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The effect of aging and caloric restriction on mitochondrial protein density and oxygen consumption

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Abstract

It has been proposed that part of the anti-aging mechanism of caloric restriction (CR) involves changes in mitochondrial function. To investigate this hypothesis, mitochondria from various tissues of male Brown Norway rats (fully fed and CR) were isolated and respiration rates determined. In mitochondria from liver, heart, brain and kidney, there were no significant effects of CR on state 4 mitochondrial respiration rate. Further experiments using liver mitochondria under a variety of incubation conditions confirmed that CR does not alter mitochondrial respiration rate in this tissue. However, the respiration rate of mitochondria from brown adipose tissue (BAT) of CR animals was approximately three-fold higher compared to mitochondria from fully fed controls. Mitochondrial protein density was significantly higher in liver tissue of CR animals; it was significantly lower in heart and unchanged in BAT. It is concluded that whilst CR results in tissue-specific changes in mitochondrial respiration rate, these effects do not explain the CR-induced changes in free radical production reported previously for these organelles.

Keywords: Caloric restriction; Aging; Mitochondria; Respiration; Metabolism; Uncoupling

1. Introduction

To date the most effective way to extend mean and maximum lifespan in mammals, retard the onset and incidence of age-related disease, and ameliorate the age-related decline in physiological function is caloric restriction (CR). This dietary intervention typically involves restriction of overall caloric intake to the order of 20–50% of fully fed control animals for the majority of lifespan. This under-nutrition without malnutrition has robust and reproducible effects on extending survival in laboratory rodents (Merry, 2002; Weindruch and Walford, 1988), various invertebrate species (Weindruch and Walford, 1988) and perhaps non-human primates (Ramsey et al., 2000a). As yet the molecular mechanisms underlying these striking effects of caloric intake on aging have remained elusive. However, increasing evidence suggests that accumulation of oxidative damage to proteins, lipids and DNA may be a key factor in normal aging, and CR may operate by slowing down the accumulation of these and other aberrant molecules (Sohal and Weindruch, 1996). There has been, therefore, an increasing focus on the role of mitochondria in the aging process, since these organelles are believed to be the primary sources of reactive oxygen species (ROS), which can give rise to oxidative damage. Whilst CR does indeed appear to lower ROS production (Gredilla et al., 2001a,b; Lopez-Torres et al., 2002; Sohal et al., 1994; Lambert and Merry, 2004), it is unclear if this phenomena is simply linked to changes in mitochondrial respiration rate. The effect of CR on mitochondrial respiration is uncertain, as the published reports are contradictory. Gredilla et al. (2001a,b) and Lopez-Torres et al. (2002) reported no effects of CR whilst Sohal et al. (1994) and Lal et al. (2001) reported significant decreases. Weindruch et al. (1980) reported significant increases or no changes depending on the conditions. While the standard metabolic rate is reported not to be significantly different in age-matched control and CR animals (McCarter and McGee, 1989; McCarter and Palmer, 1992) this observation is difficult to reconcile with those reports that CR feeding

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results in a significant depression in both states 4 and 3 mitochondrial respiration rates (Sohal et al., 1994). Hence in this report, we reassess in detail the effect that CR feeding has on mitochondrial respiration rate, and relate these findings to mitochondrial protein density and published data on the effect of CR on the standard metabolic rate.

2. Materials and methods

2.1. Animals and feeding

All animal husbandry and procedures involved were in accordance with the UK Animals (Scientific Procedures) Act 1986. Male Brown-Norway rats were purchased from Harlan UK and maintained under barrier conditions on a 12-h light/dark cycle (08:00–20:00 hours) at 22 ± 1 °C. Calorie-restricted rats were housed singly and the intake of the pelleted diet was limited such that body weights were maintained at 50–55% of the age-matched fully fed rats. CR was initiated at 60 days of age. The restricted diet was preweighed and supplied between 10:30 and 11:00 hours. CR rats consumed most of their food within about 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 10:00 and 15:00 hours, to synchronize them to the same feed/fast cycle as the rats on CR. The ages of the animals used in this study were 6.5 ± 0.7 and 16.6 ± 0.2 months (means ± standard errors). CR animals were subjected to the restricted diet continuously for 4.5 or 15 months (i.e. from 60 days of age to 6.5 or 17 months of age).

2.2. Isolation of mitochondria

Mitochondria were isolated using standard published methodologies (Lai and Clark, 1979; Mela and Seitz, 1979; Pedersen et al., 1978; Hittelman et al., 1969), with minor modifications. Briefly, livers and kidneys were removed and washed in ice-cold isolation buffer containing 210 mM mannitol, 70 mM sucrose, 5 mM HEPES, 1 mM EGTA and 0.05% (w/v) BSA, pH 7.4. The tissues were chopped and homogenized in isolation buffer, and the homogenates centrifuged at 1600*g* for 10 min. The supernatants were centrifuged at 7500*g* for 10 min to yield the crude mitochondrial pellet. The pellets were washed twice by resuspension and centrifugation, and washed once in isolation buffer without EGTA. The final mitochondrial pellets were resuspended in isolation buffer without EGTA.

Hearts were removed and washed in ice-cold isolation buffer containing 225 mM mannitol, 75 mM sucrose, 10 mM Tris HCl and 1 mM EGTA, pH 7.4. The hearts were chopped, washed and homogenized in isolation buffer containing 0.17 mg ml^{-1} nagarse, and the homogenates centrifuged at 10,000*g* for 5 min. The pellet was re-homogenized in isolation buffer and centrifuged at 480*g* for 10 min. The supernatant was centrifuged at 7700*g* for 10 min to yield the crude mitochondrial pellet. The pellet was washed twice by resuspension and centrifugation, and washed once in isolation buffer without EGTA. The final mitochondrial pellet was resuspended in isolation buffer without EGTA.

Brains were removed and washed in ice-cold isolation buffer containing 250 mM sucrose, 0.5 mM K^+EDTA and 10 mM Tris HCl, pH 7.4. The brains were chopped, washed and homogenized in isolation buffer and the homogenates centrifuged at 2000*g* for 3 min. The supernatant was centrifuged at 12,500*g* for 10 min, and the pellet resuspended in 12 ml of 3% (w/v) Ficoll, 120 mM mannitol, 30 mM sucrose, 25 μM K^+EDTA and 5 mM Tris HCl, pH 7.4. This suspension was then layered onto 50 ml of 6% (w/v) Ficoll, 240 mM mannitol, 60 mM sucrose, 50 μM K^+EDTA and 10 mM Tris HCl and centrifuged at 11,500*g* for 30 min. The pellet was resuspended in isolation buffer and centrifuged at 11,500*g* for 10 min. The final mitochondrial pellet was resuspended in isolation buffer without EDTA.

Brown adipose tissue (BAT) was removed from the inter-scapular region and washed in ice-cold isolation buffer containing 250 mM sucrose and 5 mM Tris HCl, pH 7.2. The tissue was chopped, washed and homogenized in isolation buffer and the homogenate was centrifuged at 8500*g* for 10 min. The pellet was resuspended in isolation buffer and centrifuged at 700*g* for 10 min, the supernatant was then centrifuged at 8500*g* for 10 min. The pellet was resuspended and washed in isolation buffer containing 100 mM KCl, 20 mM Tris and 0.2% BSA (fatty acid free), pH 7.2, and finally resuspended in a small volume of this buffer.

Mitochondrial protein concentration was determined by Bradford's (1976) assay using BSA as standard. All mitochondrial isolations were performed between 08:30 and 11:00 hours, before the animals were fed. Tissues were weighed prior to isolation of mitochondria, to allow the determination of yields, and calculation of mitochondrial protein density.

2.3. Measurement of mitochondrial respiration rate

Non-phosphorylating (state 4) mitochondrial respiration rates were measured polarographically using a Clark-type oxygen electrode (Rank Brothers, Cambridge). For heart, liver, kidney and brain, mitochondria were incubated at a concentration of 0.4 mg mitochondrial protein ml^{-1} in buffer containing 120 mM KCl, 5 mM K^+ HEPES, 5 mM KH_2PO_4 and 3 mM $MgCl_2{\cdot}7H_2O$ (pH 7.2 and 37 °C). Succinate (K^+ salt) was added to a concentration of 5 mM to initiate respiration. State 3 respiration rates were determined after addition of 0.1 mM ADP. Additional experiments with liver were performed under various conditions as described in the text. For BAT, mitochondria were incubated in buffer

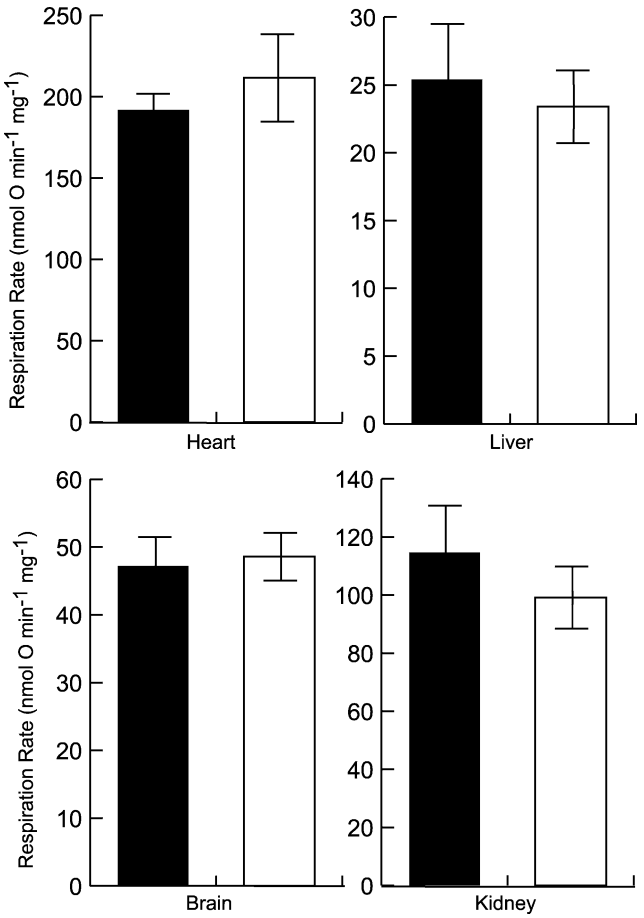

Fig. 1. Effect of CR on mitochondrial respiration rates in state 4 conditions at 6.5 months of age. Black bars represent fully fed, white bars represent CR. Values are means ± SEM, $n = 6$ for fully fed and CR (heart, liver, brain), and $n = 3$ for fully fed and CR (kidney).

containing 100 mM KCl, 20 mM Tris, 4 mM KH_2PO_4, 2 mM $MgCl_2{\cdot}7H_2O$, 1 mM EDTA and 0.05% BSA (fatty acid free), pH 7.2 and 37 °C. Glycerol-3-phosphate was added to a concentration of 10 mM to initiate respiration. The oxygen content of air-saturated buffer was assumed to be 406 nmol O ml^{-1} (Reynafarje et al., 1985).

2.4. Materials

All chemicals were purchased from Sigma.

2.5. Statistical analysis

Data are given as means ± the standard error of the mean, and differences between groups were assessed by Student's *t*-test. In all cases, *n* is the number of animals examined.

3. Results

Fig. 1 shows that at 6.5 months of age, CR had no significant effect on state 4 respiration rate in mitochondria from heart, liver, kidney and brain. As shown in Table 1, at 6.5 months of age, no significant effects of CR on state 3 respiration rates were found, thus CR did not affect the respiratory control ratio. No effect of CR was seen on state 3 respiration rates of kidney mitochondria (not shown). An additional set of experiments was conducted using liver mitochondria to investigate mitochondrial respiration in detail, and the results are displayed in Fig. 2. Under a variety of conditions (plus oligomycin, rotenone, nigericin, BSA and EGTA) and in the absence of added succinate (state 1 conditions), no differences in mitochondrial respiration rate between fully fed controls and CR animals were detected. In mitochondria from BAT (Fig. 3), there was a significant increase in mitochondrial respiration rate in CR, and this difference was still significant in the presence of GDP. In the presence of oligomycin, the mitochondrial respiration rates were also significantly higher in CR (not shown). Fig. 4 shows state 4 rates of oxygen consumption in mitochondria isolated from animals of approximately 17 months of age. Again there were no significant effects of CR in the tissues examined. As shown in Table 1, the state 3 rates were significantly lower in liver and heart mitochondria from CR animals at 17 months of age compared to mitochondria from fully fed animals. Comparison of state 4 oxygen consumption rates of mitochondria from 6.5-month-old animals to

Table 1
State 3 respiration rates and respiratory control ratios (RCR) for mitochondria from tissues of 6.5 and 16.6 month old fully fed and CR rats

	Liver		Heart		Brain	
	FF	CR	FF	CR	FF	CR
6.5 months of age						
State 3 respiration rate (nmol O min^{-1} mg^{-1})	78.1 ± 6.9	76.2 ± 2.3	480 ± 22	425 ± 28	74.0 ± 9.6	71.0 ± 4.7
RCR	3.4 ± 0.40	2.5 ± 0.4	2.5 ± 0.1	2.1 ± 0.1	1.6 ± 0.1	1.5 ± 0.1
16.6 months of age						
State 3 respiration rate (nmol O min^{-1} mg^{-1})	85.3 ± 7.5	56.0 ± 3.9[a,b]	541 ± 29	450 ± 0.04[a]	73.9 ± 4.8	83.7 ± 3.5
RCR	3.9 ± 0.2	2.8 ± 0.2[a]	2.4 ± 0.1	2.3 ± 0.1	1.6 ± 0.0	1.6 ± 0.0

Values are means ± SEM, $n = 6$. The RCR is the state 3 rate divided by the state 4 rate.
[a] Significant ($p < 0.05$) difference fully fed vs CR.
[b] Significant difference ($p < 0.005$) 6.5 months vs 16.6 months.

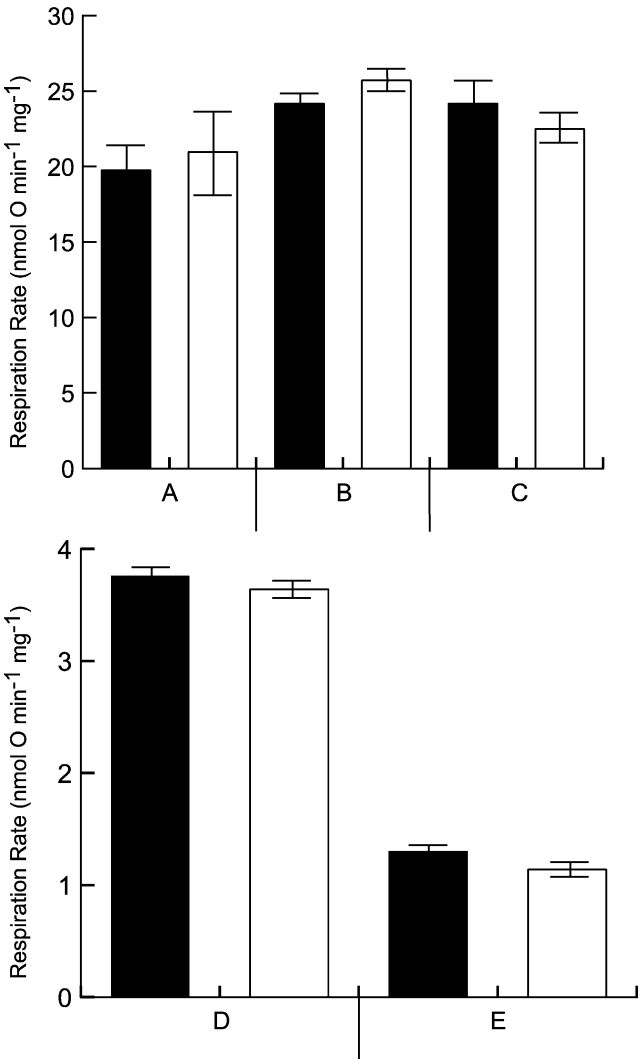

Fig. 2. Effect of CR on liver mitochondrial respiration rates under various conditions at 6.5 months of age. Black bars represent fully fed, white bars represent CR. Values are means ± SEM, $n = 2$ for fully fed and CR. Condition A is succinate only, condition B is succinate, oligomycin and nigericin, condition C is succinate, oligomycin. nigericin, EGTA, BSA and rotenone, condition D is no additions, condition E is oligomycin. nigericin, EGTA, BSA and rotenone.

those from 17-month-old animals revealed no effect of age in either the fully fed or CR groups. A decline with age in state 3 respiration rate was seen in liver mitochondria from CR animals.

Tissue mass and yield data are given in Table 2. In absolute terms, there was significantly less tissue in animals maintained on CR feeding, but when normalized to body mass, there was significantly more heart tissue in the CR animals. In absolute terms, there was significantly less mitochondrial protein in heart tissue of animals on CR, with the other tissues remain unchanged. However, when normalized to tissue mass, there was significantly more mitochondrial protein in the livers of CR animals and significantly less heart mitochondrial protein.

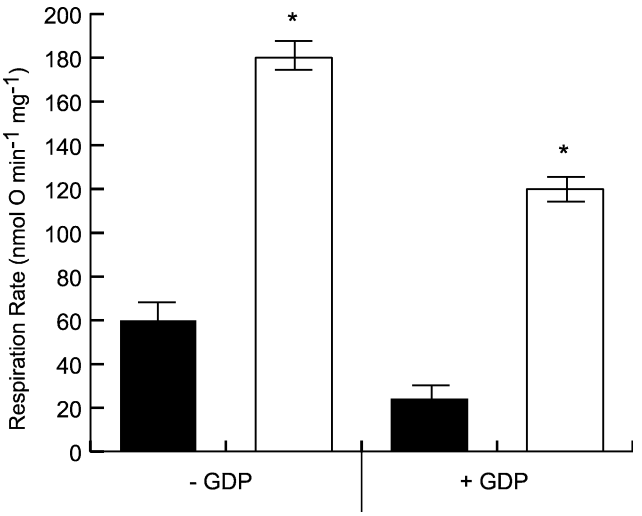

Fig. 3. Effect of CR on state IV respiration rate of mitochondria from BAT at 6.5 months of age. Black bars represent fully fed, white bars represent CR. Values are means ± SEM, $n = 6$ for fully fed and $n = 5$ for CR. * Significant difference control vs CR ($p < 0.001$).

4. Discussion

Mitochondria respiring in vivo exist in an intermediate state between states 3 and 4. We chose to focus on state 4 mitochondrial respiration rates when ROS generation is at its highest. Most mitochondria in vivo are usually closer to state 4 than to state 3 (indicated by the observation that maximum metabolic rate in animals is 3–5 times higher than the field metabolic rate (Nagy, 1987). However, state 3 respiration rates were determined to ensure that any effects of CR on maximum mitochondrial respiration rate would be detected if present. No significant effects of CR on state 4 respiration rates in mitochondria isolated from heart, liver, brain or kidney could be demonstrated. This was difficult to reconcile with certain previous reports that calorie-restricted feeding depressed state 4 respiration in heart, brain and kidney mitochondria isolated from mice (Sohal et al., 1994). State 4 respiration rates were, therefore, investigated in more detail to determine if any effects of CR could be observed under specific conditions. A variety of experimental incubation conditions were used which are frequently employed when studying mitochondrial respiration. We tested oligomycin (inhibits ATP synthesis), rotenone (prevents reverse electron flow at complex 1), BSA (binds free fatty acids), nigericin (clamps the pH difference across the inner mitochondrial membrane to zero) and EGTA (binds calcium ions). Under these various conditions, and in the absence of exogenous succinate (state 1), liver mitochondria from 6.5-month-old fully fed and calorie-restricted animals maintained the same rates of respiration. At 6.5 months of age, the CR animals had been on 55% CR for 4.5 months, (as CR was initiated at 2 months). It was possible that for certain tissues, this length of time on CR was insufficient for the full effects of

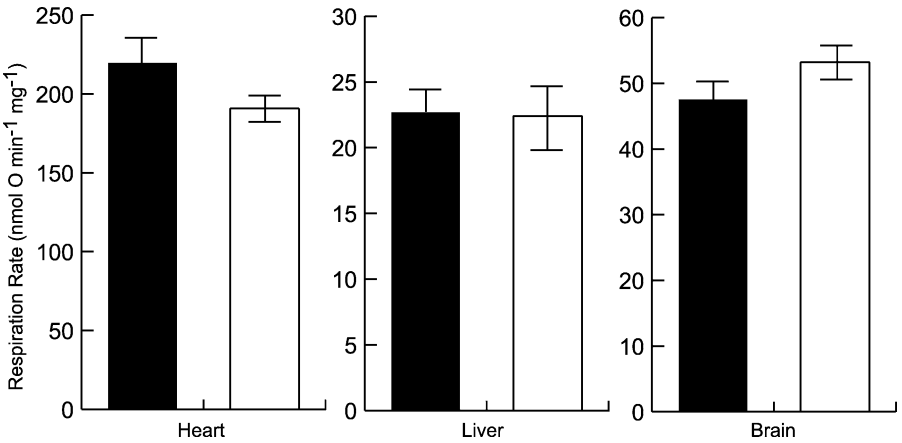

Fig. 4. Effect of CR on mitochondrial respiration rates in state 4 conditions at approximately 17 months of age. Black bars represent fully fed, white bars represent CR. Values are means ± SEM, $n = 6$ for fully fed and CR.

CR feeding on mitochondrial respiration rate to be manifest. However, this possibility was excluded since even at 16.6 months of age (about 15 months on CR), there were still no apparent effects of CR feeding on mitochondrial state 4 respiration rates.

The effect of CR on mitochondrial respiration rate may be species and/or tissue-dependent, or the effects may depend on the specific experimental conditions employed. No effect of CR on state 4 respiration rate in mitochondria from liver or brain in either adult (9–12 months) or old (23–26 months) mice had been reported previously (Weindruch et al., 1980). Similarly no effect of short- or long-term CR feeding on state 4 respiration rates in mitochondria from rat heart or liver was found (Gredilla et al., 2001a,b; Lopez-Torres et al., 2002). We conclude, therefore, that CR feeding does not depress mitochondrial respiration rates and it cannot explain the lowered rates of ROS production reported for mitochondria isolated from animals on CR feeding. If the state 4 respiration rate is unaltered under CR feeding conditions, however, lowered ROS production could still be explained by a lowering of the mitochondrial proton motive force (Δp) since the production rate of ROS is exquisitely sensitive to Δp (Korshunov et al., 1997; Liu, 1997; Votyakova and Reynolds, 2001). This would require an increase in the proton conductance of the inner mitochondrial membrane and/or a lowering of substrate oxidation, leading to a lower reduction state of the superoxide generators within the electron transport chain. (Merry, 2002). This has been demonstrated recently (Lambert and Merry, 2004) and is consistent with the suggestion that a lowered reductive state exists for complex I in mitochondria isolated from CR fed animals (Gredilla et al., 2001b).

An interesting observation of this study was that CR resulted in a marked increase in mitochondrial respiration rate from BAT. Since this difference was still present in the presence of GDP, the increase in respiration rate cannot be due solely to an increase in uncoupling protein (UCP) activity. The main physiological function of BAT in mammals is to generate heat to maintain a constant body temperature. According to the chemiosmotic theory of mitochondrial function (Mitchell and Moyle, 1967), protons pumped from the matrix to the inter-membrane space generate an electrochemical energy gradient in the form of a protonmotive force (Δp). When the protons re-enter the matrix via ATP synthase, Δp falls and the resulting free energy is coupled to the synthesis of ATP from ADP. However, in non-shivering thermogenesis, UCP-1 (which is present in BAT) catalyses the direct leak of protons from the inter-membrane space back into the mitochondrial matrix (Nicholls et al., 1986). Since Δp falls but this reaction is not coupled, the free energy is lost as heat. UCPs are inhibited by GDP, which is a useful tool to detect UCP activity. Thus, UCP-1 is inducible in response to cold-stress in

Table 2
Tissue mass and yield data for 6.5 month old fully fed and CR rats

	Liver		Heart		BAT	
	FF	CR	FF	CR	FF	CR
Tissue mass (g)	3.07 ± 0.33	1.86 ± 0.19[a]	1.13 ± 0.07	0.75 ± 0.04[a]	0.69 ± 0.06	0.48 ± 0.02[a]
Tissue mass (g/kg)	9.22 ± 0.70	10.3 ± 0.9	3.41 ± 0.34	4.16 ± 0.40[b]	2.45 ± 0.39	2.67 ± 0.13
Mitochondrial protein density (mg protein/g tissue)	15.5 ± 1.4	22.3 ± 1.8[c]	12.5 ± 0.8	9.7 ± 0.6[c]	4.4 ± 0.5	5.1 ± 0.5
Mitochondrial Protein (mg/tissue)	47.5 ± 4.5	41.4 ± 3.7	14.2 ± 1.0	7.3 ± 0.4[a]	3.0 ± 0.3	2.5 ± 0.3

Values are means ± SEM where $n = 6$ for fully fed and CR heart and liver, and $n = 5$ for fully fed and CR BAT. Significant differences between dietary groups are given as: [a] $p < 0.005$; [b] $p < 0.01$; [c] $p < 0.05$.

non-shivering thermogenesis, and part of the difference in respiration rate between control and CR may be a thermogenic response mediated by UCP-1 in BAT. This is perhaps expected as core-body temperature is lowered by CR (Cheney et al., 1983; Holehan and Merry, 1986; Rikke et al., 2003; Volicer et al., 1984). The remainder of the increase in respiration rate in response to CR may be due to an increase in basal proton leak (which is also thermogenic) or an increase in substrate oxidation, or both.

Our findings confirm earlier work that in young animals, standard metabolic rate is unaffected by CR (data not shown) apart from an initial transient lowering (McCarter and McGee, 1989; McCarter and Palmer, 1992) and this is consistent with our observations for mitochondrial respiration rates from various tissues with the exception of BAT. The problem with the interpretation of the standard metabolic rate data is that it is an average of all tissues and different tissues contribute to the standard metabolic rate to differing degrees and not just on a mass basis (Ramsey et al., 2000b). The interpretation of the effect of CR on the standard metabolic rate is confounded further when a comparison of mitochondrial protein density and total mitochondrial protein per tissue is made. It appears that for liver, the tissue metabolic rate would be similar in control and CR animals, for there was no difference in mitochondrial protein per tissue and no difference in mitochondrial respiration rate. For heart, however, CR resulted in less mitochondrial protein per tissue, since the mitochondrial respiration rates were the same, then the tissue metabolic rate would be less in CR animals. The opposite would apply to BAT, the mitochondrial respiration rate was higher in response to CR, the mitochondrial protein per tissue was unchanged, thus the tissue metabolic rate would be increased. However, caution should be applied when estimating tissue metabolic rates from measurements with isolated mitochondria for differential centrifugation may select against large and damaged mitochondria, and it is possible certain CR or age-specific soluble factors may be lost during the process of mitochondrial isolation. Our data also indicates that caution should be taken when normalising metabolic rate data of animals on restricted feeding. One common way to correct for body mass changes induced by CR is to normalise metabolic rate to lean body mass (on the assumption that fat tissue has considerably lower metabolic rate than lean tissue). However, this assumes that fat tissue has little or no contribution to the overall metabolic rate (Speakman et al., 2002), but our data indicate a clear increase in respiration rate of mitochondria from BAT of CR animals.

In summary, it appears that CR results in tissue-specific alterations in mitochondrial protein density, and tissue-specific changes in mitochondrial respiration rate. The net balance of these changes is that CR does not appear to significantly affect the standard metabolic rate (McCarter and McGee, 1989). It is clear, however, that a depression of state 4 mitochondrial respiration rate is not a consequence of CR feeding and by implication a prerequisite for inducing a lowered rate of mitochondrial ROS production.

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