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Research Article

Modulation of hepatic mitochondrial energy efficiency with age

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Abstract. This study was designed to examine the effect of youth-adulthood transition on hepatic mitochondrial energy efficiency. The changes in basal and palmitate-induced proton leak, which contribute to mitochondrial efficiency, were evaluated in mitochondria isolated from the liver of young and adult rats. Alterations in mitochondrial cytochrome oxidase and aconitase specific activities, and in adenine nucleotide translocator content were also assessed. There was no difference in basal proton leak or thermodynamic coupling and efficiency of oxidative phosphorylation in liver mitochondria between the two rat groups. On the other hand, palmitate-induced proton leak increased significantly in adult rats. The function of this uncoupling could be avoidance of elevated formation of reactive oxygen species, which are known to accelerate ageing.

Key words. Mitochondrial efficiency; basal proton leak; palmitate-induced leak; reactive oxygen species; youth-adulthood transition.

Ageing is known to be characterised by a general decline in mitochondrial function [1] and oxidative damage [2]. We have previously found that a decrease in FAD-linked respiration is the first age-linked biochemical impairment of energy-transducing pathways which could be detected in liver mitochondria from 180-day-old rats [3, 4]. This finding is in line with other results in which tissue ageing is associated with a decrease in electron transport chain enzyme activity [5]. The above decrease in liver mitochondrial respiratory activity may result in increased reactive oxygen species (ROS) production, if mechanisms that prevent ROS production are not implemented.

Mitochondrial uncoupling, by keeping the proton potential below the threshold level required to produce oxygen radicals, is functional in counteracting excess ROS production [6]. It follows that the regulation of ROS production depends on the degree of coupling of oxidative phosphorylation, and hence efficiency. Two sorts of uncoupling contribute to the efficiency of the mitochondrial machinery. Basal proton leak is present in the inner membrane of all mitochondria, is not acutely regulated and has been estimated to account for about 20% of rat standard metabolic rate [7]. Inducible proton leak is catalysed by proteins, such as uncoupling protein (UCP) 1 in brown adipose tissue mitochondria [8], adenine nucleotide translocator (ANT) [9], aspartate/glutamate antiporter [10] and other mitochondrial carriers in other tissues [11]. Mitochondrial carriers have been proposed to bind and transport negatively charged fatty acids out of the mitochondria [12]. After protonation on the cytosolic side, fatty acids could penetrate into the mitochondrial matrix, where they will be deprotonated, and thus will translocate protons into mitochondria (uncoupling effect) [13]. Therefore, inducible proton leak depends on the concentration of cellular unbound fatty acids, and can be regulated by whole-body metabolic condition.

Increased uncoupling as a mechanism to limit ROS production in the youth-adulthood transition is an attractive

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hypothesis. We were, therefore, interested to investigate if the youth-adulthood transition could cause changes in mitochondrial energy efficiency. To this purpose, in the present work we measured the parameters of oxidative phosphorylation together with changes in the degree of mitochondrial oxidative coupling in liver mitochondria from 60- and 180-day-old rats. Basal and inducible leak as well as ROS damage were also assessed in liver mitochondria of the above animals. Identification of changes in the efficiency of the mitochondrial machinery may give new insight into liver energetic adaptation with age.

Materials and methods

Animals

Two groups of eight male Wistar rats (Charles River, Calco, Como, Italy) aged 60 days (young) and 180 days (adult) were used for this study. They were kept at 24 °C under an artificial circadian 12-h light/12-h darkness cycle, with ad libitum access to water and a standard stock diet (Mucedola 4RF21; Settimo Milanese, Milan, Italy). At the time of the experiments, the animals, without any previous food deprivation, were anaesthetised with chloral hydrate (40 mg per 100 g body weight) and after blood collection from the inferior vena cava, killed by decapitation. Animal care, housing and killing met the guidelines of the Italian Health Ministry.

Preparation of isolated mitochondria

Livers were quickly removed and used for preparation of isolated mitochondria, as previously reported [14]. Briefly, tissue fragments were gently homogenised with a medium containing 220 mM mannitol, 70 mM sucrose, 20 mM HEPES, 1 mM EDTA and 0.1% (w/v) fatty-acid-free bovine serum albumin (BSA), pH 7.4, in a Potter Elvehjem homogenizer set at 500 rpm (4 strokes/min). The homogenate was then centrifuged at 1000 g_{av} for 10 min and the resulting supernatant was again centrifuged at 3000 g_{av} for 10 min. The mitochondrial pellet was washed twice and finally resuspended 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. Enzymatic and electron microscopy characterisation has shown that our isolation procedure (centrifugation at 3000 g_{av} for 10 min) results in a cellular fraction, which is constituted essentially by mitochondria [15]. The protein content of the mitochondrial suspension was determined by the method of Hartree [16] using BSA as the protein standard.

Measurement of mitochondrial respiration parameters

Mitochondrial oxygen consumption was measured polarographically with a Clark-type electrode (Yellow Springs Instruments, Yellow Springs, Ohio) in a 3 ml glass cell, at a temperature of 30 °C. Isolated mitochondria (0.3–0.4 mg protein/ml) were incubated 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, pH 7.0 and allowed to oxidise their endogenous substrates for a few minutes. In the presence of 10 mM succinate, 3.75 µM rotenone, and 0.3 mM ADP, state 3 oxygen consumption was measured. State 4 was obtained in the absence of ADP. The respiratory control ratio (RCR) was calculated according to Estabrook [17]. Oxygen consumption in the presence of oligomycin (2 µg/ml) or FCCP (1 µM) was also checked.

Cytochrome oxidase (COX) specific activity was measured polarographically with a Clark-type 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 [18]. 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-phenylenediammine (TMPD).

Measurement of basal proton leak

Mitochondrial oxygen consumption was measured polarographically with a Clark-type electrode, whereas membrane potential recordings were performed in parallel with safranin O using a JASCO dual-wavelength spectrophotometer (511–533 nm) [19]. The absorbance readings were transferred to mV membrane potential using the Nernst equation: $\Delta\psi = 61\ mV \times \log([K^+]_{in}/[K^+]_{out})$, and calibration curves made for each preparation as previously reported [20]. Measurements were carried out at 30 °C and in a medium containing 80 mM LiCl, 50 mM HEPES, 5 mM TrisPO_4, 1 mM EGTA, 0.1% (w/v) fatty-acid-free BSA, pH 7.0, 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). Oxygen consumption and membrane potential determinations were carried out by sequential additions of malonate up to 5 mM.

Measurement of inducible leak

Mitochondrial oxygen consumption and membrane potential 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. 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. [21]. Oxygen consumption and membrane potential determinations were carried out by se-

quential additions of malonate up to 600 mM. The contribution of ANT, glutamate-aspartate carrier, and other systems to the uncoupling effect of palmitate was assessed by measuring the recoupling effect of carboxyatractyloside (CAT, 10 nmol/mg protein) for ANT, glutamate (7 mM) for glutamate-aspartate carrier, and BSA (0.4%, w/v) for other systems. The membrane potential in the presence of palmitate was measured with the same procedure as above. When a steady-state level was reached, the effect of CAT, glutamate, and BSA (which were added in sequence) was tested.

Measurement of ANT content

Liver mitochondrial ANT content was determined by titrating state 3 respiration with increasing concentrations of CAT [22] 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, 10 mM succinate, 3.75 µM rotenone and 0.3 mM ADP, pH 7.0. The mitochondrial content of ANT was determined by extrapolating the linear part of the titration curve to obtain the amount of CAT required to inhibit completely state 3 respiration.

Determination of mitochondrial aconitase specific activity and serum non-esterified fatty acid levels

To determine aconitase specific activity, mitochondrial aliquots were immediately frozen on liquid nitrogen and stored at –80 °C. On the day of assay, mitochondria were sonicated and centrifugated to obtain mitochondrial extract. Aconitase was assayed spectrophotometrically by following the formation of NADPH (340 nm) at 25 °C in a mixture containing 0.2 mM $NADP^+$, 5 mM sodium citrate, 0.6 mM $MnCl_2$, 1 U/ml concentration of isocitric dehydrogenase, 50 mM Tris-HCl, pH 7.4, and 30 ml of mitochondrial extract [23].

Blood samples were centrifuged at 2000 g for 10 min. Serum was removed and stored at –20°C until the time of measurement. Serum non-esterified fatty acids (NEFAs) were measured by colorimetric enzymatic method using a commercial kit (Roche Diagnostics, Mannheim, Germany).

Statistical analysis

Data are summarised as means ± SE. Statistical analyses were performed using two-tailed unpaired Student's t test and linear or non-linear regression analysis. Probability values less than 0.05 were considered to indicate a significant difference. All analyses were performed using GraphPad Prism (GraphPad Software Inc., San Diego, Calif.).

Materials

All reagents were purchased from Sigma Chemical Co. (St. Louis, Mo.) except for CAT, which was purchased from Calbiochem (San Diego, Calif.)

Results

State 3 and 4 mitochondrial respiration, using succinate as substrate, together with COX activity (table 1) and ANT content (fig. 1) decreased significantly in 180- compared to 60-day-old rats. In addition, RCR values were similar in both groups of rats and were consistent with those of intact, functional isolated mitochondria.

We have also determined thermodynamic coupling and efficiency of oxidative phosphorylation using the methodology of Cairns et al. [24]. The degree of thermodynamic coupling symbolised by q was calculated by the following equation:

$$q = [1 - (J_{sh}/J_{unc})]^{1/2},$$

where J_{sh} represents the oxygen consumption rate in the presence of oligomycin that inhibits ATP synthase, and J_{unc} is the uncoupled rate of oxygen consumption induced by FCCP, which dissipates the transmitochondrial proton gradient.

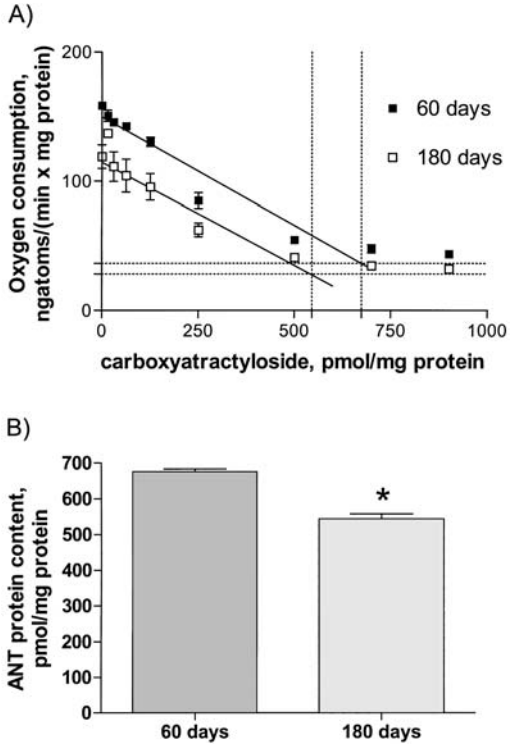

Figure 1. (*A*) Determination of ANT content in hepatic mitochondria from 60- and 180-day-old rats by titration of state 3 respiration with CAT. Horizontal lines indicate state 4 respiration values, vertical lines indicate the intercept of the regression line with the respective value of state 4, which represents the amount of CAT required for complete inhibition of state 3 respiration. (*B*) ANT contents in hepatic mitochondria from 60- and 180-day-old rats. The individual ANT contents were determined on each mitochondrial preparation by extrapolation of the linear part of the titration curve, as shown in (*A*). Results are reported as means ± SE of eight different experiments. * $p < 0.05$ compared to 60-day-old rats.

Table 1. Respiratory parameters and COX activity in liver mitochondria isolated from 60- and 180-day-old rats.

	60-day-old rats	180-day-old rats	% change
State 3	184 ± 8	116 ± 6*	–37
State 4	27 ± 2	18 ± 1*	–33
RCR	6.8 ± 0.4	6.3 ± 0.2	–7
Uncoupled State	215 ± 2	154 ± 11*	–28
State 4 plus oligomycin	29 ± 2	19 ± 2*	–34
q	0.924 ± 0.004	0.931 ± 0.008	7
η	0.448 ± 0.010	0.469 ± 0.021	5
COX activity	2027 ± 241	1297 ± 69*	–29

Values are expressed as ng atoms O/(min × mg protein) and are the means ± SE of eight different experiments. % change with respect to 60-day-old rats. State 3 and state 4 were measured in the presence of 10 mM succinate + 3.75 µM rotenone. q = thermodynamic degree of coupling; η = optimal efficiency. *$p < 0.05$ compared to 60-day-old rats.

Optimal thermodynamic efficiency of energy conversion symbolised by η was calculated using the following equation:

$$\eta = \frac{q^2}{(1+\sqrt{1-q^2})^2}$$

These parameters of coupling of mitochondrial oxidative phosphorylation q and η were similar across the experimental groups (table 1).

Figure 2 shows the kinetics of basal and inducible proton leak measured in mitochondria isolated from 180- and 60-day-old rats.The presence of fatty-acid-free BSA both during the preparation of isolated mitochondria and basal proton leak measurements is necessary to avoid contaminating free fatty acids, which are known mitochondrial uncouplers [25]. BSA chelates the free fatty acids and therefore abolishes the effect. On the other hand, as for inducible proton leak, adding exogenous palmitate stimulated it. The titration curves reported in figure 2 are an indirect measurement of the dependence of mitochondrial proton leak on membrane potential, since steady-state oxygen consumption (i.e. proton efflux rate) in non-phosphorylating mitochondria is equivalent to the proton influx rate due to proton leak [26]. No difference in the kinetics of basal proton leak occurred between hepatic mitochondria from 60- and 180-day-old rats (fig. 2A). On the other hand, liver mitochondria from 180-day-old rats displayed a higher rate of palmitate-induced proton leak than those from 60-day-old rats at all membrane potential values (fig. 2B). Concomitantly, an increase in serum NEFA levels was found in 180-day-old compared to younger rats (fig. 2C).

The recoupling effect of CAT and glutamate was measured to test the contribution of ANT and glutamate

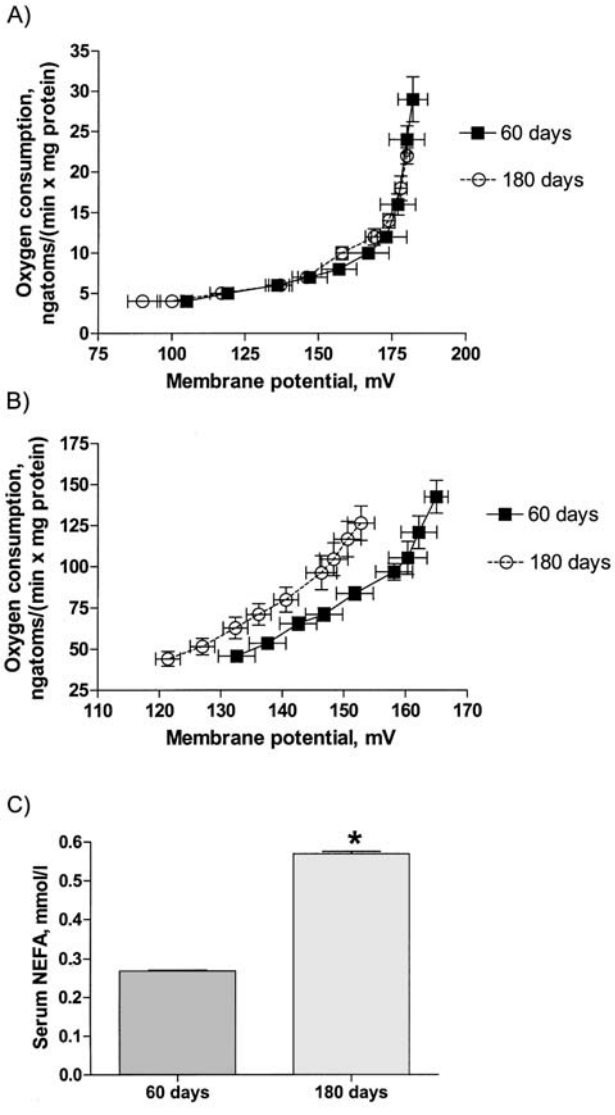

Figure 2. Kinetics of basal proton leak (*A*), palmitate-induced proton leak (*B*) and serum NEFA levels (*C*) in hepatic mitochondria from 60- and 180-day-old rats. Results are reported as means ± SE of eight different experiments. Palmitate-induced proton leak was significantly higher ($p < 0.05$) in 180- than in 60-day-old rats, as shown by fits of a non-linear regression curve. *$p < 0.05$ compared to 60-day-old rats.

carrier to palmitate-induced proton leak (table 2). The results show that the contribution of ANT decreased significantly in 180- compared to 60-day-old rats, while that of glutamate carrier increased significantly. In contrast, no variation was found across the two experimental groups in the other protein systems involved in inducible leak, as indicated by no changes in the BSA recoupling effect in either group of rats.

The specific activity of the enzyme aconitase was monitored in mitochondria isolated from the two experimental groups. This enzyme can be used to estimate indirectly the superoxide concentration, since its activity is very

Table 2. Inhibitory effect of CAT, glutamate and BSA on the palmitate-mediated decrease in membrane potential in liver mitochondria isolated from 60- and 180-day-old rats.

	60-day-old rats	180-day-old rats
Palmitate-induced decrease in membrane potential (mV)	10.1 ± 0.5	16.2 ± 0.6*
Palmitate-induced decrease in membrane potential (%) abolished by:		
CAT	25 ± 2	15 ± 1*
Glutamate	28 ± 2	35 ± 2*
BSA	47 ± 7	50 ± 4

Values are the means ± SE of eight different experiments. * $p < 0.05$ compared to 60-day-old rats.

sensitive to superoxide exposure [23]. The results showed no variation in mitochondrial aconitase activity (10.1 ± 1.4 mU/mg protein for 60-day-old rats and 10.0 ± 0.6 for 180-day-old rats) and, therefore, one can assume that superoxide radical generation is similar in mitochondria isolated from 60- and 180-day-old rats.

Discussion

In this work, we found that the decline in succinate-supported state 3 respiration previously observed in adult rats [3, 4] is due to a decrease in COX activity and ANT content. COX activity and ANT are known to exert a strong control over liver respiration [27, 28]. In addition, titration curves obtained here for ANT determination (fig. 1A) show a sharp decrease in respiratory rates with only a small percentage of inhibited translocase molecules and, therefore, confirm that ANT is an important determinant of respiratory rates in the liver, when succinate is used as a respiratory substrate.

Taking into account that basal and fatty-acid-induced proton leak contribute to mitochondrial efficiency, these parameters were measured in young and adult rats. The results showed no changes in basal proton leak kinetics in 180-day-old compared to younger rats (fig. 2). Since state 4 respiration is controlled by both proton conductance and substrate oxidation [29], we conclude that the decrease in state 4 found in adult rats is only due to the decrease in the substrate oxidation pathway, mainly in COX activity. In agreement with the finding that basal proton leakage did not change across the two experimental groups, the values of q and η, parameters of the degree of oxidative phosphorylation coupling, were similar in 60-days and 180-days old rats (table 1).

We also measured proton leak in the presence of 98 nM free palmitate, a concentration likely to be similar to that occurring in the cell, due to the presence of large amounts of various isoforms of fatty-acid-binding protein with a dissociation constant below 1 µM [30]. This concentration of palmitate does not irreversibly alter mitochondrial membrane integrity, as indicated by complete reversal of the uncoupling effect by BSA (table 2) and induces a light decrease in membrane potential of about 10–15 mV, which is less than that occurring in the transition from state 4 to state 3 (data not shown). Interestingly, we found an increased palmitate-induced proton leak in 180-day-old rats compared to younger ones, associated with increased serum NEFA levels (fig 2), which could elicit a higher cellular unbound fatty acid concentration. This situation could cause a high level of fatty-acid-mediated uncoupling in the liver cells from adult rats. As for systems involved in palmitate-induced uncoupling, our present results show that the ANT contribution significantly decreased in adult rats, consistent with decreased ANT content found in these animals.

The above increase in fatty-acid-mediated uncoupling per se implies an elevated hepatic thermogenesis, which is not in line with the decrease in whole-body energy expenditure previously found in 180-day-old rats [31]. Therefore, other tissues and organs must be involved in energy conservation in adult rats. On the other hand, liver may play a different role in order to counteract oxidative damage and, consequently, slow the ageing processes. In fact, a postulated role for incomplete mitochondrial coupling is protection against ROS [6], whose production increases when oxygen consumption rates are low and the degree of reduction of the components of the electron transport chain is high. Taking into account that in 180-day-old rats we found a decrease in oxidative rates and COX activity without any change in succinic dehydrogenase activity as previously shown [3], in adult rats, increased palmitate-induced leak possibly functions to avoid excess ROS damage. Consistent with this role, we found that in 180-day-old rats, compared to 60-day-old ones, there is no variation in the specific activity of superoxide-sensitive mitochondrial enzyme aconitase. However, changes in antioxidant defences, such as superoxide dismutase, could contribute to maintain constant aconitase activity.

In conclusion, this study reveals that the youth-adulthood transition does not elicit variations in basal proton leak or the degree of thermodynamic coupling in response to decreased respiration rate in isolated mitochondria from liver cells. On the other hand, an increase in fatty-acid-induced mild uncoupling characterises the youth-adulthood transition. The function of this uncoupling could be to avoid elevated formation of ROS, which are known to accelerate ageing. Taking into account the free radical theory of ageing [2], one can speculate that an elevated level of mitochondrial inefficiency could be important in slowing the onset of ageing.

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