions employed. The elasticity of the substrate oxidation module to Δp (εSΔp) was calculated from the normalized slope of the plot of Jp vs. Δp during titration of the Δp consumers from state IV (oligomycin present) to state III (oligomycin absent, ADP, glucose, and hexokinase present). The elasticity of the leak to Δp (εLΔp) was determined from the normalized slope of the plot of Jp vs. Δp during titration of the Δp producers with malonate in the presence of oligomycin. The elasticity of the phosphorylation system to Δp (εPΔp) was determined from the normalized slope of the plot of Jp vs. Δp during titration of the Δp producers with malonate in the absence of oligomycin, with ADP, glucose, and hexokinase present. Because total oxygen consumption during state III consists of both phosphorylation and leak components, Jp at any given Δp is calculated as Jp =
Materials. Exogenous insulin was supplied subcutaneously by implanted Alzet miniosmotic pumps, model 2002 (0.55 μl/h, 14 days). Rat plasma insulin concentrations were determined by ELISA (DRG Instruments). \(^3\)H\(_2\)O, \(^{14}\)C-\(\text{glucose}\), and \(^86\)RbCl were purchased from Amersham Pharmacia Biotech; \(^{14}\)C-TPMP\(^*\) was purchased from Moravek Biochemicals. All other chemicals were purchased from Sigma.

Statistical analysis. Data for ROS production, respiration rates, membrane potentials, elasticities, and control coefficients are given as means ± SE, and differences between groups were assessed by Student’s t-test. Unless stated otherwise, \(n = 11, 6,\) and 4 for fully fed, CR, and CR+insulin animals, respectively. Calculation of response coefficients involves combining data from individuals of two different experimental groups (fully fed and CR for example); therefore, to avoid data from any single individual biasing our calculations, the raw data from each fully fed animal was compared with that of each CR to generate a series of all possible integrated response coefficients from the empirical data [known as resampling without replacement (16)]. This set of values was used to calculate 95% confidence intervals and thus whether a response coefficient was either positive or negative, i.e., significantly different from zero and representing a real effect.

RESULTS

Plasma concentrations of insulin at 6 mo of age were as follows: fully fed 1.19 ± 0.13 (\(n = 12\)), CR 0.30 ± 0.04 (\(n = 4\)), and CR+insulin 0.48 ± 0.06 (\(n = 19\)) μg/l. It was not possible to elevate plasma insulin concentrations in CR rats above the value given without inducing symptoms of hypoglycemia. Statistical comparisons are fully fed vs. CR (\(P < 0.001\)), fully fed vs. CR+insulin (\(P < 0.001\)), and CR vs. CR+insulin (\(P < 0.02\)). The sham procedure (implantation of osmotic pumps containing saline without insulin) did not significantly alter the plasma insulin concentrations nor the ROS production rate (not shown).

Figure 2 shows the production rate of hydrogen peroxide of mitochondria from fully fed, CR, and CR+insulin rats. CR resulted in a significant decrease in mitochondrial hydrogen peroxide production rate compared with fully fed controls. This decrease was partially restored to the rate observed in mitochondria from fully fed animals by subjection of CR animals to 2 wk continuous insulin treatment.

The elasticities, flux control coefficients, and concentration control coefficients in mitochondria from fully fed, CR, and

\[ J_S - J_L \] at the same \( \Delta p \). The state III respiration rates were ~20% higher in the absence of hexokinase and glucose (not shown). The fluxes \( J_S \), \( J_L \), and \( J_P \) are given by the oxygen consumption rates driving substrate oxidation, proton leak, and phosphorylation respectively, described below.

The second step is control analysis; this provides a system-level description of the control any particular subsystem has over any other subsystem or intermediate. The flux and concentration control coefficients (\( C^i \) and \( C^m \), respectively) presented in this study were calculated from the elasticities and fluxes and not determined from a complete description of the internal control by the substrate oxidation, leak, and phosphorylation modules over substrate oxidation, leak, phosphorylation, and \( \Delta p \).

The third step is regulation analysis, to determine the magnitude of the system responses to the external effectors (CR or insulin) and to allow the routes by which the responses were effected to be quantified. The fluxes, potentials, elasticities, and control coefficients were used to calculate the partial integrated response coefficients of \( \Delta p \), \( S \), \( L \), or \( P \) to CR and insulin acting through each branch of the system (\( S_L \) or \( P_L \), \( J_L \) and \( \Delta p \) and \( S_L \) or \( J_L \) and \( \Delta p \) respectively, as described previously (26).

Measurement of \( \Delta p \), \( J \), and hydrogen peroxide production. Mitochondria were prepared by differential centrifugation from whole liver between 0830 and 1000 as described previously (41). Mitochondrial protonmotive force (\( \Delta p \)) was determined from the distribution of the potential-dependent probe triphenylmethyl phosphonium cation (TPMP\(^*\)) (6). The distribution of TPMP\(^*\) was measured using a TPMP\(^*\) electrode, constructed in conjunction with a reference electrode, both of which were connected to a voltmeter. A 5-ml magnetically stirred, thermostatically controlled incubation chamber was used with the TPMP\(^*\)/reference electrodes inserted through the lid of the chamber. The base of the chamber contained a Clark-type oxygen electrode (Rank Brothers, Cambridge, UK) to measure oxygen consumption rate (\( J_o \)). The outputs from the voltmeter and oxygen electrode allow recording of \( \Delta p \) and \( J_o \) simultaneously to a double pen chart recorder.

Mitochondria were incubated at a concentration of 1 mg mitochondrial protein/ml in buffer containing 120 mM KCl, 5 mM K\(^+\)-HEPES, 5 mM KH\(_2\)PO\(_4\), 3 mM MgCl\(_2\)-6H\(_2\)O, 5 mM MnCl\(_2\), 0.05% (wt/vol) BSA, 5 μM rotenone, and 80 mg/ml nigericin (pH 7.2 and 37°C). The fully aerated buffer was assumed to contain 406 nmol O\(_2\)/ml (42). For proton leak measurements, the incubation buffer also contained 1 μg/ml oligomycin. For phosphorylation measurements, the incubation buffer also contained 0.2 mM K\(^+\)-ADP, 20 mM glucose, and 0.4 U/ml hexokinase. The TPMP\(^*\) electrode was calibrated with sequential 1 μM additions of TPMP\(^*\) to 5 μM, after which the reaction was initiated with K\(^+\)-succinate to a concentration of 4 mM. Respiration rates and membrane potentials were titrated with 0–10 μM K\(^+\)-malonate, and at the end of each run, FCCP was added to a final concentration of 1 μg/ml to release the TPMP\(^*\) and allow for correction of any small drift in the TPMP\(^*\) electrode.

Mitochondrial TPMP\(^*\) binding corrections were measured as described previously (6). Briefly, mitochondrial matrix volumes were determined from the distribution of \(^3\)H\(_2\)O and \(^{14}\)C-succrose, and TPMP\(^*\) binding corrections were determined by comparison of the accumulation ratios of \(^{14}\)C-TPMP\(^*\) and \(^{86}\)Rb\(^+\). There was no significant effect of CR on the TPMP\(^*\) binding correction factor; the factor (mean ± SE) was 0.29 ± 0.02 (\(n = 2\)) and 0.25 ± 0.02 (\(n = 3\)) in mitochondria from fully fed and CR rats, respectively.

Hydrogen peroxide generation rates were determined fluorometrically by measurement of \( p \)-hydroxyphenyl acetic acid (HPHA) coupled to the enzymatic reduction of H\(_2\)O\(_2\) by horseradish peroxidase (19). Mitochondria were incubated at a concentration of 0.1 mg mitochondrial protein/ml in the standard “state IV” incubation buffer (described above) containing 50 μg/ml HPHA and 4 U/ml horseradish peroxidase. The increase in fluorescence at an excitation of 320 nm and emission of 400 nm was followed on a computer-controlled spectrophotometer with appropriate use of a standard curve generated using known amounts of H\(_2\)O\(_2\).

![Fig. 2. Effect of caloric restriction (CR) and CR+insulin on hydrogen peroxide production by mitochondria in “state IV” conditions. Values are means ± SE, \( n = 14, 9, \) and 3 for fully fed, CR, and CR+insulin, respectively. *\( P < 0.05\) fully fed vs. CR. †\( P < 0.05\) CR vs. CR+insulin. Animals were 6.4 mo of age.](http://ajpregu.physiology.org/doi/10.1152/ajpregu.00246.2004)
CR+insulin animals are displayed in Table 1. In state III conditions, insulin treatment resulted in significant changes in the elasticity of the leak subsystem to $\Delta p$ (C°), control over flux through three branches of the system ($C^p_3$, $C^p_4$, $C^p_5$), and a change in control over the level of $\Delta p$ by the proton leak ($C^p_7$).

As displayed in Figs. 3, 4, and 5, we compared the activities and responses of the subsystems of mitochondria from fully fed and CR animals. CR resulted in a decrease in substrate oxidation activity, i.e., at any given $\Delta p$ the rate of oxygen consumption in mitochondria from CR animals was lower than that seen in fully fed animals (Fig. 3). The partial integrated response coefficients indicate that this decreased flux was mediated mostly through the leak subsystem (Figs. 4 and 5). CR resulted in an increase in the activity of proton leak, i.e., at any given $\Delta p$ the rate of oxygen consumption driving the leak was higher than that seen in mitochondria from fully fed animals (Fig. 3). The partial integrated responses indicate that this increase was mediated mostly through the leak subsystem itself (Figs. 4 and 5). CR resulted in an increase in phosphorylation activity, i.e., at any given $\Delta p$ the rate of oxygen consumption driving phosphorylation was higher than that observed in mitochondria from fully fed animals (Fig. 3). The partial integrated responses indicate that this increase was mediated through the phosphorylation and substrate oxidation subsystems (Fig. 5). The state IV and state III membrane potentials decreased in response to

<table>
<thead>
<tr>
<th>Phosphorylating (State III)</th>
<th>Nonphosphorylating (State IV)</th>
</tr>
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<tbody>
<tr>
<td>Fully Fed</td>
<td>CR</td>
</tr>
<tr>
<td>$e_{\Delta p}^{c_p}$</td>
<td>$-14.5 \pm 3.2$</td>
</tr>
<tr>
<td>$e_{\Delta p}^{c_p}$</td>
<td>$3.1 \pm 0.6$</td>
</tr>
<tr>
<td>$C_{3}^{p}$</td>
<td>$8.5 \pm 1.2$</td>
</tr>
<tr>
<td>$C_{4}^{p}$</td>
<td>$0.35 \pm 0.04$</td>
</tr>
<tr>
<td>$C_{5}^{p}$</td>
<td>$0.23 \pm 0.04$</td>
</tr>
<tr>
<td>$C_{6}^{p}$</td>
<td>$0.42 \pm 0.02$</td>
</tr>
<tr>
<td>$C_{7}^{p}$</td>
<td>$0.17 \pm 0.04$</td>
</tr>
<tr>
<td>$C_{8}^{p}$</td>
<td>$0.94 \pm 0.01$</td>
</tr>
<tr>
<td>$C_{9}^{p}$</td>
<td>$-0.12 \pm 0.03$</td>
</tr>
<tr>
<td>$C_{10}^{p}$</td>
<td>$0.43 \pm 0.05$</td>
</tr>
<tr>
<td>$C_{11}^{p}$</td>
<td>$-0.14 \pm 0.01$</td>
</tr>
<tr>
<td>$C_{12}^{p}$</td>
<td>$0.71 \pm 0.04$</td>
</tr>
<tr>
<td>$C_{13}^{p}$</td>
<td>$0.06 \pm 0.01$</td>
</tr>
<tr>
<td>$C_{14}^{p}$</td>
<td>$-0.02 \pm 0.00$</td>
</tr>
<tr>
<td>$C_{15}^{p}$</td>
<td>$-0.04 \pm 0.01$</td>
</tr>
<tr>
<td>$C_{16}^{p}$</td>
<td>$-0.04 \pm 0.01$</td>
</tr>
</tbody>
</table>

Values are means ± SE. See materials and methods for more details. CR, calorie restricted. *Significant difference CR vs. CR+insulin, $P < 0.05$. 

Fig. 3. Effect of CR on the activities of substrate oxidation, proton leak, and phosphorylation. Closed and open symbols represent mitochondria from fully fed and CR animals, respectively. Rate of the substrate oxidation subsystem, $J_S$ (squares, short dashed lines), was measured at different values of $\Delta p$ by titration from state IV respiration (presence of oligomycin) to state III respiration (absence of oligomycin, presence of ADP, hexokinase, and glucose). Rate of the proton leak subsystem, $J_L$ (diamonds, solid lines), was measured at different values of $\Delta p$ by titration of state III respiration with malonate. Rate of the phosphorylation subsystem, $J_P$ (triangles, long dashed lines), was measured at different values of $\Delta p$ by titration of state II respiration with malonate, with subtraction of $J_L$ at the same $\Delta p$. Each point represents the mean of 11 or 6 separate animals (fully fed and CR, respectively); error bars are omitted for clarity and did not exceed more than 3% for $\Delta p$ and 10% for $J_O$ for fully fed and CR.

Fig. 4. Division of the integrated responses to CR of substrate oxidation rate, proton leak rate, and $\Delta p$ into partial integrated responses via each of the 2 subsystems in nonphosphorylating mitochondria. Partial integrated responses via the substrate oxidation and proton leak subsystems are given and represented by the relative weight of the arrows: black indicates a positive response; gray indicates a negative response.
CR (IR\text{CR} was negative, Figs. 4 and 5). These decreases in $\Delta p$ were significantly different ($P < 0.05$). The state IV and state III respiration rates were not significantly affected by CR ($39.0 \pm 1.8 \text{ nmol O}_2\text{min}^{-1}\text{mg}^{-1}$ fully fed vs. $41.4 \pm 2.5 \text{ nmol O}_2\text{min}^{-1}\text{mg}^{-1}$ CR, and $96.6 \pm 9.2 \text{ nmol O}_2\text{min}^{-1}\text{mg}^{-1}$ fully fed vs. $114.1 \pm 7.6 \text{ nmol O}_2\text{min}^{-1}\text{mg}^{-1}$ CR, respectively).

We next examined the activities and responses of the subsystems of mitochondria of CR animals subjected to insulin treatment, as displayed in Figs. 6, 7, and 8. In terms of substrate oxidation, insulin resulted in a reversal of the effects seen in CR animals compared with fully fed, i.e., the activity of substrate oxidation was shifted away from the profile seen in mitochondria from CR rats such that it resembled the profile observed in mitochondria from fully fed animals. The activity of proton leak in response to insulin was not only shifted back toward the profile seen in mitochondria from fully fed animals, but an “overshoot” effect was also observed, such that the activity of the leak in mitochondria from insulin-treated animals was even lower than that seen in fully fed. In terms of the phosphorylation system, insulin also reversed the fully fed to CR response, but the effect was relatively small compared with the effects on substrate oxidation and proton leak. The state IV and state III membrane potentials increased significantly ($P < 0.05$) in response to insulin (IR\text{INS} was positive, Figs. 7 and 8). The state IV $\Delta p$ was $182.9 \pm 2.3 \text{ mV}$, and the state III $\Delta p$ was $172.1 \pm 1.4 \text{ mV}$. The state IV respiration rate was significantly ($P < 0.05$) lowered by insulin treatment ($41.4 \pm 2.5 \text{ CR vs. } 29.4 \pm 2.1 \text{ nmol O}_2\text{min}^{-1}\text{mg}^{-1}$ CR insulin), whereas the state III respiration rate was unchanged ($114.1 \pm 7.6 \text{ CR vs. } 99.2 \pm 7.8 \text{ nmol O}_2\text{min}^{-1}\text{mg}^{-1}$ CR insulin). Examination of the partial integrated response coefficients of each subsystem (Figs. 7 and 8) indicates that insulin reversed the effects of CR by acting through the same subsystems as CR. In other words, all the corresponding gray arrows in Figs. 4 and 5 are black in Figs. 7 and 8, and the corresponding black arrows in Figs. 4 and 5 are gray in Figs. 7 and 8.

The total integrated responses to CR and insulin are given in Table 2. The change in sign for substrate oxidation and leak (from negative to positive and positive to negative, respectively) indicate the reversal of the effects of CR by insulin in
these two subsystems. The effect of CR on the phosphorylation subsystem was also reversed by insulin. The effect of CR on $\Delta p$ was reversed by insulin as indicated by the change of sign from negative to positive.

An additional set of experiments was conducted using fully fed and CR animals at 18 mo of age, shown in Fig. 9. As with mitochondria from the 6-mo-old group, the hydrogen peroxide production rate and membrane potential were significantly lower in mitochondria from the CR animals. In addition, $\Delta p$ was lower due to an increase in proton leak and a decrease in substrate oxidation. The control coefficients and integrated responses at 18 mo of age were similar to those at 6 mo (not shown).

**DISCUSSION**

In mitochondria from young fully fed animals, the membrane potentials and oxygen consumption rates used to determine the activities of substrate oxidation, proton leak, and phosphorylation are in agreement with published values for liver mitochondria from young fully fed rats (18). Our data also support previous studies showing that CR results in a lower rate of mitochondrial ROS production (14, 17, 36, 43).

The primary finding of this work is that the metabolic control analysis suggests a mechanistic explanation for the lowered ROS generation rates observed in isolated mitochondria from CR rodents. In addition, this lowering of ROS production by CR is susceptible to control by plasma insulin. The production rate of ROS is exquisitely sensitive to $\Delta p$.

**Table 2. Total IR of substrate oxidation flux, leak flux, phosphorylation flux, and $\Delta p$ to the fully fed to CR transition and the CR to CR+insulin transition**

<table>
<thead>
<tr>
<th>System Variable</th>
<th>Lower Mean</th>
<th>Upper Mean</th>
<th>Lower Mean</th>
<th>Upper Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Nonphosphorylating</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Substrate oxidation</td>
<td>$-0.89$</td>
<td>$-0.62^*$</td>
<td>$-0.36$</td>
<td>$0.48$</td>
</tr>
<tr>
<td>Proton leak</td>
<td>$0.16$</td>
<td>$0.25^*$</td>
<td>$0.35$</td>
<td>$-0.41$</td>
</tr>
<tr>
<td>$\Delta p$</td>
<td>$-0.031$</td>
<td>$-0.026^*$</td>
<td>$-0.017$</td>
<td>$0.026$</td>
</tr>
<tr>
<td><strong>Phosphorylating</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Substrate oxidation</td>
<td>$-0.34$</td>
<td>$-0.19^*$</td>
<td>$-0.05$</td>
<td>$0.01$</td>
</tr>
<tr>
<td>Proton leak</td>
<td>$0.21$</td>
<td>$0.33^*$</td>
<td>$0.44$</td>
<td>$-0.57$</td>
</tr>
<tr>
<td>Phosphorylation</td>
<td>$0.45$</td>
<td>$0.79^*$</td>
<td>$1.14$</td>
<td>$-0.25$</td>
</tr>
<tr>
<td>$\Delta p$</td>
<td>$-0.04$</td>
<td>$-0.03^*$</td>
<td>$-0.02$</td>
<td>$0.02$</td>
</tr>
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</table>

IR, integrated responses. $^*$Significant response of system variable of fully fed animals to CR, $P < 0.05$. $^+$Significant response of system variable of animals on CR to insulin, $P < 0.05$. 

**Fig. 9. Bioenergetic data and hydrogen peroxide production (inset) by mitochondria from 18-mo-old animals.** Data are from mitochondria in nonphosphorylating conditions. Closed and open symbols represent mitochondria from fully fed and CR animals, respectively. Each point represents the mean of 5 or 12 separate animals (fully fed and CR, respectively). Substrate oxidation subsystem is shown as squares with short dashed lines, the proton leak subsystem is shown as diamonds, with solid lines. Error bars are omitted for clarity. *Inset: values are means ± SE, n = 10 and 11 for fully fed and CR, respectively. $^*P < 0.05$ fully fed vs. CR.
 decreases in Δp can result in a 2- to 2.5-fold lowering of the ROS production rate (19, 30, 34). We found that CR resulted in a small but significant decrease in Δp, and this could therefore explain why a decreased rate of production of ROS is seen in mitochondria from CR animals. The CR-induced decrease in Δp was reversed by insulin treatment, which can explain the reversal of the CR-induced decrease in ROS production. The decrease in Δp induced by CR was due to a decrease in substrate oxidation activity and an increase in proton leak activity. A decrease in substrate oxidation activity means that the electron transport chain will pump protons at a slower rate; thus Δp will fall and oxygen consumption will decrease. An increase in proton leak activity means that the rate of return of protons from the intermembrane space to the matrix is increased; thus Δp will fall and oxygen consumption will increase. This explains why Δp falls in response to CR, yet the state IV respiration rate is unchanged; the increase in J$_l$ is cancelled by the decrease in J$_o$, thus J$_o$ for state IV remains the same. Insulin reversed the changes in Δp induced by CR by increasing the activity of the substrate oxidation subsystem and lowering the activity of the leak subsystem. The net effect of these changes in activity in these two subsystems will be to increase Δp. The overshoot effect of insulin on the proton leak resulted in a lowering of the state IV respiration rate; the decrease in J$_l$ was greater than the increase in J$_o$; thus J$_o$ decreases.

CR did not significantly affect the elasticities of any branch of the system to Δp. In other words, when Δp changes by a given amount, the changes in activity of each branch of the system will be the same, irrespective of whether the mitochondria are from a fully fed or CR animal. However, if the mitochondria are in state III and from a CR animal that has been subjected to insulin treatment, then the change in activity of proton leak will be much greater in response to a given change in Δp.

CR did not result in any major shifts in the internal control coefficients of mitochondria, in that the flux and concentration control coefficients were not significantly different in mitochondria from fully fed and CR animals. Therefore the distribution of control by the three branches of the system (substrate oxidation, leak, and phosphorylation) over substrate oxidation, leak, phosphorylation, and Δp is not susceptible to change by a decrease in caloric intake. The internal control of mitochondria appears to be a stable property of the network; treatment of potato tuber mitochondria with cadmium did not alter the control coefficients, despite inhibiting substrate oxidation and proton leak (27). Despite internal control being stable in response to CR, we did find that insulin treatment resulted in a significant shift in the distribution of control in phosphorylating mitochondria. Therefore it is apparent that insulin is a powerful modulator of mitochondrial bioenergetics, specifically the subsystems controlling or controlled by proton leak.

Regulation analysis indicated that CR resulted in a decrease in the activity of the substrate oxidation module. This perhaps may be expected of mitochondria from animals that essentially are restricted in substrate supply, and there is evidence that CR results in a shift away from glycolysis and substrate oxidation toward gluconeogenesis (13). In addition the activity of the individual complexes of substrate oxidation appear to be lowered by CR (12, 47). This decrease in substrate oxidation was mediated primarily through the leak and phosphorylation subsystems and insulin treatment reversed this decrease by acting through the same subsystems. The decrease in substrate oxidation activity with normal aging is usually considered to be a negative effect; here it appears that it is a positive effect in that it reduces Δp and ROS production in mitochondria from CR animals. We postulate that the decrease in substrate oxidation activity in response to CR is a regulated change, in that it is an adaptation to reduced caloric intake. Conversely the decrease in substrate oxidation with aging is likely to be unregulated, perhaps due to damage to the individual complexes of the electron transport chain.

An increase in the activity of proton leak was also observed in mitochondria from CR animals. As with substrate oxidation, insulin treatment reversed this effect by acting through the same subsystem. There is no evidence to indicate that liver mitochondria contain a GDP-sensitive uncoupling protein (UCP) (15), so it is unlikely that the increase in proton leak rate was due to an increase in UCP activity. However, the increase in proton leak activity could be the result of an increase in inner membrane surface area, changes in fatty acid composition, or a change in some other protein or carrier that catalyzes a proton leak. The precise effects of CR on mitochondrial proton leak are unclear and may be tissue and/or age specific. Lal et al. (32) reported that in skeletal muscle mitochondria from rats at 30 mo of age, the rate of proton leak was lower than the rate observed in mitochondria from fully fed animals, but this interpretation of this data has been questioned (40). As yet there is no other published evidence of how CR effects proton leak or mitochondrial morphology.

An increase in the activity of the phosphorylation module was seen in mitochondria from animals on CR. The phosphorylation system comprises numerous components, including the ATPase, adenine nucleotide translocator (AdN), the phosphate carrier, the adenine nucleotide pool, and matrix magnesium. Changes seen in the activity of phosphorylation may be due to an increase in one or more of the activities of these components. Overall this indicates that at a given proton motive force the mitochondria will synthesize ATP at a faster rate. This perhaps reflects the need of the CR animals to produce energy equivalents at a faster rate, because the CR animals are limited in caloric intake but do not show a depression in whole body metabolic rate (38). Again the effects of CR were reversed via the same pathways by insulin treatment.

In terms of the effects of aging, we found an increase in mitochondrial hydrogen peroxide production rate between 6 and 18 mo, and this increase was consistent with a significant increase in membrane potential. These effects were observed in animals from both fully fed and CR groups. There is of course another set of response coefficients within the data, namely the responses of the animals on each diet to age (S, L, P, or R$_{Age}$, see Table 1). However, we chose to focus on the effects of CR to attempt to elucidate its mechanism of action, as opposed to merely showing a set of age-related changes that are or are not affected by diet.

Insulin and insulin-like signaling are believed to play a role in the nematode, fly, and rodent models of aging. For example, C. elegans, with mutations in thedaf-2 insulin receptor-like gene, live approximately twice as long as wild-type controls (48). Similarly, in D. melanogaster, the chico mutation in the insulin-like signaling pathway extends life span (10). In mammals, the Ames and Snell Dwarf mice, both long-lived, also display altered insulin signaling (4,
23. It is known that insulin itself stimulates hydrogen peroxide (and presumably other ROS) production (37). It is possible that reduced insulin signaling could lead to reduced substrate oxidation and increased proton leak, the net effect of this will be to lower Δp and lower the production rate of ROS. This in turn leads to a lower accumulation rate of oxidative damage, and hence a slower rate of aging. Further experiments are required to ascertain whether what is observed for liver mitochondria represents a general case for all tissues in CR rodents and is part of the mechanism underlying increased survival in long-lived mutants with altered insulin signaling.

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REFERENCES


