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The mechanism of stimulation of respiration in isolated hepatocytes from rats fed an energy-dense diet

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We measured oxygen consumption in hepatocytes isolated from rats fed an energy-dense diet. We conceptually divided the respiratory pathway into blocks of reactions that produced or consumed mitochondrial membrane potential, and then measured the kinetic response of these blocks of reactions to this potential. The results show increased hepatic respiration rates in rats fed an energy-dense diet accounted for by an increased rate of the substrate oxidation reactions at any particular mitochondrial membrane potential, but no change in the kinetic response to potential of the reactions that consume it, resulting in a small rise in this potential in the rats fed an energy-dense diet. The increased hepatic oxygen consumption is in line with the increased energy expenditure that occurs in rats fed an energy-dense diet. © Elsevier Science Inc. 1996 (J. Nutr. Biochem. 7:571–576, 1996.)

Keywords: hepatocytes; energy-dense diet; oxygen consumption; top-down elasticity analysis

Introduction

Previous results have shown that young rats provided with an energy-dense diet become hyperphagic, but fail to exhibit excess weight gain through a facultative increase in energy expenditure (diet-induced thermogenesis, DIT).[1–3] The same results were previously obtained feeding rats the ''cafeteria diet,'' and brown adipose tissue was initially suggested as the main site of DIT.[4,5] However, a contribution to the increased energy expenditure found in hyperphagic rats could be given by the liver, because it constitutes only about 4% of the total body weight and consumes about 20% of the total oxygen used by the organism.[6,7] This hypothesis is supported by the results of Berry et al.,[8] which showed increased respiration rates in hepatocytes from hyperphagic rats fed a ''cafeteria diet.'' In line with this finding, it was suggested that an increase in liver metabolism contributes to the increased energy expenditure of hyperphagic rats.[6,9]

In view of the above observation, it appeared of interest to investigate whether changes in respiration rates of isolated hepatocytes occur in hyperphagic rats fed an energy-dense diet. Any changes in respiration rates in isolated hepatocytes may reflect changes either in the leak of proton across the mitochondrial inner membrane or in the activity of the phosphorylating system (ATP synthase, Pi transporter, adenine nucleotide carrier, and extramitochondrial ATP-consuming reactions) or in the reactions that oxidize substrates (cellular catabolic reactions, the citric acid cycle, and the electron transport chain).[10] The application of the top-down elasticity analysis allows the identification of the blocks of reactions, which are sites of action of external effectors within metabolic pathways.[11,12] This approach involves dividing the system under consideration into blocks of reactions that produce and that consume a common intermediate, which is the mitochondrial membrane potential ($\Delta\Psi_m$) in the case of respiration in isolated hepatocytes.[11] We can then consider three blocks of reactions: 1) the reactions that produce $\Delta\Psi_m$, i.e., the substrate oxidation pathway that consists of all of the steps from endogenous substrate to the production of $\Delta\Psi_m$; 2) the reactions that consume $\Delta\Psi_m$-producing ATP and all cellular ATP-consuming reactions, called ''phosphorylating system,'' and 3) the reactions that consume $\Delta\Psi_m$ without ATP synthesis, called ''proton leak.'' The top-down elasticity analysis involves

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measuring the kinetic response of the above blocks of reactions to $\Delta\Psi_m$. This approach has been previously used to identify the significant sites of action of thyroid hormones in rats on subsequently isolated hepatocytes.[13]

Therefore, we thought it would be interesting to apply top-down elasticity analysis to identify the groups of reactions that could be affected in isolated hepatocytes from rats fed an energy-dense diet.

Methods and materials

Treatment of animals

Male Wistar rats of about 30 days of age (bred in the animal house of the Department of Biochemistry, University of Cambridge, UK) were divided into two groups with the same mean body weight (about 80 grams). One group (CD rats) was fed a control diet (percentage of total metabolizable energy: 29 protein, 10.6 lipid, and 60.4 carbohydrate; 15.88 kJ gross energy/g), whereas the other group (ED rats) was fed an energy-dense diet (28% control diet, 39.5% lyophilized meat, 17.8% butter, 12% alphacel, 0.7% AIN vitamin mix, 2% AIN mineral mix, g/g: percentage of total metabolizable energy: 29 protein, 50 lipid, and 21 carbohydrate; 19.85 kJ gross energy/g).[14] The diet treatment lasted 15 days. The rats were housed individually in suspended wire-bottom cages at 24°C under an artificial circadian 12-hr light/12-hr dark cycle. Body weights and food intakes were monitored daily to allow calculations of body weight gain and gross energy intake. At the end of the experimental period these rats were fasted overnight and were used for preparations of isolated hepatocytes. Other male Wistar rats of 30 days of age (Charles River Italia, Calco, Como, Italy) were kept at 24°C under an artificial circadian 12-hr light/12-hr dark cycle in the animal house of the Department of General and Environmental Physiology, University of Naples, Italy. The rats were divided into two groups, which were fed either the control diet or the energy-dense diet for 15 days. At the end of the experimental period, these rats were fasted overnight and were used for energy balance measurements.

Energy balance measurements

Body weights and food intakes were monitored daily to allow calculations of body weight gain and gross energy intake. Digestible energy intake (taking into account the food spillage) was obtained by subtracting the energy measured in the feces from the gross energy intake as measured from daily food consumption. Metabolizable energy (ME) intake was expressed as digestible energy intake × 0.96.[15] The gain in energy was obtained by subtracting the energy content of an initial group (five rats killed for energy content at the beginning of the study) from that of each of the two experimental groups. At the end of the experiment the animals were anesthetized by the intraperitoneal injection of chloral hydrate (40 mg/100 g BW) and were killed by decapitation. Then, after gut content removal, the carcasses were autoclaved, chopped into small pieces, thoroughly mixed, and homogenized in water (volumes equal to twice the carcass weight) with a Polytron. Samples of homogenates, as well as samples of feces, were dessicated into a dry powder from which small pellets (about 200 mg) were made. The energy content of the pellets and the gross energy content of control and energy-dense diet were measured with a Parr adiabatic calorimeter calibrated with dry benzoic acid standard. Energy expenditure was calculated from the difference between ME intake and energy gain.

Preparation and incubation of cells

Hepatocytes were prepared by a modification[16] of the method of Berry and Friend.[17] Dry cell weight was determined by drying a known volume of cell suspension to a constant weight at 70°C; from this value, we then subtracted the dry weight of an equal volume of isolation medium (118 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4 \cdot 7H_2O$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 2.5 mM $CaCl_2$). The cells that were suspended in isolation medium were then diluted 6.6 fold into the incubation medium (106 mM NaCl, 5 mM KCl, 25 mM $NaHCO_3$, 0.41 mM $MgSO_4 \cdot 7H_2O$, 10 mM Na_2HPO_4, 2.5 mM $CaCl_2$, and 2.25% (w/v) defatted bovine serum albumin). Stock 9% bovine serum albumin was defatted by the method of Chen[18] and dialysed against 153 mM NaCl and 11 mM KCl. The final cell suspension was counted with trypan blue (0.3% w/v) in a Neubauer chamber to assess the viability (routinely >90%). The gas above the cell suspension during incubations was 95% air and 5% CO_2 to allow equilibration of the medium to a pH of 7.4.

Measurement of respiration rate

Incubations of cell suspensions were routinely carried out using approximately 3 mL of suspension (6 to 9 mg dry weight/mL) in 20-mL stoppered glass vials. To allow cells to establish ion gradients after being stored on ice, cells were preincubated at 37°C in the shaking water bath for 10 min before the addition of any inhibitors or isotopes. After a further incubation period of 20 min, oxygen consumption was measured in duplicate 2-mL aliquots of cell suspension using two Clark-type oxygen electrodes (Rank Brothers, Bottisham, Cambridge, UK) maintained at 37°C. Respiration rates were determined approximately 3 to 5 min after the addition of the cell suspension to the chambers, i.e., once stable linear rates were obtained. Mitochondrial respiration was calculated by subtracting the non-mitochondrial respiration from the cellular respiration rate. To determine the rate of non-mitochondrial oxygen consumption, cells were incubated with maximal concentrations of myxothiazol (1.0 μM).

Measurement of mitochondrial membrane potential ($\Delta\Psi_m$)

The $\Delta\Psi_m$ was measured simultaneously with oxygen consumption using the distribution of the lipophilic cation, triphenylmethylphosphonium ($TPMP^+$). The relationship between mitochondrial membrane potential (mV) and $TPMP^+$ distribution at 37°C is:

$$\Delta\Psi_m = -61.5 \cdot \log \left| \frac{V_c \cdot a_m}{V_m \cdot a_c} \left[\frac{[Cl^-]_{tot} \cdot [TPMP]_{tot} \cdot a_c \cdot (V_c + V_m)}{[Cl^-]_e \cdot [TPMP]_e \cdot a_e \cdot V_c} - 1 \right] \right|$$

Where V = volume, a = apparent $TPMP^+$ activity coefficient, and subscripts c, m, e, and tot represent cytoplasmic + nuclear, mitochondrial, extracellular, and total, respectively. Appropriate corrections were applied for plasma membrane potential, cytoplasmic and mitochondrial binding of $TPMP^+$, and other factors as described by Harper and Brand[13] ($V_c = 0.82$, $V_m = 0.18$, $a_m = 0.43$, $a_c = 0.17$, $a_e = 0.71$). Mitochondrial membrane potential can be calculated knowing the extent of the accumulation of $TPMP^+$ into the whole cell ($[TPMP^+]_{tot}/[TPMP^+]_e$) and into the cytoplasm in relation to the external medium ($[Cl^-]_e/[Cl^-]_{tot}$). The distribution of $^{36}Cl^-$ was also used to calculate the plasma membrane potential.[19] Because no differences in plasma membrane potential were observed between cells from CD and ED rats, a standard value of $[Cl^-]_e/[Cl^-]_{tot} = 0.30$[13] was assumed.

Hepatocytes were incubated at 37°C in the presence of $3.7 \cdot 10^4$ Bq of ^3H_2O/mL and $3.7 \cdot 10^3$ Bq/mL of [^{14}C]methoxyinulin/mL for measurement of cell volume; $3.7 \cdot 10^3$ Bq of [^{3}H]$TPMP^+$/mL for measurement of $TPMP^+$ accumulation; or $3.7 \cdot 10^3$ Bq of $^{36}Cl^-$/

mL and $3.7 \cdot 10^4$ Bq of ^3H_2O/mL for measurement of $^{36}Cl^-$ distribution. Incubation with ^3H_2O allowed the total pellet volume to be determined, whereas [^{14}C]methoxyinulin allowed the calculation of extracellular volume in pellets; the cellular volume was then calculated as the difference between the total pellet volume and the extracellular volume. Carrier inulin (0.1 mg/mL) and TPMP bromide (0.1 μM) were added to all incubations.[20] At the end of each incubation triplicate aliquots (0.70 mL) were centrifuged for 2 min, and were processed according to Harper and Brand[13] to determine the radioactivities of the supernatant and pellet. The apparent volume of pellet available to each isotope (its space in μL) was calculated as dpm in total pellet divided by dpm/μL of supernatant sample. The [^{3}H]TPMP$^+$ accumulation ratio ($[TPMP^+]_{tot}/[TPMP^+]_e$) was calculated as ([^{3}H]TPMP$^+$ space – [^{14}C]methoxyinulin space)/(^3H_2O space – [^{14}C]methoxyinulin space). For greater precision, correction for total pellet volume was made in each pellet containing $^{36}Cl^-$; thus, $^{36}Cl^-$ distribution $[Cl^-]_{tot}/[Cl^-]_e$, was calculated as [(^3H_2O space – [^{14}C]methoxyinulin space) – (^3H_2O space – [^{36}Cl] space)/(^3H_2O space – [^{14}C]methoxyinulin space)].

Top-down elasticity analysis

Top-down elasticity analysis is derived from control analysis.[11] It contains no broad assumptions and uses a very simple criterion of "what happens to y when we change x a little bit" involving only the ability to change x and to measure x and y before and after the change. Elasticity analysis simply measures the kinetic response of some catalyst to some variable, and asks if this response is changed by the test condition (here diet). If it is, then the test condition affects the catalyst directly, if it is not, it doesn't. In top-down analysis the catalyst is a grouped set of enzymes, carriers, etc., but that doesn't alter the theory and it makes the experiments simpler to do and to interpret.[11,12]

The kinetic responses of the proton leak, phosphorylation system and substrate oxidation pathways to $\Delta\Psi_m$ were measured in isolated hepatocytes from CD and ED rats by using inhibitors (oligomycin and myxothiazol), which are very specific and have primary sites of action within mitochondrial respiratory system.

Hepatocytes were incubated in a medium without glucose and without addition of any substrates. If we manipulate the mitochondrial proton conductance with oligomycin (0.025 μg/mL) or FCCP (1.5 μM), then the relationship between the rate of respiration and $\Delta\Psi_m$ will reflect the kinetic response of all of the reactions involved in the substrate oxidation pathways to $\Delta\Psi_m$.

The kinetic response of the proton leak pathway to the $\Delta\Psi_m$ should be obtained by titrating the activity of the respiratory chain with myxothiazol in the presence of saturating amounts of oligomycin to prevent ATP synthesis. However, when $\Delta\Psi_m$ and respiration were titrated with oligomycin, low doses of oligomycin caused $\Delta\Psi_m$ to rise, but when sufficient oligomycin was added to almost completely abolish oxidative phosphorylation $\Delta\Psi_m$ began to fall again and respiration was further inhibited, presumably because there was no glycolytic ATP to activate the fatty acids, which are presumably the major fuel in starved cells, so that there may be no substrate for respiration.[21,10] Due to the fall in the $\Delta\Psi_m$, it was difficult to examine the kinetic response of the proton leak pathway to the $\Delta\Psi_m$. Control experiments were done titrating with myxothiazol in the presence of different concentrations of oligomycin to see when the kinetic curve so obtained, overlapped with the kinetic curve obtained titrating with oligomycin (0.05 to 7.5 μg/mL) (*Figure 1*). We found overlapped curves when the myxothiazol titration (0.10 to 1.0 μM) was done in the presence of 1 μg/mL of oligomycin. This means that starting from this concentration of oligomycin, the curve obtained titrating with oligomycin overlaps with the kinetic curve of the proton leak response to the $\Delta\Psi_m$. So the overlapped kinetic curves obtained can be assumed to represent the kinetic response of the proton leak pathway to the $\Delta\Psi_m$.

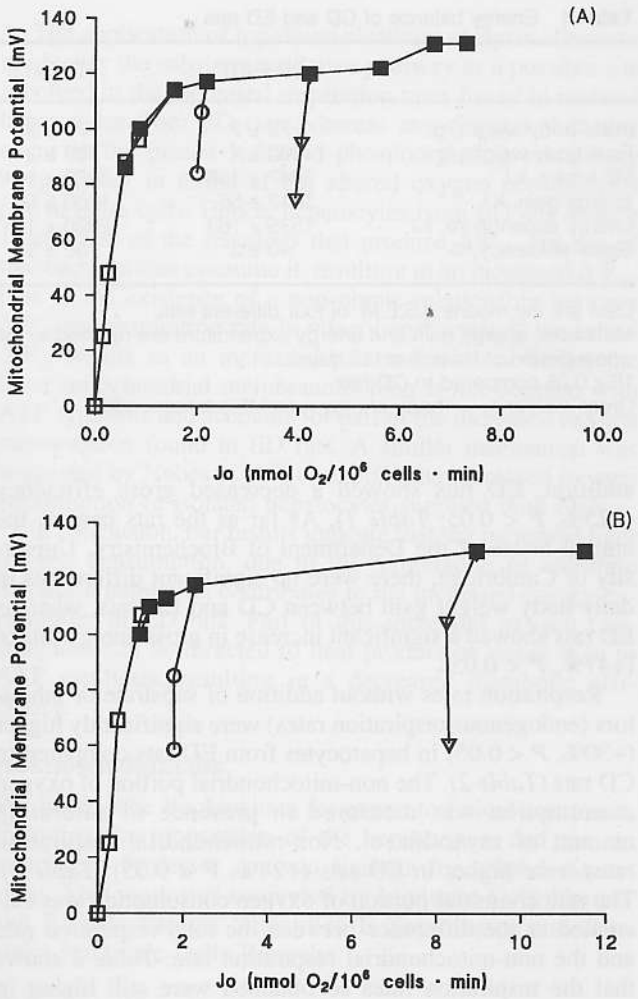

Figure 1 Titration with myxothiazol in presence of different concentrations of oligomycin in isolated hepatocytes from CD (A) and ED rats (B). ■ titration with different concentrations of oligomycin (0.05 to 7.5 μg/mL); ▽, ○, □ titrations with different concentrations of myxothiazol (0.10 to 1.0 μM) in presence of either 0.1 μg/mL (▽) or 0.5 μg/mL (○) or 1.0 μg/mL (□) of oligomycin. Data are from a single representative experiment. The non-mitochondrial oxygen consumption was subtracted from each value.

If the activity of the respiratory chain is titrated with myxothiazol (0.10 to 0.25 μM) without oligomycin and corrections are later made for the amount of oxygen required to balance the rate of proton leakage at each $\Delta\Psi_m$ measured, the relationship between the rate of respiration and $\Delta\Psi_m$ will reflect the kinetic response of the phosphorylating system to $\Delta\Psi_m$.

Results

Table 1 shows the results of energy balance measurements in CD and ED rats. Mean initial and final body weights were not significantly different between the two groups, yielding a daily body weight gain of about 7 g in both groups. The ME intake and energy expenditure of ED rats during the whole period of treatment were 30% ($P < 0.05$) and 51% ($P < 0.05$) higher, respectively, than in CD rats (*Table 1*). In

Table 1 Energy balance of CD and ED rats

	CD RATS	ED RATS
Initial body weight, g	75 ± 2	75 ± 2
Final body weight, g	190 ± 4	189 ± 2
ME intake, kJ	2547 ± 156	3320 ± 111*
Energy gain, kJ	1012 ± 56	1000 ± 55
Energy expenditure, kJ	1535 ± 163	2320 ± 165*
Gross efficiency, %	40 ± 3	30 ± 3*

Data are the means ± S.E.M. of four different rats.
ME intake, energy gain and energy expenditure are referred to the whole period of treatment (15 days).
*$P < 0.05$ compared to CD rats.
Gross efficiency = (body energy gain/ME intake) × 100.

addition, ED rats showed a decreased gross efficiency (–25%, $P < 0.05$; *Table 1*). As far as the rats bred in the animal house of the Department of Biochemistry, University of Cambridge, there were no significant differences in daily body weight gain between CD and ED rats, whereas ED rats showed a significant increase in gross energy intake (+47%, $P < 0.05$).

Respiration rates without addition of substrate or inhibitors (endogenous respiration rates) were significantly higher (+30%, $P < 0.05$) in hepatocytes from ED rats compared to CD rats (*Table 2*). The non-mitochondrial portion of oxygen consumption was measured in presence of saturating amount of myxothiazol. Non-mitochondrial respiration rates were higher in ED rats (+24%, $P < 0.05$) (*Table 2*). The mitochondrial portion of oxygen consumption was calculated as the difference between the total respiration rate and the non-mitochondrial respiration rate: *Table 2* shows that the respiration rates so obtained were still higher in hepatocytes from ED rats compared with CD rats (+32%, $P < 0.05$).

Figure 2 shows the kinetic responses of the proton leak, phosphorylation system, and substrate oxidation pathways to $\Delta\Psi_m$ in isolated hepatocytes from CD and ED rats. For the analysis of the kinetic responses of the three blocks of reactions to $\Delta\Psi_m$ (*Figure 2*), the non-mitochondrial portion of oxygen consumption was subtracted from the total cellular oxygen consumption. There are no significant differences in the rate of the proton leak and phosphorylating system at any given value of $\Delta\Psi_m$ between CD and ED rats

Table 2 Endogenous respiration rates in hepatocytes from CD and ED rats

	CD RATS	ED RATS
Total	11.53 ± 0.27	14.98 ± 0.30*
Non-mitochondrial	3.28 ± 0.16	4.08 ± 0.15* (23%)
Mitochondrial	8.25 ± 0.27	10.9 ± 0.3* (77%)

Data are expressed in nmol $O_2/10^6$ cell · min and are the means ± S.E.M. of four different experiments.
The values in brackets represent the % contribution to the increased respiration rate.
$P < 0.05$ compared to CD rats.

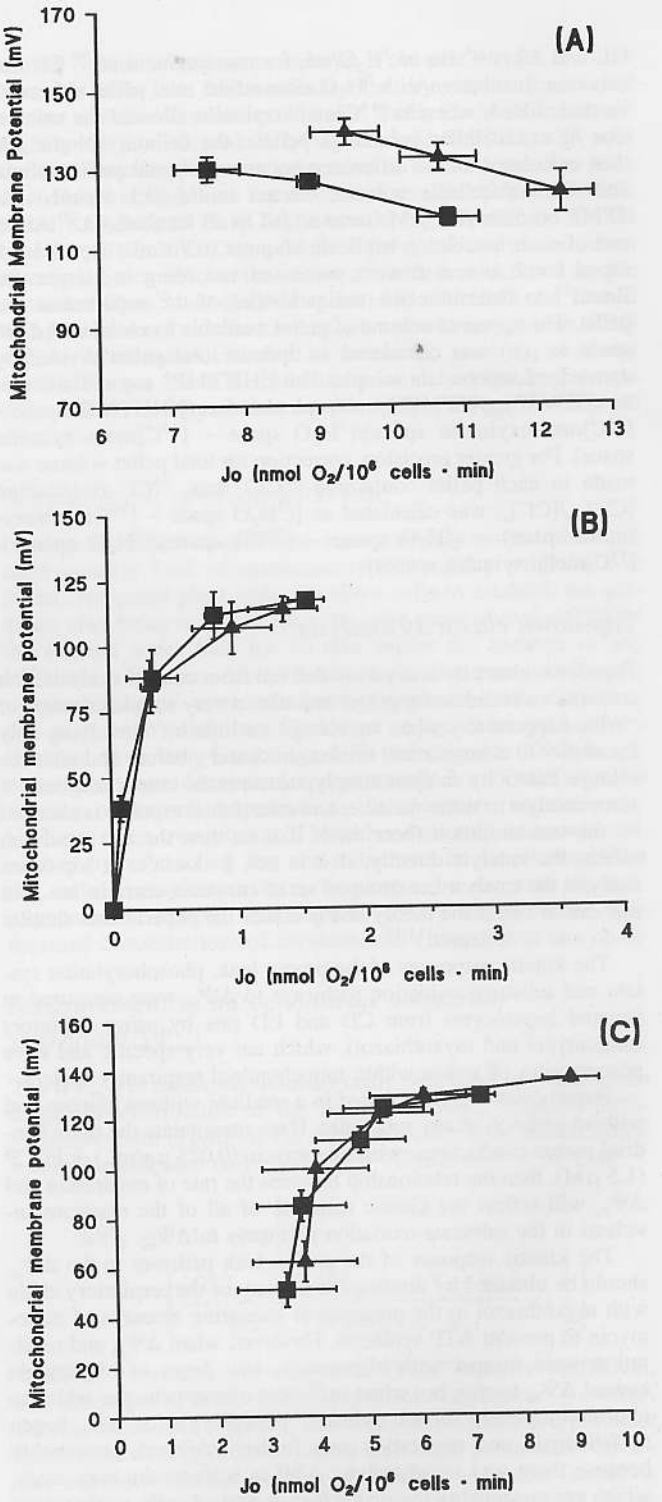

Figure 2 Comparison of the kinetic responses of the substrate oxidation (A), proton leak (B) phosphorylating system (C) to $\Delta\Psi_m$ in hepatocytes from CD (■) and ED (▲) rats. (A) Kinetic response of substrate oxidation to $\Delta\Psi_m$. The middle point represents the endogenous respiration rate, the point on the right represents the respiration rate in presence of FCCP (1.5 μM) and the one on the left represents the respiration rate in presence of oligomycin (0.025 μg/mL). (B) Kinetic response of the mitochondrial proton leak to $\Delta\Psi_m$: myxothiazol titration (0.10 to 1.0 μM) of oligomycin-inhibited respiration (1 μg/mL). (C) Kinetic response of the phosphorylating system to $\Delta\Psi_m$: myxothiazol titration (0.10 to 0.25 μM) of respiration in the absence of oligomycin minus the myxothiazol titration in the presence of oligomycin. Each point represents the mean ± SEM of four cell preparations. The non-mitochondrial oxygen consumption was subtracted from each value.

(*Figure* 2B,C). On the other hand, there is a clear difference in the rate of the reactions involved in the substrate oxidation pathway at the same values of $\Delta\Psi_m$ between CD and ED rats. In fact, the plot of mitochondrial oxygen rate in isolated hepatocytes from ED rats against $\Delta\Psi_m$ lies above the plot obtained with hepatocytes from CD rats, such that, at any given $\Delta\Psi_m$ the oxygen consumption is higher in hepatocytes from ED rats than in hepatocytes from CD rats (*Figure* 2A). This result indicates that substrate oxidation reactions are stimulated in hepatocytes from ED rats compared with CD rats.

Discussion

In the present study, energy balance measurements have shown that in ED rats ME intake and energy expenditure significantly increased compared to CD rats without any change in body weight gain (*Table 1*). In addition, gross efficiency was significantly reduced in ED rats (*Table 1*). These results are in agreement with our previous ones obtained in ED rats exhibiting DIT.[1–3] Our results also indicate that the increase in energy expenditure found in ED rats is partly due to an increase in hepatic metabolism, as shown by significantly increased rates of oxygen consumption in isolated hepatocytes from ED rats (*Table 2*). The increase in liver oxygen consumption is due both to an increased mitochondrial oxygen consumption (77%) and to an increased non-mitochondrial oxygen consumption (23%) (*Table 2*). The mechanisms responsible for the changes in non-mitochondrial oxygen consumption are not known, but they may involve altered microsomal and/or peroxisomal oxidative pathways.

To gain insight into the mechanism of stimulation of mitochondrial oxygen consumption in ED rats, we applied top-down elasticity analysis to identify which groups of reactions involved in mitochondrial respiration are affected in hepatocytes from ED rats. The higher respiratory rates found in hepatocytes from ED rats could be due to variations in the activity of proton leak, phosphorylating system, and/or substrate oxidation reactions. The results show that the kinetic response of mitochondrial proton leak to $\Delta\Psi_m$ is not affected in isolated hepatocytes from ED rats (*Figure* 2B). Because we found no variation in mitochondrial protein mass in ED rats,[1] we can exclude variations in intrinsic permeability of the mitochondrial inner membrane. Similarly, the kinetic response of the phosphorylating system to $\Delta\Psi_m$ is unchanged (*Figure* 2C). On the other hand, the kinetic response of the substrate oxidation reactions to $\Delta\Psi_m$ is significantly increased in hepatocytes from ED rats (*Figure* 2A). The stimulation of the substrate oxidation pathway can be due to an increase in the pool of metabolites in the mitochondria, as well as in oxidative enzyme activity. Because rats fed an energy-dense diet exhibited high circulating free fatty acid (FFA) levels,[1] a greater flow of FFA to the liver and an increased FFA availability for liver oxidation can occur. In addition, it is known that carnitine palmitoyltransferase I, the main regulatory step of mitochondrial fatty acid oxidation,[22] is increased by a high-fat diet.[23] An increased FFA oxidation may contribute to reduce the availability of lipids for storage in adipose tissue thus counteracting the development of obesity.

The application of top-down elasticity analysis allows us to identify the substrate oxidative pathway as a possible site involved in the increased respiration rates found in isolated hepatocytes from ED rats, whereas any changes that may occur in the proton leak and phosphorylating system are insignificant in terms of the altered oxygen consumption rate in these cells. Hence, hepatocytes from ED rats show a stimulation of the reactions that produce $\Delta\Psi_m$, but not of the reactions that consume it, resulting in an increased $\Delta\Psi_m$. Due to the existence of a non-ohmic relationship between $\Delta\Psi_m$ and respiration rate in intact hepatocytes,[24] the raised $\Delta\Psi_m$ results in an increased proton backflow across the inner mitochondrial membrane, which is not coupled with ATP synthesis and accounts for part of the increased oxygen consumption found in ED rats. A similar mechanism was suggested by Nobes et al.[21] to explain the increased oxygen consumption in isolated hepatocytes supplied with FFA.

In conclusion, our results indicate that the increased liver oxygen consumption, due to the stimulation of substrate oxidation pathways, contributes to the increased energy expenditure of ED rats. Part of this increased oxygen consumption can be directed to heat production rather than to ATP synthesis, resulting in a decreased metabolic efficiency.

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