Mitochondrial Respiration and Triiodothyronine Concentration in Liver from Postpubertal and Adult Rats

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The purpose of this study was to investigate the decline in rat liver mitochondria respiration found in adult rats compared to younger ones, and to find a link between this respiratory impairment and a tissue hypothyroidism state. To this end, hepatic concentration and serum levels of triiodothyronine were measured in postpubertal rats (60 days old) and adult rats (180 days old). In addition, in these rats we measured oxidative phosphorylation in homogenate together with coupled and uncoupled respiration in isolated mitochondria using succinate or durohydroquinone as substrate. We found that mitochondria from adult rats consumed less oxygen compared to younger rats due to lower electron transport chain and phosphorylating system activity. In addition, we found that in state 4 condition, mitochondria from adult rats consumed less oxygen than mitochondria from young rats. Finally, we found a decrease in liver triiodothyronine concentration in adult rats. In conclusion, the results of this study show that hepatic mitochondria in adult rats have a decreased ATP synthesis capacity and proton permeability, both consistent with the tissue hypothyroidism found in the liver of adult rats.

■ Key words: Homogenate Respiration – Mitochondrial Protein Mass – Uncoupled Respiration – Cytochrome Content – Serum Triiodothyronine Level

Introduction

Thyroid hormones are important factors in the regulation of hepatic metabolism and mitochondria could be considered as targets of thyroid hormone action in view of their central role in energy metabolism. In fact, thyroid hormones regulate many of the mitochondrial metabolic functions [1] and influence oxidative phosphorylation [2]. In particular, it has been shown that in liver mitochondria from hypothyroid rats, oxygen consumption and ATP synthesis are lower than those of liver mitochondria from normal rats [3]. Interestingly, our previous results showed that the rate of FAD-linked respiration significantly declined in mitochondria from 180-day-old rats compared with those from younger rats [4]. In contrast to the above results, the rate of NAD-linked respiration was found undiminished with age. It seems that the age-related decrease in mitochondrial respiration is substrate-specific. One possible explanation may be that with NAD-linked substrates, the entry of metabolites into the electron transport chain is rate limiting for respiration, so that the decrease in respiration becomes masked. Therefore, FAD-linked respiration allowed us to detect at the liver level the first age-linked biochemical impairment of energy-transducing pathways. In order to have information about a possible implication of thyroid hormones in this respiratory impairment, variations in serum free triiodothyronine (T_3) levels were checked in postpubertal (60 days) and adult rats (180 days). Since tissue hypothyroidism may occur in the presence of normal or modestly decreased serum thyroid hormone levels [5], we also measured hepatic T_3 concentration.

In addition, we were interested in identifying the sites responsible for the decreased respiration rate found in isolated mitochondria from 180-day-old rats. Changes in oxygen consumption in isolated mitochondria may essentially reflect modifications in the features of respiratory chain and/or ATP synthesis system, as well as in the supply of reducing equivalents into the respiratory chain [6]. In this study, we measured the respiration rates using succinate or durohydroquinone as substrate. Durohydroquinone was used to eliminate the regulation of substrate supply, namely of dicarboxylate carrier and substrate dehydrogenase. The cytochrome content was also determined in order to verify if any changes in the components of the electron chain occurred. Finally, we assessed uncoupled respiration to evaluate the maximal electron flow through the respiratory chain. The respiration measurements were carried out in liver homogenate and isolated mitochondria from postpubertal and adult rats.

Materials and Methods

Animals

Male Wistar rats were obtained from Charles River (Calco, Como, Italy). The rats were housed at 24 °C under an artificial circadian 12-h light/12-h dark cycle, with *ad libitum* access to

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water and a standard stock diet (Mucedola 4RF21, Settimo Milanese, Milan, Italy) before being sacrificed. Two groups of nine rats were used: postpubertal and adult, which were 60 and 180 days old at time of sacrifice, respectively. Animal care, housing, and killing were carried out according to the guidelines of the Italian Health Ministry.

Preparation of homogenates and isolated mitochondria

The rats, without any previous food deprivation, were anaesthetised with chloral hydrate (40 mg/100 g body weight). Blood was collected via the inferior cava vein, and serum samples were stored at – 20 °C. Livers were then freed of blood by *"in situ"* perfusion using 150 ml of a cold solution of 220 mM mannitol, 70 mM sucrose, 20 mM Hepes, pH 7.4, 1 mM EDTA, and 0.1 % (w/v) fatty acid free bovine serum albumin (BSA). After perfusion, livers were quickly removed, blotted, weighed, and aliquots were immediately frozen in liquid nitrogen for the determination of liver T_3 concentration. The remainder of the liver was finely minced, gently homogenised with the above medium (1 : 4, w/v) in a Potter Elvehjem homogeniser set at 500 rpm (4 strokes/min), and filtered through sterile gauze. After withdrawal of aliquots of the fresh homogenate for respiration measurements, it was further processed for preparation of isolated mitochondria as previously reported [7]. The protein content of the mitochondrial suspension was determined according to the method of Hartree [8], using bovine serum albumin as the protein standard.

Polarographic measurement of respiration in liver homogenates and mitochondria

Mitochondrial oxygen consumption in homogenates was estimated using a Clark-type electrode (Yellow Springs Instruments, Yellow Springs, OH, USA), maintained in water jacketed chamber at 30 °C. Aliquots of the homogenate were added to 3 ml of the respiratory medium containing 80 mM KCl, 50 mM HEPES, pH 7.0, 5 mM KH_2PO_4, 1 mM EGTA, 0.1 % (w/v) BSA, and state 3 and 4 respiratory rates were measured as previously reported [9]. Oxygen consumption in isolated mitochondria was measured polarographically with the above-mentioned Clark-type electrode. Isolated mitochondria (1 mg protein) were incubated in 3 ml of the above respiratory medium at 30 °C. Measurements were made within 2 h following the isolation of the mitochondria. The mitochondria were allowed to oxidise their endogenous substrates for few minutes. State 4, coupled oxygen consumption (State 3), and uncoupled oxygen consumption were then measured in the presence of succinate 10 mM + rotenone 3.75 µM or durohydroquinone 4.5 mM + rotenone 3.75 µM as substrates. State 4 oxygen consumption rate was determined by adding oligomycin (2 µg/mg of protein) to prevent ATP synthesis. Coupled respiration rate was measured by adding ADP at a final concentration of 0.3 mM. Uncoupled respiration rate was determined by adding carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) at the final concentration of 1 µM in the presence of oligomycin (2 µg/mg of protein). Respiratory control ratio (RCR) and ADP/O ratios were calculated according to Estabrook [10]. ATP production rate during coupled respiration was measured monitoring the disappearance of inorganic phosphorus in the above respiratory medium in the presence of 5 mM ADP [11].

Determination of cytochrome content and enzyme activity

Cytochrome content of isolated mitochondria was determined as previously reported [12]. Succinic dehydrogenase (SDH) (E.C.1.3.99.1) activity was measured by the method described by Lee and Lardy [13] in homogenate and isolated mitochondria.

Determinations of liver T3 concentration and serum free T3 level

To extract T_3 from liver samples, we used the procedure of Morreale de Escobar et al. [14]. Recovery of the extracted iodothyronine was carried out in a similar way to that reported by Morreale de Escobar et al. [14] (50 – 75 %). To measure the concentration of the extracted T_3, a commercial radioimmunoassay kit (ICN Pharmaceuticals, Diagnostic Division, New York, USA) was used. The standard curve was constructed by using calibrator made adding pure T_3 to phosphate buffer so that T_3 concentration ranges from approximately 100 to 1000 pg/ml. The same radioimmunoassay kit was also used for determination of serum free T_3 levels. Measurements of liver T_3 concentration and serum free T_3 level were carried out in a single assay to remove inter-assay variations.

Statistical analysis

Data are given as means ± SEM from nine different rats. Statistical analyses were performed by two-tailed unpaired Student's *t*-test or two-way analysis of variance (ANOVA) for repeated measures. Probability values less than 0.05 were considered to indicate a significant difference. All analyses were performed using GraphPad Prism (GraphPad Software, San Diego, CA).

Materials

ADP, rotenone, succinate, FCCP, and duroquinone were purchased from Sigma Chemical Co., St. Louis, USA. Very high specific activity ^{125}I-labeled T_3 was purchased from NEN Life Science Products, Inc, Boston, USA. All other reagents were of the highest purity commercially available.

Results

Hepatic mitochondrial respiratory rates in liver homogenates from 60- and 180-day old rats using succinate as the substrate are reported in Table **1**. The measurements made in liver homogenates allow us to link mitochondrial oxidative activities with the effective mitochondrial protein mass. State 3 did not significantly change at different ages, while state 4 significantly decreased in 180-day-old rats compared to younger rats. The mitochondrial protein mass (mg/g liver) was calculated by dividing the values of the SDH activity in liver homogenates by the values of the SDH activity in isolated liver mitochondria. This calculation can be done since our mitochondrial preparations are virtually pure as shown by the results of control experiments, in which marker enzyme activities of plasma membrane and organelles have been determined. Mitochondrial protein mass was significantly higher in 180-day-old rats (Table **1**).

Table 1 Liver mitochondrial respiration in homogenate, succinic dehydrogenase (SDH) activity, and mitochondrial protein mass in 60-day-old and 180-day-old rats

	60-day-old rats	180-day-old rats	% change
State 3, natoms O/(min × g liver)	9992 ± 432	8249 ± 831	– 17
State 4, natoms O/(min × g liver)	1191 ± 66	865 ± 56*	– 27
SDH activity in homogenate, µmol/(min × g liver)	10.4 ± 0.8	11.2 ± 0.5	8
SDH activity in isolated mitochondria, nmol/(min × mg protein)	258 ± 24	221 ± 17*	– 14
Mitochondrial protein mass, mg/g liver	40 ± 3	50 ± 2*	25

Values are the means ± SEM of nine different rats. % change referred to 60-day-old rats; *$P < 0.05$ compared to 60-day-old rats (two-tailed Student's *t*-test).

ADP- and FCCP-stimulated respiration (coupled and uncoupled respiration, respectively) measured in isolated mitochondria using succinate as the substrate is presented in Fig. **1 a**. The values of RCR (6.3 ± 0.4 and 6.1 ± 0.6 in 60- and 180-day-old rats, respectively) and ADP/O ratio (1.9 ± 0.1 and 1.7 ± 0.1 in 60- and 180-day-old rats, respectively) found in this study indicate the high quality of the mitochondrial preparation. A significant decrease ($p < 0.05$) with age was found in ADP/O ratio. As shown in Fig. **1 a**, coupled and uncoupled respiration significantly decreased in 180-day-old rats compared to 60-day-old rats. Uncoupled respiration was not significantly different from coupled respiration in 60-day-old rats. In 180-day-old rats, however, uncoupled respiration was significantly higher than coupled respiration. Fig. **1 b** shows coupled and uncoupled respiration obtained using durohydroquinone as the substrate. Electrons from durohydroquinone and succinate enter the mitochondrial respiratory chain at the level of coenzyme Q, but the synthetic substrate durohydroquinone is rapidly transported across the mitochondrial membrane without the need of carriers. Coupled and uncoupled respiration significantly decreased in 180-day-old rats compared to younger rats. In addition, uncoupled respiration was significantly higher than coupled respiration both in 60-day-old rats and in 180-day-old rats. State 4 respiratory rates in isolated mitochondria from 60- and 180-day old rats using succinate and durohydroquinone as substrates are presented in Fig. **1 c**. Mitochondria from 180-day-old rats had significantly lower state 4 respiration than 60-day-old-rats, whatever the substrate utilised.

Fig. **2** shows that ATP production during state 3 respiration both in homogenate and isolated mitochondria significantly decreased in 180-day-old rats compared to younger ones.

The content of cytochromes b,c_1 (complex III) was 0.443 ± 0.026 and 0.432 ± 0.032, the content of cytochrome c was 0.187 ± 0.015 and 0.183 ± 0.015; finally, the content of cytochromes a,a_3 (complex IV) was 0.294 ± 0.023 and 0.262 ± 0.023 for 60- and 180-day-old rats, respectively. The results show that no difference due to age was found in complex III

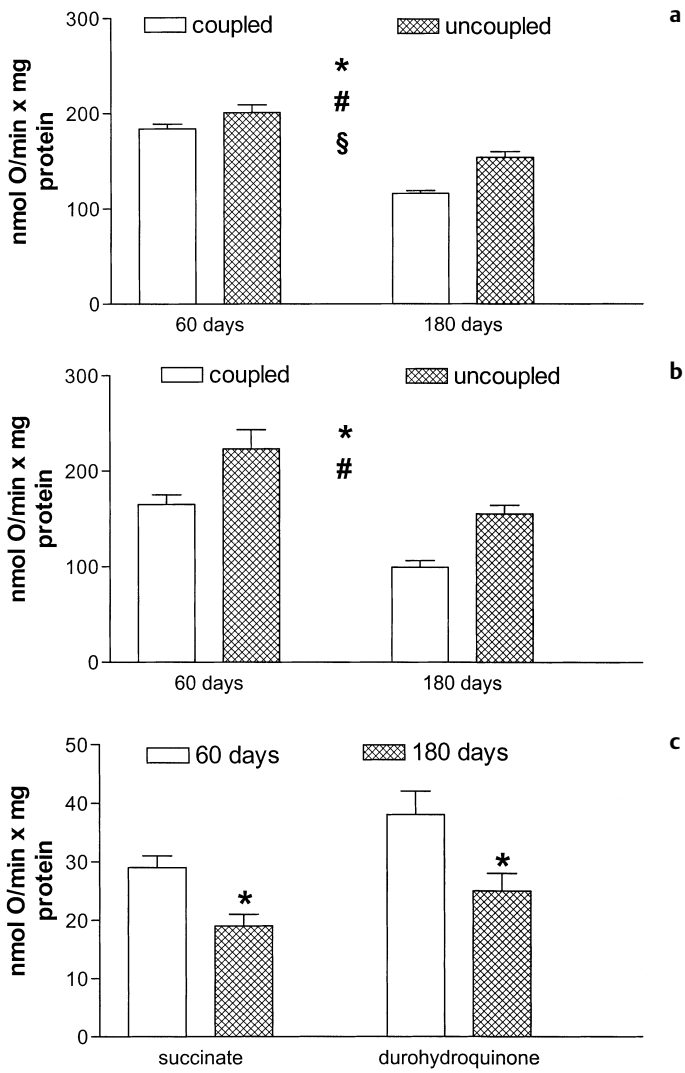

Fig. 1 Coupled and uncoupled respiratory rates in isolated mitochondria from 60- and 180-day-old rats using succinate (**a**) and durohydroquinone (**b**) as substrates. State 4 respiratory rates in isolated mitochondria from 60- and 180-day-old-rats using succinate and durohydroquinone as substrates (**c**). Values are the means ± SEM of nine different rats. In **a** and **b** statistical significance by ANOVA ($p < 0.05$): * effect of age, # effect of uncoupling, § interaction. In **c**: * $p < 0.05$ compared to 60-day-old rats (two-tailed Student's t-test).

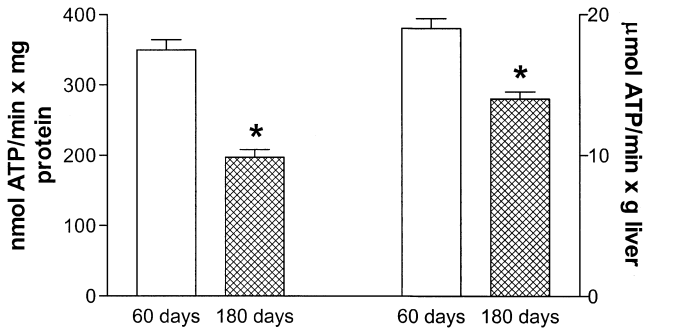

Fig. 2 ATP production in isolated mitochondria nmol/(min × mg protein) and in homogenate nmol/(min × g liver). Values are the means ± SEM of nine different rats. *$p < 0.05$ compared to 60-day-old rats (two-tailed Student's *t*-test).

Table 2 Serum free T_3 levels and liver T_3 concentrations in 60-day-old and 180-day-old rats

	60-day-old rats	180-day-old rats	% change
Serum free T_3 levels (pg/100 ml)	290 ± 33	246 ± 26	– 15
Liver T_3 concentration (ng/g liver)	3.70 ± 0.25	2.44 ± 0.33*	– 34

Values are the means ± SEM of nine different rats. % change referred to 60-day-old rats. * $p < 0.05$ compared to 60-day-old rats (two-tailed Student's *t*-test).

or cytochrome c, while a tendency to decrease was found in complex IV content from adult rats compared to younger rats.

Table **2** shows the results obtained measuring serum free T_3 levels and liver T_3 concentration. Serum free T_3 levels showed a slight tendency to decrease in 180-day-old rats compared with younger rats. On the other hand, a significant decrease was found in liver T_3 concentration in 180-day-old rats compared to 60-day-old rats.

Discussion

In this study, we showed that a decrease in liver mitochondrial respiratory activity overlaps liver hypothyroidism in adult rats, which occurs despite serum thyroid hormone levels only being slightly decreased compared to younger rats.

As for mitochondrial respiration, we found that the rates of succinate-supported coupled respiration significantly declined in mitochondria isolated from 180-day-old rats compared to 60-day-old rats, in agreement with our previous work [4]. The higher mitochondrial protein mass found in adult rats can account for the observation that homogenate state 3 respiration rates did not significantly vary between the two experimental groups. On the other hand, a significant fall in homogenate ATP production during state 3 respiration occurred in adult rats, despite the higher mitochondrial protein mass found in these animals. This result is due to the decreased ATP production and ADP/O ratio found in mitochondria isolated from adult rats.

Maximal mitochondrial respiratory chain activity was assessed by measuring oxygen consumption in the presence of excess uncoupler, FCCP. Since the values of respiration, measured with succinate as substrate, in uncoupled and coupled states are similar in 60-days old rats but significantly different (increased uncoupled respiration compared to coupled) in adult rats, it is conceivable that the ATP synthesis system activity in these animals is lower than that of younger rats. In fact, while the ATP synthesis system activity in 60-day-old rats is not rate-limiting for coupled mitochondrial respiration, decreased coupled respiration in adult rats may be partly due to a decline in this activity. However, the uncoupled rate in adult rats is lower than that of 60-day-old rats, therefore the decrease in respiration found in adult rats is not completely due to a fall in the ATP synthesis system activity.

To have information about other factors that can be involved in the decline in coupled respiration found in adult rats, we utilised the artificial proton donor, durohydroquinone. In adult rats compared to younger ones, we found significantly decreased both coupled and uncoupled rates of respiration. Since the percentage decrease in coupled respiration with durohydroquinone and succinate as the substrates was similar in adult rats (about 40%), it can be suggested that the rate limiting step under these conditions is the same and not due to decreased dicarboxylate carrier and/or SDH activity.

By a process of elimination, another factor that can be involved in the decline in coupled respiration found in adult rats when succinate is oxidised is the electron transport chain activity itself, namely activity of complex III, cytochrome c, and complex IV. The lower activity of the above complexes could be due, in principle, to a change in enzyme mass. To assess this, the cytochrome content of mitochondrial preparations from the two experimental groups was measured. The results show that the content of cytochromes bc_1 and cytochrome c was practically the same in both mitochondria from 60- and 180-days-old rats, whereas in mitochondria from older rats the content of cytochrome aa_3 tended to decrease. Therefore, the decline in succinate-supported respiration observed in mitochondrial preparations from older rats does not appear to be dependent on a decrease in the mass of both enzyme complex III and cytochrome c. On the other hand, a decrease in the mass of cytochrome oxidase may be associated with decreased mitochondrial coupled respiration found in 180-day-old rats. It should be noted that cytochrome oxidase is considered an important factor in the regulation of mitochondrial respiration [15].

As for state 4 mitochondrial respiration, it significantly declined in homogenate and isolated mitochondria from 180-day-old rats compared to 60-day-old rats. State 4 respiratory rates in the presence of saturating amounts of oligomycin, which prevents any ATP synthesis by proton flux through ATP synthase, can give an indirect measurement of the proton leak of mitochondrial inner membranes [16]. Therefore, the decreased state 4 respiratory rates found in mitochondria isolated from 180-day-old rats may indicate that there is a reduction in mitochondrial proton leak at this developmental stage. This observation is in line with lowered T_3 concentration found in liver from adult rats. In fact, it is well known that thyroid hormone status influences membrane permeability. Mitochondria from hyperthyroid rats have an increased leak to proton across the inner membrane, while hypothyroidism decreases the proton leak [17].

Taken together, the above results indicate that in the transition from puberty to adulthood, there are structural and functional changes in the hepatic mitochondrial compartment. Mitochondria from older animals consumed significantly less oxygen under coupled and uncoupled conditions, consistent with a lower activity of the electron transport chain from complex II onward and phosphorylating system. In addition, since mitochondria from adult rats in state 4 conditions consumed significantly less oxygen than mitochondria from young animals, it can be suggested that mitochondrial permeability decreased. Interestingly the above changes in hepatic mitochondrial compartments are characteristics of hypothyroid state [3,17]. In agreement, we have found significantly decreased T_3 concentration in liver, while only a tendency to decrease was observed in T_3 serum concentration. The achievement of adulthood seems to be associated with tissue hypothyroidism in the presence of modestly decreased serum thyroid hormone lev-

els. Since there is abundant evidence that T_3 is transported across the plasma membrane of target cells by carrier-mediated processes that are energy dependent [5], we suggest that the reduced intracellular energy production that occurred in adult rats can lower T_3 uptake into the liver.

In conclusion, hepatic mitochondria from adult rats are different from younger ones in both declined ATP synthesis capacity and decreased proton permeability of the inner mitochondrial membrane. These features are consistent with a tissue hypothyroidism found in liver of adult rats.

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