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Abstract

To examine the effect of 50% food restriction over a period of 3 days on mitochondrial energy metabolism, liver mitochondria were isolated from ad libitum and food restricted rats. Mitochondrial enzyme activities and oxygen consumption were assessed spectrophotometrically and polarographically. With regard to body weight loss (-5%), food restriction decreased the liver to body mass ratio by 7%. Moreover, in food restricted rats, liver mitochondria displayed diminished state 3 (-30%), state 4-oligomycin (-26%) and uncoupled state (-24%) respiration rates in the presence of succinate. Furthermore, "top-down" elasticity showed that these decreases were due to an inactivation of reactions involved in substrate oxidation. Therefore, it appears that rats not only adapt to food restriction through simple passive mechanisms, such as liver mass loss, but also through decreased mitochondrial energetic metabolism.

Abbreviations : DCPIP, 2,3-dichlorophenollindophenol; PMS, phenazine methosulfate; RCR, respiratory control ratio; TPMP, triphenylmethylphosphonium.
1. Introduction

Most human and non-human studies show that energy restriction decreases energy expenditure [1]. Moreover, in some but not all studies, there is an adaptation to energy restriction, i.e. energy expenditure becomes lower than that expected for body mass [1]. However, the precise mechanisms responsible for this adaptation remain unclear. A decline in organ mass may be an explanation. Indeed, although internal organs (liver, kidney, heart, gastrointestinal tract…) represent less than 10% of body weight, they contribute 50-70% of energy expenditure [2] and resting energy expenditure per gram of tissue in internal organs is several-fold higher than that of muscle or adipose tissue. During starvation, weight loss initially involves tissues with a high metabolic rate, namely the liver and gastrointestinal tract, then other organs or tissues [3-5]. The same chronological pattern is observed during energy restriction [1]. It is therefore possible that changes in liver mass could be responsible for much of the initial change observed in whole-animal energy expenditure with food restriction.

In addition to this, arterio-venous differences in oxygen concentration (a proxy measure of organ energy expenditure) are reduced by 35-60% in the liver and splanchnic tissues while they are only reduced by 5% in muscle tissue [6-8]. Together, these studies suggest that caloric restriction induces a “passive” adaptation of energy metabolism (because of organ weight loss) as well as a “dynamic” adaptation (visceral organs energy expenditure becomes more reduced than expected for their given weight loss). This metabolic adaptation therefore implicates decreased mitochondrial oxygen consumption as found during medium-term (several weeks) food restriction [9-11]. This decrease in mitochondrial respiration could be partly explained by a reduced Na⁺-K⁺-ATPase activity [9], or by a decrease in cytochrome a + a₃ content [10]. However, few studies exist and all concern the effects of medium-term energy restriction only.
Adaptation of whole-body energy metabolism is well described after a period of several weeks but less so after short periods of energy restriction ranging between 3 and 10 days [12-15]. However, in human clinical practice, many patients are exposed to short-term caloric restriction. In such circumstances, data suggests that a decrease in energy expenditure [1] and an adaptation of energy metabolism occur due to food restriction [16,17]. However, there is no information regarding the relative contribution of visceral organ weight loss and changes in cellular energy conversion during short-term food restriction. Therefore, the aim of this study was to investigate changes in 1) liver weight and 2) liver mitochondrial metabolism following a short-term (3 days) 50% food restriction using top-down metabolic control analysis.
2. Materials and methods

2.1. Animals

The present investigation was performed in accordance with the guiding principles in the care and use of animals. Sixteen male Sprague-Dawley rats, born and bred in our animal facilities, were housed in individual cages at 9 weeks of age (300-350g). Animals were provided with water *ad libitum* and a standard diet (U.A.R A04) consisting (by weight) of 16\% protein, 3\% fat, 60\% carbohydrate and 21\% water, fiber, vitamins and minerals. The metabolizable energy content was 2.9 kcal/g. Half of the rats (control) were fed *ad libitum*, while the other half were food-restricted and received 50\% of the food given to the control group. The experimental design was conducted for 3 days. On the 4\textsuperscript{th} day, the animals were fasted overnight before they were killed by decapitation. This overnight fasting is used to reduce liver glycogen content to minimal levels. Liver was removed rapidly and weighed. Some tissue samples were immediately used for respiration measurements and the remainder were frozen in liquid nitrogen and stored at -80\textdegree C in order to measure enzyme activity levels.

2.2. Liver water content determination

A portion of liver (1-2 g) was weighed and placed in an oven at 85\textdegree C to dry for 24 hours. Dry weights were measured and the wet to dry weight ratio was calculated for the determination of liver water content.

2.3. Liver mitochondria preparations
The liver was removed, weighed and immediately placed in an ice-cold isolation medium consisting of 250 mM sucrose, 1 mM EGTA and 10 mM Tris/HCl, pH 7.4. Liver mitochondria were isolated by modifications of the differential centrifugation procedure used previously by Krahenbuhl et al. [18]. The liver (5-6 g) was minced with scissors and homogenized using a Potter-Elvehjem homogeniser (7 strokes) in the isolation medium (8 ml/g tissue). The homogenate was centrifuged at 600 g for 10 min. The resulting supernatant was filtered through cheesecloth, then centrifuged at 7 000 g for 10 min. The pellet was resuspended in the isolation medium (10 ml/g tissue) and then centrifuged at 3 500 g for 10 min. The resulting pellet was resuspended in a minimal volume of respirator medium, consisting of 120 mM KCl, 5 mM KH₂PO₄, 1 mM EGTA, 2 mM MgCl₂, 3 mM Hepes (pH 7.4) supplemented with 0.3% (w/v) bovine serum albumin (BSA). Protein concentration was determined by using the Bicinchoninic acid Assay kit (Interchim, Montluçon, France) with BSA used as a standard.

2.4. Mitochondrial respiration

Oxygen was measured with a Clark oxygen electrode (oxygraph Hansatech), in a 2 ml glass cell, thermostatically controlled at 30°C, with constant stirring. The mitochondria (0.5 mg protein/ml) were incubated in the same respiratory reaction medium as described above and saturated with room air. Substrate concentrations were 5 mM succinate and 5 mM ascorbate + 0.5 mM N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD). Inhibitor concentrations included 5 µM rotenone (to inhibit complex I of the respiratory chain), and 3 µM myxothiazole (to inhibit complex III). The active state of respiration (state 3) was initiated by the addition of 150 µM ADP. The basal non-phosphorylating respiration rate (state 4) was obtained by the addition of 3 ng/µl of oligomycin. The uncoupled state of respiration was initiated by the addition of 2 µM of carbonyl cyanide p-
trifluoromethoxyphenylhydrazone (FCCP). RCR refers to the ratio of oxygen consumed after adding ADP to that consumed in the presence of oligomycin.

2.5. Mitochondrial membrane potential

The mitochondrial membrane potential was determined from the distribution of lipophilic cation triphenylmethylphosphonium (TPMP\(^+\)) using a TPMP-sensitive electrode. The TPMP electrode was calibrated with sequential 1 μM additions up to 4 μM TPMP\(^+\), then respiratory substrate was added to start the reaction. After each run, 2 μM FCCP was added to dissipate the membrane potential and release all TPMP back into the medium for baseline correction. Noted that the assay medium was supplemented with 80 ng/ml nigericin to collapse any pH difference across the inner membrane. Membrane potential was calculated as described previously by Brand [19], assuming a TPMP binding correction of 0.42 (μl/mg of protein\(^{-1}\)) for liver mitochondria [20].

2.6. Experimental approach to top-down elasticity analysis

To quantitatively determine the effect of food restriction on oxidative phosphorylation processes in liver mitochondria, we used top-down elasticity analysis. As previously described [21], we divided the oxidative phosphorylation system into three blocks of reactions linked by the common intermediate membrane potential (ΔΨ): one block contains the reactions that produce ΔΨ (the substrate oxidation block, including substrate translocases, dehydrogenases, respiratory chain complexes) and two blocks that consume the ΔΨ, the phosphorylation block (including the ATP synthase and the adenine nucleotide and phosphate translocases) and the proton leakage block (including the passive permeability of the mitochondrial inner membrane to proton and any cation cycling reactions).
This analysis involves measuring the kinetics responses of each block of the system to changes in the common intermediate, the mitochondrial membrane potential. By comparing the kinetic curves for the all three reaction blocks in control and food-restricted conditions, it is easy to identify which blocks have a changed kinetic response to $\Delta \Psi$ and so can be identified as primary site affected by the experimental condition.

The kinetic response of the proton leak to $\Delta \Psi$ was obtained in non-phosphorylating mitochondria in the presence of oligomycin (1 $\mu$g/ml) as the respiration rate was titrated by the sequential addition of malonate up to 4 mM (Region A in Fig. 1). The kinetic response of the substrate oxidation block to $\Delta \Psi$ was determined from the slope of a plot of respiration rate against $\Delta \Psi$ by manipulating the ATP synthesis capacity from state 3 (500 $\mu$M ADP) to resting respiration measured in the presence of oligomycin (1 $\mu$g/ml) (Region B in Fig. 1). The kinetic response of the phosphorylation block to $\Delta \Psi$ was determined in phosphorylating mitochondria in the presence of the saturating amount of ADP (500 $\mu$M). The state 3 respiration rate was titrated by the sequential addition of malonate up to 0.25 mM and, at any given $\Delta \Psi$, the value of the respiration rate for the proton leak was subtracted (Region C in Fig. 1).

In all measurements, the respiration rate and membrane potential were assessed simultaneously using electrodes sensitive to oxygen (Oxygraph Rank Brothers Ltd) and to the potential-dependent probe TPMP$^+$. The liver mitochondria (1 mg/ml) were incubated in respiratory reaction medium (see above section 2.3) supplemented with nigericin (80 ng/ml) and rotenone (5 $\mu$M) before the TPMP electrode was calibrated with 4 $\mu$M TPMP$^+$. When required, oligomycin or ADP were also added before the addition of TPMP$^+$. Respiration was then initiated by the addition of 4 mM succinate.

2.7. Mitochondrial enzyme activities
Frozen liver (10-30 mg) was thawed and homogenized with a Potter-Elvehjem homogeniser (7 strokes) in an isolation medium consisting of 220 mM mannitol, 75 mM sucrose, 10 mM Tris and 1 mM EGTA, pH 7.2. Each homogenate was centrifuged at 600 g for 10 min and the resulting supernatants were filtered through cheesecloth. All procedures were performed at 4°C. Activities of citrate synthase, succinate dehydrogenase and complexes I, III and IV were measured spectrophotometrically at 37°C in the supernatant fraction through modified procedures of Malgat et al. [22] in agreement with the Mitochondrial Diseases group of Association Française de Myopathie.

The activity of citrate synthase was measured in a reaction medium consisting of 100 mM Tris/HCl, 40 µg/ml 5,5'-dithio-bis(2-nitrobenzoic acid), 1 mM oxaloacetate, 0.3 mM acetyl CoA and 4 % of Triton X 100, pH 8.1. After 3 min of incubation, the reaction was initiated by adding the homogenate (20 to 50 µg proteins) and the change in optical density at 412 nm was recorded for 3 min.

The activity of succinate dehydrogenase was measured by following the reduction of 2,6-dichlorophenolindophenol (DCPIP), in the presence of phenazine methosulfate (PMS) at 600 nm. Homogenate (20 to 50 µg proteins) was preincubated in a buffer containing 50 mM KH$_2$PO$_4$, 16 mM succinate, 1.5 mM KCN, 100 µM PMS, pH 7.5 for 5 min. The reaction was initiated by the addition of 103 µM DCPIP and the optical density was recorded for 3 min.

The activity of complex I was determined by monitoring the oxidation of NADH at 340 nm. Homogenate (40 to 100 µg proteins) was preincubated for 3 min in 820 µl of distilled water. Following this, 10 mM KH$_2$PO$_4$, 2 mM KCN, 5 µg/ml antimycin A, 100 µM decylubiquinone, 1.3 mg/ml BSA, 5 mM MgCl$_2$, pH 7.5 were added. The reaction was initiated by adding 200 µM NADH and the change in the optical density was analysed for 3 min. In parallel, the NADH decylubiquinone reductase activity was also measured in the presence of 12.7 µM rotenone. The specific activity of complex I was the difference between NADH oxidation activity with and without the rotenone.
The activity of complex III was determined by monitoring the reduction of cytochrome c at 550 nm. Homogenate (20 to 50 µg proteins) was incubated, for 30 sec, in a reaction medium consisting of 35 mM KH$_2$PO$_4$, 5 mM MgCl$_2$, 2.5 mg/ml BSA, 1.8 mM KCN, 125 µM oxidized cytochrome c, 12.5 µM rotenone and 62.5 mM EDTA, pH 7.5. The reaction was initiated by adding 80 µM decylubiquinol and the optical density was measured for 3 min. The nonenzymatic reduction of cytochrome c was measured under the same conditions after the addition of 10 µg/ml antimycin A. The specific activity of complex III was calculated by subtracting the activity of the nonenzymatic reaction from that of the total activity of complex III.

The activity of complex IV was measured by monitoring the oxidation of reduced cytochrome c at 550 nm. A 50 µM solution of reduced cytochrome c (92 to 97% reduced using dithionite) in 10 mM KH$_2$PO$_4$, pH 7.0 was preincubated for 5 min. The reaction was initiated by adding the homogenate (20 to 50 µg proteins) and the change in optical density was measured for 1.5 min.

2.8. Statistical analysis

Results were expressed as mean ± SD. Means were compared using either ANOVA for repeated measurement or Student’s $t$ test where appropriate. A $P$ value < 0.05 was considered significant.
3. Results

3.1. Animals

Food restricted and control groups did not differ with respect to body weight at the beginning of the experimental procedure (281 ± 23 g vs. 285 ± 37 g in the food restricted and the control group). Food restriction slowed the growth rate of the rats, leading to a significant 5% loss in their initial body mass after 3 days of treatment. At the same time, the control rats had an increased body mass of 8%. Food restriction significantly decreased liver mass by 17% (7.6 ± 0.6 g vs. 9.1 ± 1.0 g in food restricted and control rats) and the liver to body weight ratio by 7% (3.02 ± 0.21 g/100 g vs. 3.24 ± 0.22 g/100 g in food restricted and control rats). Liver water content (g water / g liver) was not affected by food restriction (66.0 ± 8.4 vs. 62.1 ± 4.3 % per liver in food restricted and control rats).

3.2. Effect of food restriction on liver mitochondrial enzyme activities and respiratory parameters.

We tested the quality of mitochondrial preparations by measuring the specific citrate synthase activities in fresh mitochondria with or without Triton X 100. We found 95.8 ± 0.6 and 95.2 ± 1.8 % (P=0.438) of intact mitochondria in liver preparations from control and food restricted rats.

There was a slight and almost statistically significant increase (+26%) in the enzyme activity of complex IV in food restricted rats (P = 0.057) (Table 1). We did not find any significant differences in the specific activities of citrate synthase, succinate dehydrogenase, and complexes III and I between food restricted and control rats (Table 1).
With succinate as substrate, oxygen consumption in conditions of phosphorylation (state 3) as well as in minimum respiratory activity (state 4-oligomycin), was significantly decreased by 30% in food restricted rats (Table 2, see also Figure 1, region A). Uncoupled state respiration with succinate was significantly decreased by 24% by food restriction. Table 2 also shows that state 3 respiration in the presence of ascorbate/TMPD was marginally decreased by food restriction (-17%, P=0.051). Whatever the substrate used, RCR was not affected by food restriction (Table 2).

3.3. Kinetics responses of proton leak, substrate oxidation and phosphorylation system to changes in ΔΨ.

Figure 1 depicts the kinetic responses of the proton leak (region A), the substrate oxidation block (region B) and the phosphorylation block (region C) to changes in ΔΨ in liver mitochondria from food restricted and control rats. It shows that the substrate oxidation system is less active in food restricted mitochondria than in control ones. Indeed at any given value of ΔΨ, the amount of oxygen used to support the activity of the substrate oxidation reactions is lower in food restricted animals. In contrast, the proton flux and the phosphorylating system were the same in food restricted and control mitochondria at any given ΔΨ.
4. Discussion

The present study shows that 3 days of caloric restriction results in 1) a decrease in the relative liver mass, and 2) a decrease in state 4-oligomycin and state 3 oxygen consumption rates in isolated liver mitochondria, which can be explained by a decreased activity of the substrate oxidation system as measured by top-down metabolic control analysis.

Three days of food restriction led to a significant weight loss (-5%) in the rats. Such a reduction in body size generally results in a lowering of whole-body energy expenditure, because of reduced maintenance requirements [1]. More interesting than body mass change, which is a poor indicator of metabolic adaptation during restriction [13], is the change in organ mass. In the present study, food-restricted rats had a significant decrease in liver weight and liver-to-body weight ratio when compared to their ad libitum counterparts. This is consistent with the fact that the liver and gastrointestinal tract, with their high metabolic rates, are the first to lose mass following energy restriction or total starvation [1]. This change in liver mass does not appear to be related to a decrease in water mass, as liver hydratation (g water /g liver) was not affected by food restriction. In consequence, it is unlikely that a change in glycogen content was sufficient to fully explain the loss in liver mass. Such a change in internal organ mass may therefore contribute to a decrease in whole energy expenditure in a food restricted animal.

The main finding reported in this work is that three days of food restriction significantly decreased phosphorylative as well as non-phosphorylative mitochondrial oxygen consumption rate in the presence of succinate. Our results agree with data obtained from medium-term (2-week) food restriction studies, which reported a decreased oxygen consumption in state 3 (-27%) and state 4 (-18%) respiration with succinate as substrate [10]. As shown in figure 1, this lower oxygen consumption is due to a decrease in the rate of the
reactions that generate proton motive force, and does not involve changes in the activity of the proton leak pathway or the reactions that form and export ATP. The substrate oxidation system includes the activities of the substrate translocases, the dehydrogenases and the respiratory chain complexes. A change in substrate dehydrogenase activity can be excluded since there was no difference in mitochondrial succinate dehydrogenase activity. The mitochondrial enzyme activities were not decreased, therefore suggesting that the decrease in uncoupled state respiration is the consequence of either altered succinate transport or decreased electron transfer along the respiratory chain (complex II, ubquinone, complex III, cytochrome c and complex IV). Oxygen consumption with ascorbate/TMPD as substrate was decreased to a lesser extent than that using succinate (-15% vs. -30% with ascorbate/TMPD and succinate). This result suggests that the effect on the rate of substrate oxidation reactions caused by food restriction is at least partly located at the level of cytochrome c oxidase. Moreover, the decrease in oxygen consumption rates with ascorbate/TMPD could have been attenuated by the slight increase in the enzyme activity of complex IV. Therefore, a decrease in the content of cytochromes a + a₃ could be an explanation to the decrease in the rate of substrate oxidation reactions. Such a change in cytochromes a + a₃ concentration, associated with a decrease in oxygen consumption, has already been found in the liver of food restricted rats after 2 weeks [10]. Although it is not sure that 3 days of food restriction is sufficient to modify the content of the electron transport chain content it remains a possibility.

Collectively, these results show that the liver mitochondria of food restricted rats pumped less protons under both resting (state 4-oligomycin respiration) and phosphorylating conditions (state 3 respiration). This decrease in substrate oxidation activity [10] is therefore apparent after only 3 days of reduced food intake. It seems therefore that a continuum exists regarding the effects of food restriction on liver mitochondrial oxygen consumption. This decrease in the capacity of substrate entry and/or electron transport within the respiratory chain tends to lower membrane potential. A decrease in mitochondrial membrane potential
causes a decreased formation of ROS [23], occurrences that have been proposed as mechanisms to explain the extended life span from long-term energy restriction [24], and decreased proton leak and redox slipping [25,26]. The latter occurrences that have never been studied in the liver of long-term food restricted rats, in turn may result in decreased thermogenesis and increased oxidative phosphorylation efficiency. Consequently, the present results may help to aid in the understanding of the biochemical mechanisms involved in decreased whole-body energy expenditure as induced by food restriction. Indeed, this decrease in substrate oxidation could represent a notable energy saving in a situation where energy supply becomes more critical, as could be the case during more severe (higher in intensity or duration) food restriction than that used in the present study. On the contrary, the present short-term adaptation could be dramatic in a situation where energy demand suddenly becomes more important. Indeed, according to the thermodynamic theory, energy homeostasis relies on both the rate and efficiency of oxidative phosphorylation. Therefore, the consequence of this expected higher oxidative phosphorylation efficiency would be a low rate of ATP synthesis. This could have negative consequences in the face of a sudden increase in energy demands (for example an infection occurring while energy restricted).

In conclusion, 3 days of a 50% reduction in food intake is sufficient to induce significant passive (decreased liver mass) and active (decreased mitochondrial oxygen consumption) adaptation of liver energy metabolism, which occur further in medium-term food restriction. The decrease in respiration rate is the consequence of a reduced substrate oxidation activity. Further experiments are necessary however to specify the exact mechanisms involved in short-term food restriction.
Acknowledgements

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References


Fig. 1. Kinetic responses of the proton leak (region A), the substrate oxidation block (region B) and the phosphorylation block (region C) to changes in membrane potential ($\Delta \Psi$) in liver mitochondria from food restricted ($\bullet$) and control rats ($\circ$).
Table 1

Effects of food restriction on liver mitochondrial enzyme activities

<table>
<thead>
<tr>
<th>Enzymatic activity (nmol/min per mg of protein)</th>
<th>Food restricted rats</th>
<th>Control rats</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citrate Synthase</td>
<td>158 ± 23</td>
<td>152 ± 26</td>
<td>0.609</td>
</tr>
<tr>
<td>Succinate Dehydrogenase</td>
<td>107 ± 30</td>
<td>96 ± 19</td>
<td>0.358</td>
</tr>
<tr>
<td>NADH-ubiquinone reductase (Complex I)</td>
<td>18 ± 6</td>
<td>19 ± 10</td>
<td>0.684</td>
</tr>
<tr>
<td>Ubiquinol-cytochrome c reductase (Complex III)</td>
<td>86 ± 52</td>
<td>101 ± 81</td>
<td>0.665</td>
</tr>
<tr>
<td>Cytochrome c oxidase (Complex IV)</td>
<td>126 ± 29</td>
<td>100 ± 21</td>
<td>0.057</td>
</tr>
</tbody>
</table>

*Enzymatic activities were determined at 37°C in liver 600 g homogenate as described in the Materials and methods section. Values are expressed as mean ± SD for eight animals.
Table 2
Effects of food restriction on respiratory parameters in liver isolated mitochondria\(^a\)

<table>
<thead>
<tr>
<th>Respiratory substrates</th>
<th>Parameters</th>
<th>Food restricted rats</th>
<th>Control rats</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(natomes of oxygen/min per mg of protein)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Succinate</td>
<td>State 3</td>
<td>69 ± 7 ††</td>
<td>99 ± 16</td>
<td>0.002</td>
</tr>
<tr>
<td></td>
<td>State 4</td>
<td>13.7 ± 2.3 ††</td>
<td>18.4 ± 2.3</td>
<td>0.004</td>
</tr>
<tr>
<td></td>
<td>RCR</td>
<td>5.1 ± 1.3</td>
<td>5.4 ± 0.8</td>
<td>0.763</td>
</tr>
<tr>
<td></td>
<td>Uncoupled state</td>
<td>92 ± 31†</td>
<td>121 ± 21</td>
<td>0.013</td>
</tr>
<tr>
<td>Ascorbate/TMPD</td>
<td>State 3</td>
<td>123 ± 14</td>
<td>148 ± 27</td>
<td>0.051</td>
</tr>
<tr>
<td></td>
<td>State 4</td>
<td>81 ± 16</td>
<td>94 ± 16</td>
<td>0.178</td>
</tr>
<tr>
<td></td>
<td>RCR</td>
<td>1.5 ± 0.2</td>
<td>1.6 ± 0.1</td>
<td>0.578</td>
</tr>
<tr>
<td></td>
<td>Uncoupled state</td>
<td>163 ± 30</td>
<td>190 ± 40</td>
<td>0.179</td>
</tr>
</tbody>
</table>

\(^a\)Mitochondria were isolated from liver as described in the Materials and methods section. Respiration rate was determined at 30°C with succinate and TMPD/ascorbate as substrates. Values are expressed as mean ± SD for eight animals. State 3 was the ADP-stimulated respiration. State 4 was the control state of respiration measured in presence of oligomycin. Uncoupled state was the FCCP-stimulated respiration. RCR was the respiratory control ratio.
Fig. 1

Membrane potential (mV) vs. Respiration rate (nmol O/min.mg protein).

- Line A
- Line B
- Line C