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Rat liver mitochondrial respiratory capacities in the transition from weaning to adulthood

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Abstract

In the present study we investigated the changes in hepatic mitochondrial function in the transition from weaning to adulthood in the rat. We measured mitochondrial respiration using FAD- and NAD-linked substrates in 25 and 60 day old rats. The results show that adult rats exhibited significantly higher respiratory rates with all the substrates used except pyruvate. Our results indicate that the transition from weaning to adulthood induces important changes in hepatic mitochondrial function. © 1998 Elsevier Science Ireland Ltd.

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1. Introduction

In growing rats, significant modifications to liver mitochondrial activity take place. Immediately after birth, there is an improvement of the respiratory mitochondrial capacity compared to the foetal mitochondria. In fact a decrease in the passive proton permeability of mitochondrial membranes (Valcarce et al., 1990) and an increase in the state 3 respiration rate has been shown (Aprille and Asimakis,

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1980; Valcarce et al., 1988). This increase in state 3 has been reported to occur during the first postnatal hour with no further changes until adult rates of respiration were attained (Valcarce et al., 1988). On the other hand, other authors showed that the state 3 respiration rate reached an higher value during the suckling/weaning period than during the perinatal one (Quant et al., 1991). Finally, a lot of studies reported a decrease in the state 3 respiration rate in aging rats (Hansford, 1983; Alemany et al., 1988; Kim et al., 1988; Tummino and Gafni, 1991). However, it is not clear whether any other changes in mitochondrial respiration rates occur in the transition from weaning to adulthood.

In the light of the above considerations, we thought it would be interesting to study functional variations of the liver mitochondrial compartment in 25 (post-weaning) and 60 day old (mature) rats. Studies of this nature should help in determining the age at which a complete maturation of the mitochondrial respiratory capacity is reached. To this purpose, we measured hepatic mitochondrial respiratory activity in isolated mitochondria. Different respiratory substrates were used, to involve different dehydrogenases, different carrier systems and different sites of the electron transport chain.

2. Materials and methods

2.1. Animals

Male Wistar rats (Charles River Italia, Calco, Como, Italy) were used for the experiments and their care, housing and killing met the guidelines of the Italian Health Ministry. They were housed individually in grid-bottomed cages 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).

Rats of 25 days of age and about 60 g body weight or 60 days of age and about 280 g body weight were killed by decapitation and the livers were rapidly removed.

2.2. Preparation of isolated mitochondria

After removal, the livers were finely minced and washed with a medium containing 220 mM mannitol, 70 mM sucrose, 20 mM Tris, pH 7.4, 1 mM EDTA, and 0.1% (w/v) fatty acid free bovine serum albumin. Tissue fragments were gently homogenized with the same medium (1:10, w/v) in a Potter Elvehjem homogenizer set at 500 rpm (4 strokes/min). The homogenate was filtered through sterile gauze and freed of debris and nuclei by centrifugation at $1000 \times g$ for 10 min; the resulting supernatant was centrifuged at $3000 \times g$ for 10 min, the mitochondrial pellet was washed twice and finally resuspended in a medium containing 80 mM KCl, 50 mM Hepes, pH 7.0, 5 mM KH_2PO_4, 0.1% (w/v) fatty acid free bovine serum albumin. Enzymic and electron microscopy characterization of mitochondria isolated by centrifugation at $3000 \times g$ has shown that they are virtually pure

(Goglia et al., 1988). The protein content of the mitochondrial suspension was determined by the method of Hartree (1972) using bovine serum albumin as the protein standard.

2.3. Mitochondrial respiration and enzyme activities

Mitochondrial oxygen consumption was measured polarographically with a Clark-type electrode (Yellow Springs Instruments, Yellow Springs, OH), maintained in a 3 ml water jacketed chamber at 30°C. Mitochondria (1 mg of protein) were incubated in 3 ml of the above suspension medium. Measurements were made within 2 h following the isolation of the mitochondria. The mitochondria were allowed to oxidize their endogenous substrates for a few minutes. Substrates were then added at the concentration reported in the Tables to determine the state 4 oxygen consumption rate. Six min later, ADP (at a final concentration of 0.3 mM) was added and the state 3 rate was measured. The ratio between states 3 and 4 (RCR) was calculated according to Estabrook (1967).

Succinic dehydrogenase (EC 1.3.99.1) and mitochondrial α-glycerophosphate dehydrogenase (EC 1.1.1.8) activity were measured by the method of Lee and Lardy (1965).

2.4. Statistical analysis

Data are given as means $\pm$ S.E.M. Statistical significance between the means was examined by a two-tailed Student's t-test. $P < 0.05$ were considered to indicate a significant difference.

2.5. Materials

ADP, pyruvate, malate, glutamate, succinate, rotenone, α-glycerophosphate, palmitoylcarnitine, carnitine, palmitoylCoA, phenazine methosulfate and iodonitrotetrazolium violet were purchased from Sigma (St. Louis, MO). All other reagents used were of the highest purity commercially available.

3. Results

Table 1 shows the results on hepatic mitochondrial respiration using two NAD-linked substrates, namely glutamate + malate and pyruvate + malate. The states 3 and 4 respiration with glutamate + malate significantly increased (64% and 31%, respectively) in 60 day old rats compared to 25 day old rats (Table 1). With pyruvate + malate as substrate, a significant increase (41%) in the state 4 but not in the state 3 respiration was found in 60 day compared to 25 day old rats (Table 1).

Respiratory and enzymic activities using FAD-linked substrates are reported in Table 2. The states 3 and 4 respiration with succinate + rotenone, as well as succinic dehydrogenase specific activity, significantly increased (61%, 32% and 68%, respec-

Table 1
Respiratory activity with NAD-linked substrates in isolated mitochondria from 25 and 60 day old rats

	25 day	60 day	% Change
Glutamate + malate			
State 3	53 ± 6 (38–67)	87 ± 12 (48–125)*	64
State 4	7.4 ± 0.2 (6.8–8.2)	9.7 ± 0.7 (6.1–12.7)*	31
RCR	7.2 ± 0.9 (4.9–9.4)	9.0 ± 0.4 (7.9–10.3)	25
Pyruvate + malate			
State 3	34 ± 3 (29–38)	34 ± 2 (28–40)	0
State 4	5.4 ± 0.4 (4.1–6.4)	7.6 ± 0.3 (6.2–9.1)*	41
RCR	6.3 ± 0.7 (4.7–8.5)	4.5 ± 0.2 (3.9–4.9)*	−28

Values are the means ± S.E.M. of four different rats. 95% Confidence intervals are in parenthesis. States 3 and 4 respiratory rates are expressed as nmol O/min × mg protein. Substrate concentrations: glutamate 10 mM, pyruvate 10 mM, malate 2.5 mM.
* $P < 0.05$ compared to 25 day rats.

tively) in 60 day old rats compared to 25 day old rats, while no variation was found in mitochondrial α-glycerophosphate dehydrogenase specific activity (Table 2).

Hepatic mitochondrial respiratory rates with lipid substrates are reported in Table 3. The states 3 and 4 oxygen consumption with palmitoylcarnitine + malate significantly increased (74% and 50%, respectively) in 60 day old rats compared to 25 day old rats (Table 3). Similarly, significantly higher states 3 and 4 respiratory rates with palmitoyl–CoA + carnitine + malate (115% and 45%, respectively) were found in 60 day old rats than in 25 day old rats (Table 3). It should be noted that the high RCR values found in this work indicate the high quality of the mitochondrial preparations. In addition, these values were not significantly different in the two sets of mitochondria, except when pyruvate + malate and palmitoyl–CoA + carnitine were used as substrate.

Table 2
Respiratory and enzymic activities with FAD–linked substrates in isolated mitochondria from 25 and 60 day old rats

	25 day	60 day	% Change
Respiratory rate with succinate			
State 3	135 ± 11 (107–162)	218 ± 11 (182–254)*	61
State 4	25 ± 2 (19–30)	33 ± 3 (25–42)*	32
RCR	5.5 ± 0.4 (4.4–6.4)	6.6 ± 0.2 (5.8–7.3)	20
SDH activity	190 ± 10 (155–220)	320 ± 20* (246–393)	68
α–GPDH activity	38 ± 2 (32–47)	40 ± 2 (33–48)	5

Values are the means ± S.E.M. of four different rats. 95% Confidence intervals are in parenthesis. States 3 and 4 respiratory rates were measured in the presence of succinate 10 mM + rotenone 3.75 μM and are expressed as nmol O/min × mg protein. Succinic dehydrogenase (SDH) and α–glycerophosphate dehydrogenase (α–GPDH) activities are expressed as nmol/min × mg protein.
* $P < 0.05$ compared to 25 day rats.

Table 3
Respiratory activiy with lipid substrates in isolated mitochondria from 25 and 60 day old rats

	25 day	60 day	% Change
Palmitoylcarnitine + malate			
State 3	54 ± 4 (43–65)	94 ± 6 (75–112)*	74
State 4	10 ± 1 (8–12)	15 ± 1 (11–20)*	50
RCR	5.4 ± 0.4 (4.4–6.5)	6.3 ± 0.4 (5.0–7.5)	16
Fatty acid utilization	2.5 ± 0.1 (1.9–2.9)	3.6 ± 0.2 (3.4–5.1)*	44
PalmitoylCoA + carnitine + malate			
State 3	40 ± 3 (31–49)	86 ± 4 (63–109)*	115
State 4	11 ± 1 (8–13)	16 ± 1 (12–20)*	45
RCR	3.6 ± 0.2 (3.0–4.4)	5.4 ± 0.4 (4.2–6.6)*	50
Fatty acid utilization	1.8 ± 0.1 (1.4–2.2)	3.9 ± 0.2 (2.8–5.0)*	116

Values are the means ± S.E.M. of four different rats. 95% Confidence intervals are in parenthesis. States 3 and 4 respiratory rates are expressed as nmol O/min × mg protein. Fatty acid utilization rates are expressed as nmol/min × mg protein. Substrate concentrations: palmitoylcarnitine 40 μM, palmitoyl-CoA 40 μM, carnitine 2 mM, malate 2.5 mM.
* $P < 0.05$ compared to 25 day rats.

4. Discussion

The present study showed that mitochondrial respiratory capacity increased in 60 day old rats compared to 25 day old rats. This finding is different from a previous one which showed that during the first hour of postnatal life a complete maturation of respiratory capacity occurred (Valcarce et al., 1988). However, another study has found an increase in respiratory rates in suckling and weaning rats compared to neonatal ones (Quant et al., 1991).

Hepatic mitochondrial respiratory capacity was determined by measuring the states 3 and 4 respiration with various substrates, to involve different dehydrogenases, different carriers and different sites of entry of reducing equivalents into the respiratory chain.

Two NAD-linked substrates were used: glutamate + malate and pyruvate + malate (Table 1). As for the state 3 respiration rate, we found a significant increase with glutamate + malate, but no variation with pyruvate + malate. These results suggest an increase in the specific dehydrogenases involved in the oxidation pathway for glutamate. However, the increased glutamate-supported respiration could also be due to an increase in the activity of the respiratory chain and/or ATP synthetase, which does not influence pyruvate-supported respiration: in fact, respiratory rates with pyruvate are always lower than those with glutamate, suggesting that the initial step of pyruvate dehydrogenase and/or pyruvate carrier regulates pyruvate-supported respiration (Liverini et al., 1990).

We have also measured hepatic mitochondrial respiratory rates with a FAD-linked substrate, succinate (Table 2), as well as the specific activity of two FAD-linked dehydrogenases: succinic dehydrogenase and mitochondrial α-glyc-

erophosphate dehydrogenase (Table 2). The significantly higher respiratory rates with succinate found in 60 day old rats could be due to the significant increase found in succinic dehydrogenase specific activity (Table 2); however, we cannot exclude an increase in the activity of the respiratory chain from complex II onwards and/or ATP synthetase. On the other hand, no variation was found in mitochondrial α-glycerophosphate dehydrogenase specific activity. This finding is in agreement with our previous result showing no variation in triiodothyronine (T_3) serum levels between 30 and 60 day old rats (Barletta et al., 1980), since mitochondrial α-glycerophosphate dehydrogenase specific activity is strictly related to T_3 serum levels (Lee and Lardy, 1965).

We have also found an increase in state 4 respiratory rates with both NAD- and FAD-linked substrates (Tables 1–3) in 60 day old rats, compared to 25 day old rats. With the limitation that state 4 rates can give only a rough indication of the activity of the proton leak pathway Brand (1990), we suggest that H^+ permeability of the inner mitochondrial membrane increases in 60 day old rats.

Lipid-supported respiration was measured with palmitoylcarnitine + malate and palmitoyl–CoA + carnitine + malate, which produce NADH and $FADH_2$ through the β-oxidative pathway. Palmitoyl–CoA-supported respiration reflects the activity of carnitine–palmitoyltransferase (CPT) I, CPT II and the intramitochondrial β-oxidation pathway, while respiration with palmitoylcarnitine, which bypasses the step catalyzed by CPT I, represents an index of fatty acid oxidation per se (Escriva et al., 1986). Since the measurements were made in the presence of malate, acetylCoA is directed towards citrate synthesis and fatty acid utilization rates can be calculated by dividing the rate of oxygen consumption by 22, which is the ratio of oxygen consumption/fatty acid utilized for palmitoylcarnitine (Brady and Hoppel, 1983). Significant increases in lipid-supported respiratory rates were found in 60 day old rats (Table 3). Moreover, state 3 respiration and fatty acid utilization rates with palmitoyl–CoA were about 25% lower than those with palmitoylcarnitine in 25 day old rats, while no difference between the two substrates was found in 60 day old rats (Table 3); therefore, CPT I activity limits palmitoyl–CoA oxidation only in 25-day old rats. This result is in agreement with the observation that hepatic fatty acid oxidation capacity dramatically decreases at weaning, due to a decrease in the activity of CPT I and of the other enzymes involved in the β-oxidation pathway (Decaux et al., 1988a,b; Asins et al., 1995). This can be due to the fact that the switch from the high-fat–low-carbohydrate diet represented by maternal milk to high-carbohydrate–low-fat laboratory chow is accompanied by modifications of glucose and fatty acid metabolism (Decaux et al., 1986, 1988a,b), which are regulated by alterations in hormonal secretion (Girard et al., 1977; Walker et al., 1980). The results also indicate an increase in hepatic lipid oxidation capacity in 60 day old rats, which is partly due to an increase in CPT I activity. However, the increase found with palmitoylcarnitine indicates that there is also increased activity of other enzymes, such as those of the β-oxidation pathway. Accordingly, it has been shown that hepatic palmitoyl–CoA dehydrogenase activity steadily increased during postnatal development in the rat Carroll et al. (1989) in addition, a 3-fold increase in the mRNA levels for medium chain acyl-CoA dehydrogenase has been

found between 35 and 70 days of age in the rat (Kelly et al., 1989). In summary, our present results obtained with lipid substrates indicate that the reduction in the capacity for fatty acid oxidation induced by weaning is reversed in post-pubertal rats.

Taken together, our results clearly indicate that in the transition from weaning to adulthood liver mitochondria improve their oxidative capacity. So, maturity is associated with elevated rates of oxygen utilization. Then, with aging, the mitochondrial respiratory capacity declines as shown by various authors (Hansford, 1983; Alemany et al., 1988; Kim et al., 1988; Tummino and Gafni, 1991). We suggest that adulthood can be the developmental stage in which the liver mitochondrial compartment achieves the maximum capacity to oxidize lipid and non lipid substrates.

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