Metabolic efficiency of liver mitochondria in rats with decreased thermogenesis

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Received 26 February 2003; revised 14 April 2003; accepted 28 April 2003

First published online 12 May 2003

Edited by Vladimir Skulachev

Abstract **We have studied changes in hepatic mitochondrial efficiency induced by 24-h fasting or acclimation at 29°C, two conditions of reduced thermogenesis. Basal and palmitate-induced proton leak, which contribute to mitochondrial efficiency, are not affected after 24-h fasting, when serum free triiodothyronine decreases significantly and serum free fatty acids increase significantly. In rats at 29°C, in which serum free triiodothyronine and fatty acids decrease significantly, basal proton leak increases significantly, while no variation is found in palmitate-induced proton leak. The present results indicate that mitochondrial efficiency in the liver is not related to a physiological decrease in whole body thermogenesis.**

Key words: Mitochondrial efficiency; Fasting; Basal proton leak; Palmitate-induced uncoupling

1. Introduction

Whole body thermogenesis changes in response to physiological conditions. Food availability and environmental temperature are two important factors which influence thermogenesis. Resting metabolism is lower in fasted than in fed animals [1] and is lower at thermoneutrality than at cooler temperatures [2].

Changes in thermogenesis at the whole body level must reflect parallel changes at the level of organs and tissues, which are the major contributors to standard metabolic rate. At the cellular level, important sites of thermogenesis are the mitochondria, due to their incomplete coupling between substrate oxidation and ATP production [3]. It has been calculated that basal proton leak, one of the mechanisms responsible for mitochondrial uncoupling, accounts for about 20% of resting energy expenditure of mammals [4]. In addition, it is well known that free fatty acids (FFA) are responsible for so-called mild uncoupling in mitochondria [5,6] due to a non-ionic diffusion coupled with the fatty acid anion transport out of the mitochondria through various mitochondrial carriers, such as the adenine nucleotide (ANT) and glutamate/aspartate antiporter [7]. While basal proton leak essentially depends on the characteristics of the mitochondrial inner membrane [8] and is not influenced by external factors, fatty acid-induced leak also depends on the concentrations of unbound cellular FFA, and therefore on whole body metabolic conditions. The combination of basal leak and fatty acid-induced uncoupling could thus determine the efficiency of the mitochondrial system.

It has been shown that in liver mitochondria basal proton leak and fatty acid-mediated uncoupling vary in parallel to changes in thermogenesis induced by hypo- or hyperthyroidism [9,10]. In addition, we have previously found that the efficiency of skeletal muscle subsarcolemmal mitochondria is lowered in overeating rats exhibiting diet-induced thermogenesis [11]. On the other hand, less is known about changes of the above parameters in response to a physiological decrease in resting energy expenditure.

We were therefore interested in investigating mitochondrial energy coupling and activity in two physiological situations characterised by reduced thermogenesis, namely fasting and thermoneutrality. To this end, in 24-h fasted rats and in rats acclimated to 29°C, we measured basal and palmitate-mediated proton leak in mitochondria isolated from the liver, since this tissue accounts for about 20% of whole body energy expenditure in rats [3], and is involved in the changes of thermogenesis due to physiological stimuli [12–14].

2. Materials and methods

2.1. Animals and experimental design

Male Wistar rats (about 200 g) were acclimated at 24 or 29°C for 10 days before being killed. Then, rats acclimated at 24°C were divided into two groups, one killed in the fed state and the other killed after 24-h fasting, while rats acclimated at 29°C were killed in the fed state. Treatment, housing, and killing met the guidelines of the Italian Health Ministry.

2.2. Preparation of isolated liver mitochondria

The rats were anaesthetised with chloral hydrate (40 mg per 100 g body weight). After blood collection from the inferior vena cava, livers were quickly removed and used for preparation of isolated mitochondria [12]. The protein content of the mitochondrial suspension was determined by the method of Hartree [15], while the percentage of intact mitochondria was evaluated by detecting oxygen consumption in the presence of NADH as substrate (2.5 mM) and was found to be about 90% in the three conditions.

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Abbreviations: ANT, adenine nucleotide translocator; BAT, brown adipose tissue; CAT, carboxyatractyloside; COX, cytochrome oxidase; FFA, free fatty acids; ROS, reactive oxygen species; T_3, triiodothyronine

0014-5793/03/$22.00
doi:10.1016/S0014-5793(03)00490-3

2.3. Mitochondrial oxygen consumption and cytochrome oxidase-specific activity

State 3 mitochondrial oxygen consumption was measured polarographically with a Clark-type electrode (Yellow Springs Instruments, Yellow Springs, OH, USA) at 30°C in a medium containing 80 mM KCl, 50 mM HEPES, 5 mM KH_2PO_4, 1 mM EGTA, 0.1% (w/v) fatty acid-free bovine serum albumin (BSA), pH 7.0 in the presence of succinate 10 mM, rotenone 3.75 μM, and ADP 0.3 mM.

Cytochrome oxidase (COX)-specific activity was measured polarographically with a Clark electrode at 30°C in a medium containing 30 μM cytochrome *c*, 4 μM rotenone, 0.5 mM dinitrophenol, 10 mM Na-malonate, 75 mM HEPES, pH 7.4 [16]. Mitochondria were diluted in modified Chappel–Perry medium (1 mM ATP, 100 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 5 mM EGTA, 50 mM HEPES, pH 7.4) containing Lubrol PX (225 μg/mg protein) and incubated for 30 min in ice to unmask enzyme activity. At the end of the incubation, COX-specific activity was measured as oxygen consumed in the presence of 4 mM ascorbate+0.3 mM tetramethyl-*p*-phenylenediamine.

2.4. Measurements of basal proton leak

Oxygen consumption was measured polarographically with a Clark-type electrode at 30°C, in a medium containing 80 mM LiCl, 50 mM HEPES, 5 mM Tris-PO_4, 1 mM EGTA, 0.1% (w/v) fatty acid-free BSA, pH 7.0. Titration of respiration was carried out by sequential additions of malonate up to 5 mM in the presence of succinate (10 mM), rotenone (3.75 μM), oligomycin (2 μg/ml), safranin O (83.3 nmol/mg) and nigericin (80 ng/ml). Mitochondrial membrane potential recordings were performed in parallel with safranin O using a Jasco dual-wavelength spectrophotometer (511–533 nm) [17]. The absorbance readings were transformed into mV membrane potential using the Nernst equation: $\Delta\psi = 61$ mV log ($[K^+]_{in}/[K^+]_{out}$) and calibration curves made for each preparation as previously reported [18].

2.5. Measurement of ANT and glutamate/aspartate antiporter contribution to the uncoupling effect of palmitate and palmitate-induced proton leak

Mitochondrial membrane potential and oxygen consumption were measured as above in the presence of succinate (10 mM), rotenone (3.75 μM), oligomycin (2 μg/ml), safranin O (83.3 nmol/mg) and 85 μM palmitate. This dose does not irreversibly alter membrane integrity (as indicated by complete reversal of the uncoupling effect by BSA), and causes a decrease in membrane potential (about 10–15 mV), which is less than that occurring in the transition from state 4 to state 3. Due to the presence of 0.1% BSA in the incubation medium, the above concentration of palmitate corresponds to 98 nM free (not bound) fatty acid, calculated using the equation of Richieri et al. [19]. The contributions of ANT and glutamate/aspartate antiporter were assessed by measuring the recoupling effect of specific inhibitors of these carriers, such as carboxyatractyloside (CAT) (10 nmol/mg protein) for ANT, glutamate (7 mM) for glutamate/aspartate antiporter and BSA (0.4%, w/v) for the other systems (which were added in sequence). A typical trace of membrane potential measurements is reported in Fig. 1. Palmitate-induced proton leak was estimated from titration of respiration by sequential additions of malonate up to 600 μM and parallel recordings of membrane potential.

2.6. Measurement of ANT content

State 3 respiration was titrated with increasing concentrations of CAT [20] in a medium containing 80 mM KCl, 50 mM HEPES, 5 mM KH_2PO_4, 1 mM EGTA, 0.1% (w/v) fatty acid-free BSA, succinate 10 mM and rotenone 3.75 μM, ADP 0.3 mM, pH 7.0. The mitochondrial content of ANT was determined by extrapolation of the linear part of the titration curve to obtain the amount of CAT required to inhibit state 3 respiration completely. The turnover number of ANT was calculated from the slope of the inhibitory region, after conversion of respiration rate into phosphorylation rate with the ADP/O ratio [21].

2.7. Determination of serum FFA and free triiodothyronine (T_3) levels

Serum free T_3 levels were measured using commercial radioimmunoassay kits (ICN Pharmaceuticals, Diagnostic Division, New York, NY, USA). Serum FFA were measured by a colorimetric enzymatic method using a commercial kit (Roche Diagnostics, Italy).

2.8. Statistical analysis

Data are given as means ± S.E.M. Statistical analyses were performed by one-way analysis of variance (ANOVA) followed by Dunnett's post-hoc test. Correlation between selected parameters was performed by linear or non-linear regression analysis. Probability values less than 0.05 were considered to indicate a significant difference.

2.9. Materials

All reagents were purchased from Sigma, St. Louis, MO, USA, except for CAT (Calbiochem, San Diego, CA, USA).

3. Results and discussion

In the present study, we found that mitochondrial state 3 respiratory rates, COX activity and ANT content were not affected by 24-h fasting (Table 2, Fig. 2). Therefore, the liver does not appear to contribute to energy conservation in response to 24-h fasting. The present result is in agreement with our previous one [22], showing that isolated hepatocyte oxygen consumption increased significantly in 24-h fasted rats. One explanation could be that the specific metabolic function of the liver is to synthesise glucose in response to fasting-induced hypoglycaemia [23]. Since gluconeogenesis is an ATP-requiring pathway, mitochondrial activity must supply the required metabolic energy. Accordingly, Ma and Foster found no reduction in blood flow going to the liver after 16-h fasting [24]. We also found that in 24-h fasted rats there is no reduction in basal (Fig. 3A) and palmitate-induced proton leak (Fig. 3B), as well as in the systems involved in palmitate-induced uncoupling (Table 3). Therefore, the increase in FFA levels in the serum (Table 1) and intracellularly [25] is not associated with changes in the mitochondrial sensitivity to the uncoupling effect of palmitate. However, this increase per se implies a higher FFA-mediated leak, with a following decrease in the degree of mitochondrial coupling. This result is apparently in contrast with the purpose of energy conservation, which characterises fasting. However, as pointed out by Soboll and Stucki [26] and Skulachev [27], decreased coupling in the fasted state would allow liver cells to attain faster rates of ATP production, since there is an inverse relationship between degree of coupling and rate of ATP production.

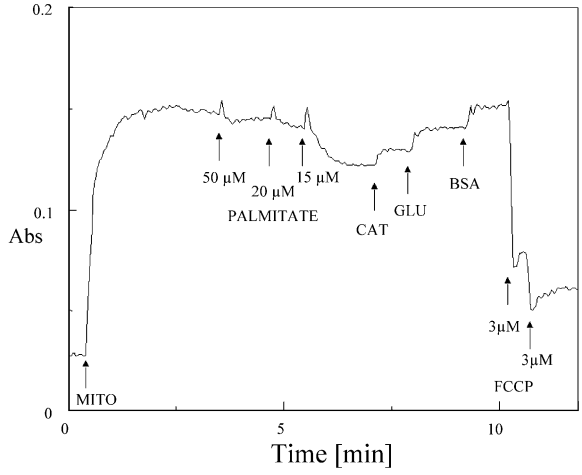

Fig. 1. Typical membrane potential responses of liver mitochondria to uncouplers and recouplers. Additions: mitochondria (mito) (0.5 mg), palmitate (85 μM), CAT (10 nmol/mg), glutamate (glu) (7 mM), BSA (4 mg/ml), FCCP (6 μM).

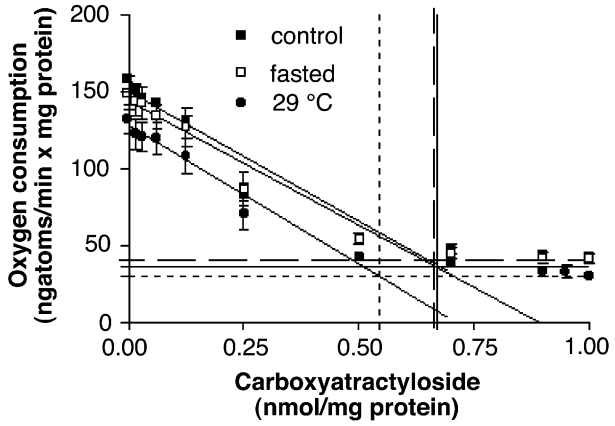

Fig. 2. Determination of ANT content by titration of state 3 respiration with CAT in hepatic mitochondria from control, fasted and 29°C rats. Results are reported as means ± S.E.M. of four different rats. For statistical analysis of the means see legend to Table 2. Horizontal lines indicate state 4 respiration values, vertical lines indicate the intercept of the regression line with the respective value of state 4, which represents the amount of CAT required for complete inhibition of state 3 respiration and therefore ANT content. Solid line, control rats; dotted line, 29°C rats; dashed line, fasted rats.

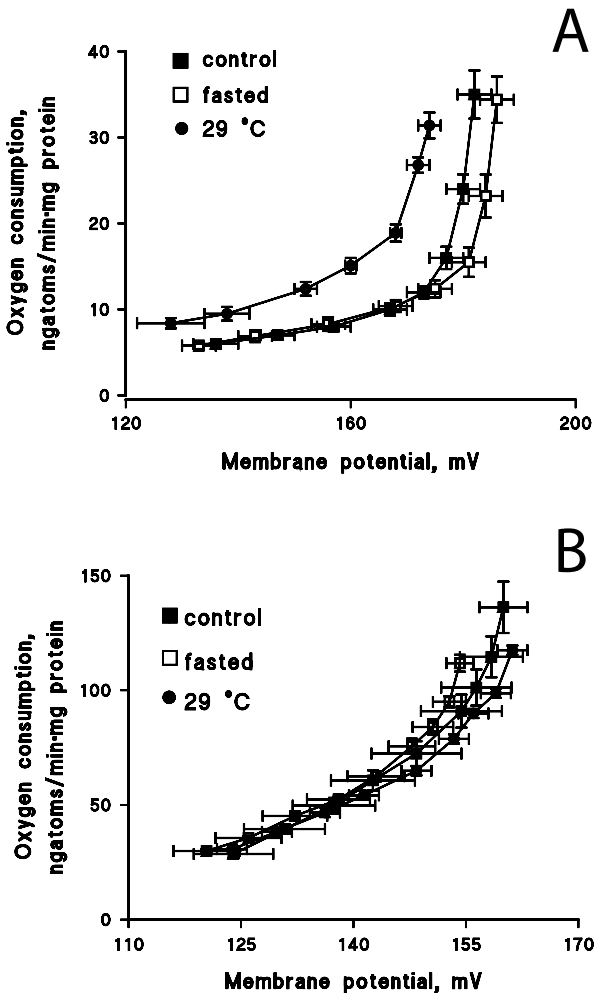

Fig. 3. Kinetics of basal (A) and palmitate-induced (B) proton leak in hepatic mitochondria from control, fasted and 29°C rats. Results are reported as means ± S.E.M. of four different rats. Basal proton leak was significantly ($P < 0.05$) higher in rats at 29°C than in controls, as shown by non-linear regression curve fits.

Another physiological condition characterised by decreased thermogenesis is thermoneutrality. Rats acclimated to 29°C do not need to produce additional heat to maintain body temperature, as rats acclimated to 24°C must do [28]. One of the major sites of energy conservation at thermoneutrality is brown adipose tissue (BAT), which is completely inactive, compared to its partial activation at room temperature [29]. However, no information on the behaviour of other metabolically active organs is available. Here we show that hepatic mitochondrial activity and efficiency vary in response to changes in environmental temperature. Hepatic mitochondria from rats acclimated to 29°C display decreased state 3 respiration (Table 2) and ANT content (Table 2, Fig. 2). It is known that in liver cells ANT exerts a strong control over state 3 respiration [30], as also confirmed by our present titration curves (Fig. 2), showing a sharp decrease in respiratory rates with only a small percentage of inhibited translocase molecules. The high control exerted by ANT on state 3 respiration led us to suggest that decreased ANT content and, therefore, activity is the main determinant of the decreased state 3 respiration found in hepatic mitochondria from rats acclimated to 29°C.

Liver mitochondria from rats acclimated to 29°C show no variation in palmitate-induced proton leak, as well as in the systems involved in palmitate-induced uncoupling (Fig. 3B, Table 3), despite the decrease in serum FFA levels (Table 1). On the other hand, an increased basal proton leak is found in rats at 29°C, compared to those at 24°C (Fig. 3A). Thus, basal proton leak is differently influenced in liver than in skeletal muscle mitochondria, since we have previously found no variation in rats at 29°C compared to those at 24°C [18]. It has previously been shown that basal proton leak in liver mitochondria is sensitive to changes in thyroid state of the animal. Basal proton leak is lower in liver mitochondria from hypothyroid rats and higher in liver mitochondria from hyperthyroid ones, compared to euthyroid controls [9]. This result has led to the conclusion that T_3 is an important determinant of mitochondrial efficiency. However, it should be noted that this conclusion was based on experimental conditions which could be considered pathological, such as undetectable levels of circulating T_3 or administration of pharmacological doses of T_3 [9]. In contrast with the above conclusions, here we show that when serum levels of T_3 are physiologically decreased (Table 1), such as at thermoneutrality and during fasting, basal leak of liver mitochondria is

Table 1
Serum free T_3 and FFA in control, fasted and 29°C rats

	Control	Fasted	29°C
Serum free T_3 (pg/100 ml)	383 ± 41	199 ± 5*	266 ± 10*
Serum FFA (mmol/l)	0.215 ± 0.01	0.450 ± 0.02*	0.143 ± 0.01*

Values are the means ± S.E.M. of four different rats. *$P < 0.05$ compared to controls (one-way ANOVA followed by Dunnett's post-hoc test).

Table 2
State 3 respiration, COX activity, ANT content and activity in control, fasted and 29°C rats

	Control	Fasted	29°C
State 3 respiration (ngatoms O/min/mg protein)	192 ± 10	171 ± 7	159 ± 9*
COX-specific activity (ngatoms O/min/mg protein)	1178 ± 77	1238 ± 34	1103 ± 19
ANT content (nmol/mg protein)	0.67 ± 0.01	0.66 ± 0.02	0.54 ± 0.02*
ANT turnover number (s^{-1})	335 ± 32	324 ± 28	349 ± 30
ANT-specific activity (nmol/min/mg protein)	226 ± 10	214 ± 9	188 ± 8*

Values are the means ± S.E.M. of nine (state 3) or four different rats. *$P < 0.05$ compared to control rats (one-way ANOVA followed by Dunnett's post-hoc test). State 3 respiration was measured using succinate (10 mM), rotenone (3.75 μM) and ADP (0.3 mM). Individual ANT contents were determined on each mitochondrial preparation by extrapolation of the linear part of the titration curve, as shown in Fig. 2.

differently affected, i.e. increased or not changed. Therefore, it appears from the present results that basal proton leak is not regulated by physiological changes in serum T_3 levels. The increased basal leak in rats acclimated to 29°C could have a role in controlling reactive oxygen species (ROS) production. In fact, it has been suggested that an important physiological role for incomplete mitochondrial coupling is protection against ROS [31], whose production increases when oxygen consumption rates are low and the degree of reduction of the components of the electron transport chain is high. Therefore, it is possible that in rats at 29°C, when state 3 respiratory rate is depressed, increased basal leak is functional in avoiding excess ROS production. In addition, it cannot be excluded that heat produced by increased basal leak could also try to compensate, at least in part, the decrease in metabolic rate due to inactivation of BAT thermogenesis [29] and decrease in T_3-mediated obligatory thermogenesis. The latter suggestion would point to basal proton leak in the liver as a way to produce heat, which is alternative to the classic mechanisms which are generally accepted as being involved in the control of body temperature, i.e. those acting in BAT and skeletal muscle [3]. In support of this notion, we found that in rats acclimated to cold (4°C) for 15 days, when thermoregulatory thermogenesis in BAT and skeletal muscle is maximally activated, basal proton leak in liver mitochondria decreases significantly compared to rats at 24°C (data not shown). Therefore, an inverse relationship between basal proton leak in liver mitochondria and environmental temperature appears to exist.

In conclusion, this study reveals that mitochondrial efficiency in the liver is not related to a physiological decrease in whole body thermogenesis. Cellular sites of energy conservation during fasting and at thermoneutrality must therefore be located in other tissues and organs. In addition, there is dissociation between changes in serum FFA levels and the mitochondrial sensitivity to the uncoupling effect of palmitate. Finally, the increased basal proton leak in rats at 29°C could be mainly involved in avoiding ROS production and/or it could represent an alternative way of metabolic heat production.

Table 3
Inhibitory effect of CAT, glutamate and BSA on palmitate-mediated decrease in membrane potential and increase in oxygen consumption in control, fasted and 29°C rats

	Control	Fasted	29°C
Palmitate-induced decrease in membrane potential (%) abolished by:			
CAT	24.0 ± 2.7	26.3 ± 3.2	27.1 ± 3.2
Glutamate	40.0 ± 3.5	35.0 ± 2.3	37.4 ± 3.0
BSA	36.0 ± 4.7	38.7 ± 4.7	35.5 ± 4.3
Palmitate-induced increase in oxygen consumption (%) abolished by:			
CAT	17.5 ± 1.9	16.5 ± 1.8	17.7 ± 2.3
Glutamate	14.6 ± 1.2	15.6 ± 1.5	17.6 ± 1.5
BSA	67.9 ± 7.1	67.9 ± 6.8	64.7 ± 6.8

Values are the means ± S.E.M. of four different rats.

Acknowledgements: This work was supported by a grant from the Università 'Federico II' of Naples.

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