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Oxidative Activity in Mitochondria Isolated from Rat Liver at Different Stages of Development

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The purpose of this study was to evaluate the oxidative capacities in hepatic mitochondria isolated from prepubertal, young adult and adult rats (40, 90 and 180 days of age, respectively). In these rats, mitochondrial respiratory rates using FAD- and NAD-linked substrates as well as mitochondrial protein mass were measured. The results show that only the oxidative capacity of FAD-linked pathways significantly declined in mitochondria from 180-day-old rats compared with those from younger animals. When we consider FAD-linked respiration expressed per g liver, no significant difference was found among rats of different ages because of an increased mitochondrial protein mass found in 180-day-old rats. However, when FAD-linked and lipid-dependent respiratory rates were expressed per 100 g body weight, significant decreases occurred in 180-day-old rats. Therefore, the decrease in liver weight expressed per 100 g body weight rather than an impaired hepatic cellular activity may be the cause of body energy deficit in 180-day-old rats. © 1998 John Wiley & Sons, Ltd.

KEY WORDS — developing rats; liver mitochondria; respiratory rates

INTRODUCTION

It has been reported that a general decline in mitochondrial function is associated with the aging process.[1–7] In fact, cellular energy deficits caused by mitochondrial function impairments may damage the cell's ability to adapt to physiological stresses and this damage seems to be one of the most profound manifestations of biological ageing.[8] In addition, if the mitochondrial ATP production decreases and falls below the minimum energy levels necessary for oxidative tissues and organs to function, certain diseases can result. In fact, a variety of age-related pathological alterations has been generally associated with an inhibition of oxidative phosphorylation.[9–11]

Most studies conducted to date on the effect of age on mitochondrial oxidative activity have examined adult and old animals.[6,10] In an attempt to gain further insights into the complex physiological processes that may be involved in the age-related decline of mitochondrial oxidative activity, investigations of their development in young animals, from puberty to adulthood, may prove to be of critical importance. The specific objective of the present study was to determine when age-related impairments of the mitochondrial respiratory system begin to appear. To this end, state 3 and state 4 respiration rates were studied in isolated mitochondria from prepubertal (40 days), young adult (90 days), and adult rat (180 days). To stimulate mitochondrial respiration, NAD- and FAD-linked substrates were used so that different pathways of oxidation could be tested. Hepatic mitochondrial protein mass was also determined. This measurement allows us to link the oxidative capacities measured in isolated mitochondria with the effective liver mitochondrial protein mass. So, more physiological evidence may be obtained.

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MATERIALS AND METHODS

Animals

Male Wistar rats obtained form Charles River (Calco, Como, Italy) just after weaning were used for the experiments. At the start of the study the rats (aged 25 days) were divided into three groups, each composed of four rats, with similar mean body weights. 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) before being killed. Animal care, housing and killing met the guidelines of the Italian Health Ministry.

One group of rats was killed at 40 days of age, another at 90 days of age and the last one at 180 days of age.

Preparation of Isolated Mitochondria

The rats, without any previous food deprivation, were anaesthetized with chloral hydrate (40 mg per 100 g body weight) and then decapitated. Livers were rapidly removed, 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 per cent (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 r.p.m. (4 strokes min^{-1}). Liver mitochondria were isolated from the homogenate as previously reported.[12,13] Briefly, the homogenate was filtered through sterile gauze and freed of debris and nuclei by centrifugation at 1000 g_{av} for 10 min; 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 KCL, 50 mM Hepes, pH 7·0, 5 mM KH_2PO_4, 0·1 per cent (w/v) fatty acid-free bovine serum albumin. It should be noted that enzymic and electron microscopy characterization 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. [14,15] The protein content of the mitochondrial suspension was determined by the method of Hartree[16] using bovine serum albumin as the protein standard.

Mitochondrial Respiration and Enzyme Activities

Mitochondrial oxygen consumption was measured polarographically with a Clark-type electrode (Yellow Springs Instruments, Yellow Springs, OH, USA), maintained in a water-jacketed chamber at 30°C. Mitochondria (1 mg 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 Tables 2, 3 and 4 to determine state 4 oxygen consumption rate. Six minutes later, ADP (at a final concentration of 0·3 mM) was added and state 3 rate was measured. The ratio between state 3 and 4 (RCR) was calculated according to Estabrook.[17]

Succinic dehydrogenase (E.C. 1.3.99.1) and mitochondrial α-glycerophosphate dehydrogenase (E.C. 1.1.1.8) activities were measured by the method described by Lee and Lardy.[18]

Statistical Analysis

Data are given as means ± SEM of four different rats. Statistical analyses were performed by one-way analysis of variance (ANOVA). *Post-hoc* comparison between group pairs were made with the Tukey test after ANOVA had established significant differences among groups. Probability values less than 0·05 were considered to indicate a significant difference.

Materials

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

RESULTS

Body Weight, Liver Weight and Mitochondrial Protein Mass

Table 1 shows that rat body weights increased with age, although with decreasing rate. Liver weights significantly increased in rats of 90 days of age compared with rats of 40 days of age, but

Table 1. Body and liver weights, liver weight/body weight ratio, and mitochondrial protein mass at different ages.

	40 days	90 days	180 days
Body weight (g)	180 ± 3	406 ± 8*	490 ± 8*†
Liver weight (g)	8·2 ± 0·3	14·5 ± 1·3*	14·8 ± 0·5*
Liver weight/body weight (g/100 g)	4·5 ± 0·1	3·6 ± 0·2*	3·0 ± 0·1*
Mitochondrial protein mass, mg per g liver	44·3 ± 0·4	38·6 ± 1·4	55·5 ± 4·7†

Values are the means ± SEM of four different rats.
* $p < 0.05$ compared to 40 days; † $p < 0.05$ compared to 90 days.

remained constant thereafter (Table 1). In addition, the relative liver weights (g liver per 100 g body weight) significantly decreased with age (Table 1).

The mitochondrial protein mass (mg per g liver) was determined as previously reported using a mitochondrial marker enzyme such as succinic dehydrogenase (SDH).[19,20] It was calculated by dividing the values of the SDH activity in liver homogenates (10·2 ± 0·2, 14·3 ± 0·4, 12·2 ± 0·7 μmol min^{-1} g^{-1} liver in 40-, 90- and 180-day-old rats, respectively) by the values of the SDH activity in isolated liver mitochondria (Table 2). This calculation can be done since our mitochondrial preparation are virtually pure as previously shown.[14,15] The highest value of mitochondrial protein mass was found at 180 days of age (Table 1).

Respiratory and Enzymic Activities with FAD-linked Substrates

Respiration rates using succinate as substrate as well as SDH and α-glycerophosphate dehydrogenase (α-GPDH) specific activities measured in isolated mitochondria are reported in Table 2. State 3 and state 4 respiratory rates as well as α-GPDH-specific activity were significantly decreased (about −26 per cent, −24 per cent and −29 per cent, respectively) in 180-day-old rats compared with younger rats (Table 2). On the other hand, the SDH-specific activity was significantly increased (+61 per cent) in 90-day-old rats compared with the younger ones. Subsequently it returned to values exhibited by 40-day-old rats (Table 2).

Respiratory Activities with NAD-linked and Lipid Substrates

Table 3 shows the results of hepatic mitochondrial respiration using two NAD-linked substrates, namely glutamate plus malate and pyruvate plus malate. State 3 and state 4 respiratory rates with the above NAD-linked substrates did not significantly change over the age range of this study (Table 3).

Hepatic mitochondrial respiratory rates with lipid substrates are reported in Table 4. State 3 and state 4 respiratory rates with palmitoylcarnitine plus malate did not significantly change at different ages (Table 4). On the other hand significantly lower state 3 and 4 respiratory rates with palmitoyl-CoA plus carnitine plus malate were found in 180-day-old rats than in younger animals (Table 4). In fact, the state 3 respiratory rate decreased by 24 per cent compared with 40-day-old rats, whereas the state 4 respiratory rate decreased by 20 per cent compared with 90-day-old rats (Table 4).

Table 2. Hepatic mitochondrial respiratory and enzymic activities with FAD-linked substrates in rats at different ages.

	40 days	90 days	180 days
Succinate plus rotenone			
State 3	199 ± 11	193 ± 9	145 ± 11*†
State 4	34 ± 1	32 ± 1	25 ± 1*†
RCR	5·9 ± 0·3	6·0 ± 0·4	5·8 ± 0·1
SDH-specific activity in isolated mitochondria	0·23 ± 0·01	0·37 ± 0·01*	0·22 ± 0·03†
α-GPDH-specific activity	36 ± 2	34 ± 3	25 ± 2*†

Values are the means ± SEM of four different rats.
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^{-1} mg^{-1} protein.
SDH, succinic dehydrogenase. SDH-specific activity in isolated mitochondria is expressed as μmol min^{-1} mg^{-1} protein.
α-GPDH, α-glycerophosphate dehydrogenase. α-GPDH-specific activity is expressed as nmol min^{-1} mg^{-1} protein.
* $p < 0.05$ compared to 40 days; † $p < 0.05$ compared to 90 days.

Table 3. Hepatic mitochondrial respiratory activity in rats at different ages using NAD-linked substrates.

	40 days	90 days	180 days
Glutamate plus malate			
State 3	87 ± 2	86 ± 3	94 ± 4
State 4	9·4 ± 1	10·6 ± 0·4	8·5 ± 0·9
RCR	9·2 ± 0·4	8·1 ± 0·3	11·0 ± 1·4
Pyruvate plus malate			
State 3	29 ± 3	34 ± 2	32 ± 3
State 4	6·5 ± 0·4	8·1 ± 0·6	7·0 ± 0·7
RCR	4·5 ± 0·3	4·2 ± 0·4	4·6 ± 0·3

Values are the means ± SEM of four different rats. States 3 and 4 respiratory rates are expressed as nmol O min^{-1} mg^{-1} protein. Substrate concentrations: glutamate 10 mM, pyruvate 10 mM, malate 2·5 mM.

Table 4. Hepatic mitochondrial respiratory activity in rats at different ages using lipid substrates.

	40 days	90 days	180 days
Palmitoyl-carnitine plus malate			
State 3	79 ± 2	73 ± 3	71 ± 4
State 4	13·9 ± 0·5	14·0 ± 0·4	12·6 ± 0·6
RCR	5·7 ± 0·3	5·2 ± 0·4	5·6 ± 0·5
Palmitoyl-CoA plus carnitine plus malate			
State 3	86 ± 4	75 ± 4	65 ± 5*
State 4	13·7 ± 0·6	14·8 ± 0·3	11·8 ± 0·6†
RCR	6·3 ± 0·3	5·1 ± 0·4	5·5 ± 0·2

Values are the means ± SEM of four different rats. State 3 and 4 respiratory rates are expressed as nmol O min^{-1} mg^{-1} protein. Substrate concentrations: palmitoylcarnitine 40 μM, palmitoyl-CoA 40 μM, carnitine 2 mM, malate 2·5 mM.
* $p < 0.05$ compared to 40 days; † $p < 0.05$ compared to 90 days.

RCR values, with all the substrates used, were not significantly different in mitochondria isolated from rats of 40, 90 and 180 days of age (Tables 2, 3 and 4). It should be noted that the RCR values found in this work indicate the high quality of the mitochondrial preparations.

State 3 Respiratory Rates Expressed per g Liver and per 100 g Body Weight

When the respiratory activities were expressed as μmoles O min^{-1} g^{-1} liver, state 3 respiratory rates using succinate, palymitoylcarnitine, or palmitoyl-CoA as substrate were not significantly changed over the age range of this study (Figure 1A). On the other hand, when pyruvate or glutamate were used as substrate, state 3 respiratory rates increased in the oldest rats compared with both 40-day-old (+42 per cent and +49 per cent, respectively) and 90-day-old (+31 per cent and +67 per cent, respectively) rats (Figure 1A). In an opposite way, when respiratory activities were expressed as μmoles O min^{-1} 100 g^{-1} body weight, state 3 respiratory rates using succinate, palymitoylcarnitine, or palmitoyl-CoA as substrate, significantly decreased in 90-day-old (−30 per cent, −36 per cent and −37 per cent, respectively) and 180-day-old (−38 per cent, −26 per cent and −39 per cent, respectively) rats compared with 40-day-old animals (Figure 1B). On the other hand, no variations were found in state 3 respiratory rates expressed per 100 g body weight when glutamate or pyruvate were used as substrate (Figure 1B).

DISCUSSION

In this work oxidative activity of mitochondria isolated from livers of rats in various developmental stages has been studied. The oxidative capacity was determined in two different conditions: state 3 and state 4 respiratory rates. State 3 represents a condition where oxidative phosphorylation is not rate-limited by ADP and maximum oxidative capacity can be measured, whereas state 4 is a condition where the level of ADP limits oxidative phosphorylation. Both NAD- and FAD-linked substrates have been chosen to stimulate the mitochondrial respiratory system so that different dehydrogenases, different carriers, and different sites of entry of reducing equivalents will be involved.

When hepatic mitochondrial respiratory rates were supported with an FAD-linked substrate, such as succinate which enters the electron transport chain at complex 2, both state 3 and state 4 respiratory rates significantly decreased by about 25 per cent in 180-day-old rats compared with younger animals. Consequently, the RCR value did not change in 180-day-old rats compared with younger rats. It should be noted that in previous work where succinate-supported state 3 and state 4 respiratory rates were measured in older rats (12 and 29 months of age), only the state 3 mitochondrial respiration was found to decline in animals of 29 months of age.[8] So, in older animals the RCR values also declined. With the limitation that the state 4 respiratory rate can give only a rough indication of the proton leak pathway activity of mitochondrial inner membranes,[21] the decreased state 4 respiratory rate found in 180-day-old rats may indicate that at this developmental stage, the

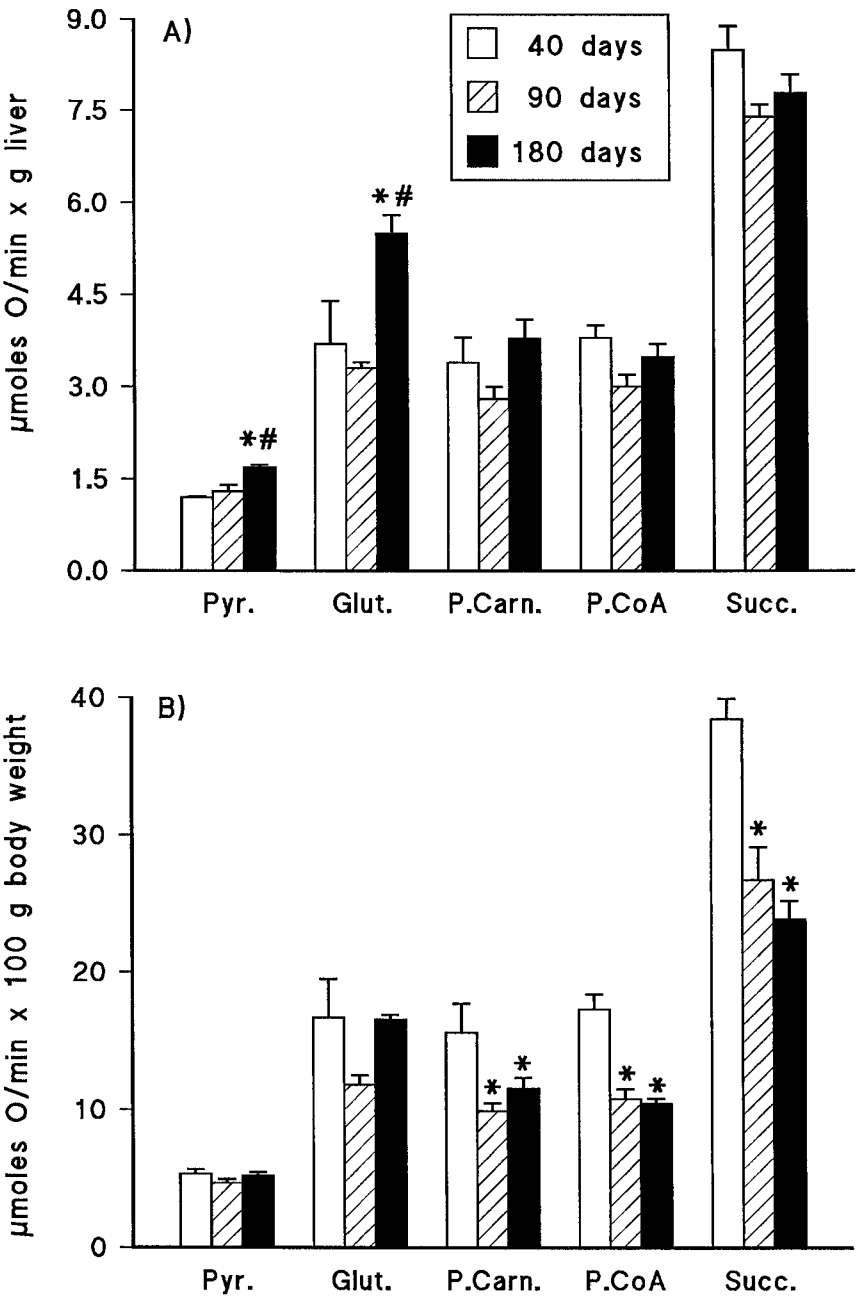

Figure 1. Hepatic mitochondrial state 3 respiratory rates in rats at different ages expressed as µmoles O min^{-1} g^{-1} liver (A) and as µmoles O min^{-1} 100 g^{-1} body weight (B). Values are obtained from those reported in Tables 2, 3 and taking into account the mitochondrial protein mass and the liver weight/body weight ratio reported in Table 1. Pyr., pyruvate + malate; Glut., glutamate + malate; P. Carn., palmitoylcarnitine + malate; P. CoA, palmitoylCoA + carnitine + malate; Succ., succinate + rotenone. * $p < 0{\cdot}05$ compared to 40 days. † $p < 0{\cdot}05$ compared to 90 days.

proton leak pathway declines. This hypothesis is in line with the results that state 4 respiratory rates using other substrates also showed a tendency to decrease in 180-day-old rats. However, we recognize the need for more work in order to substantiate the above hypothesis.

The specific activity of two FAD-linked dehydrogenases, SDH and mitochondrial α-GPDH, was also measured. With respect to the SDH-specific activity, the highest value was reached at 90 days of age, while in 180-day-old rats the SDH-specific activity significantly decreased compared with 90-day-old rats. If a comparison between succinate-supported state 3 respiratory rate and SDH-specific activity is made it can be noted that at 180 days of age, despite state 3 respiratory rate being lower than that found at 40 days of age, SDH-specific activity was the same as that found in the youngest rats. It follows therefore that the decreased succinate-supported state 3 respiration in 180-day-old rats could be due either to a decrease in the activity of the dicarboxylate carrier, or the electron transport chain from complex II onward, and/or the phosphorylation system, but not to changes in SDH-specific activity. This suggestion is in agreement with the statement that SDH-specific activity is not a rate-controlling step in the succinate oxidation pathway.[22]

With regard to the α-GPDH, a significant decrease in the activity of this enzyme was found in 180-day-old rats compared with the younger animals. A similar result has been shown in previous work.[3] Since this enzyme is rate limiting for the α-glycerophosphate shuttle which exchanges one cytosolic NADH molecule for one mitochondrial $FADH_2$ molecule, it can be suggested that in 180-day-old rats a smaller fraction of cytoplasmic NADH is transported into mitochondria through the above shuttle as $FADH_2$. This suggestion is in line with the decreased FAD-linked respiration found in the present work. In addition, the finding of the decreased α-GPDH-specific activity is in agreement with our previous results showing a strong decrease in hepatic tissue T_3 concentration in rats of about 180 days of age.[23] In fact, mitochondrial α-GPDH activity is strictly related to T_3 levels.[18]

Glutamate plus malate or pyruvate plus malate were chosen as NAD-linked substrates to stimulate the mitochondrial respiratory system. Glutamate enters the citric acid cycle at α-oxoglutarate and pyruvate can be converted to acetyl-CoA. Both the substrates are NAD-linked and electrons enter at site 1 of the electron transport chain. The results show that the state 4 respiratory rate with both the substrates tended to decrease in 180-day-old rats, even if no significant variation has been found. In addition, the state 3 respiratory rate remained unchanged in 40-, 90-, and 180-day-old rats, despite the decreased activity of the electron transport chain from complex 2 onwards and/or the phosphorylation system, as suggested above for the impaired succinate-supported respiration. The decreased activity of such factors is without effect on NAD-linked respiratory rates because they are always lower than those with succinate even if they were expressed as proton efflux rates calculated by multiplying the oxygen consumption by the H^+/O stoichiometry, using the stoichiometric values of 9 or 10 for NAD-linked substrate, and 6 for succinate.[24,25] Therefore, during the oxidation of pyruvate or glutamate only their membrane permeation and dehydrogenases as far as complex 2 of the respiratory chain may be rate limiting. It should be noted that there are some reports in the literature that show in old rats a decline in the rate of state 3 respiration when supported by NAD-linked substrates.[1,10] It follows that 180 days of age are not sufficient to elicit mitochondrial age-linked variations in the NAD-linked oxidation pathways.

Since the liver is a central organ playing a major role in lipid metabolism,[26] the mitochondrial respiratory activity was also stimulated with two lipid substrates: palmitoylcarnitine plus malate or palmitoyl-CoA plus carnitine plus malate. Palmitoyl-CoA-supported respiration reflects the activity of carnitine-palmitoyltransferase (CPT) I, CPT II and the intramitochondrial β-oxidation pathway, which produces both NADH and $FADH_2$. On the other hand, respiration with palmitoylcarnitine, which bypasses the main regulatory step of fatty acid oxidation (i.e. CPT I),[27–29] may represent an index of fatty oxidation *per se*.[30] The results indicate that when hepatic mitochondrial respiration was supported with palmitoyl-CoA plus carnitine plus malate, a significant decrease both in state 3 and state 4 respiratory rates in 180-day-old rats compared with younger rats was found. When respiration was supported by palmitoylcarnitine, no variation in 40-, 90-, and 180-day-old rats was found in state 3, while the state 4 rate tended to decrease in 180-day-old rats. On the basis of the above results, it can be suggested that the reduction in the capacity for fatty acid oxidation found in 180-day-old rats in this work can be due to a fall in CPT I activity. This

suggestion is in line with the results of previous work which has shown an age-associated decrease in CPT I activity in heart mitochondria.[31]

Our results on mitochondrial respiration, supported by NAD- and FAD-linked substrates, indicate that in 180-day-old rats only the oxidative capacity of FAD-linked pathways significantly declines. This change in oxidative capacity may represent the first age-linked biochemical lesion of energy-transducing pathways. However, when we consider succinate-supported mitochondrial respiration expressed per g liver we see that there is no significant difference in state 3 respiratory rates between rats of 40, 90, and 180 days of age. This finding is due to the higher mitochondrial protein mass per g liver found in 180-day-old rats. In agreement with this result is a previous study showing that proliferation of mitochondria reached a maximum value at 180 days of age.[32] In addition, due to the increased mitochondrial protein mass, NAD-linked oxidative capacities significantly increased in 180-day-old rats compared with younger animals. Taken together the above results indicate that liver cells from 180-day-old rats exhibit similar mitochondrial oxidative capacities to those found in younger animals, or even better. However, this situation is the result of an increased mitochondrial protein mass which tends to compensate for the reduced oxidative capacity found for FAD-linked substrates at 180 days of age.

Our present results could have additional metabolic implications, if we consider that liver weight expressed per 100 g body weight declined in rats with increasing age. In fact, Figure 1B shows that a general decrease in FAD-linked and lipid-dependent state 3 respiratory rates expressed per 100 g body weight occurred in 90- and 180-day-old rats compared with 40-day-old rats, whereas no variation was found in NAD-linked state 3 respiratory rates. Therefore, similar decreases in mitochondrial oxidative capacities occurred when they were expressed either per mg of mitochondrial protein or per 100 g of body weight. Since the liver consumes about 20 per cent of the total oxygen used by the organism,[33] the above decline of liver respiratory activity when expressed per 100 g body weight could partly account for the decrease in body energy requirements found in adult rats compared with younger animals.[4,34] In conclusion, the body energy deficit in adult rats may be caused by the relative liver weight decrease which occurs in rats with increasing age rather than by an impaired hepatic cellular activity.

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