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Postnatal changes in rhodamine-123 stained mitochondrial populations are sensitive to protein synthesis inhibitors but mimicked in vitro by ATP

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Abstract

The incubation of term fetus mitochondria with ATP mimicked in vitro the increase in the respiratory control index and in the percentage of the rhodamine-123-low fluorescence population that occurred in vivo immediately after birth, suggesting that both phenomena are closely associated. The administration of streptomycin inhibited the increase in the percentage of the low fluorescence population that occurred immediately after birth, while the administration of cycloheximide even reversed these changes. These results suggest that the in vivo interconversion between mitochondrial forms depends on both cytosolic and mitochondrial protein synthesis.

Key words: Mitochondrion; Rhodamine-123; Flow cytometry; Postnatal

1. Introduction

The transition from fetus to neonate involves a shift from a relatively anaerobic environment to an aerobic one. This situation requires the rapid postnatal acquisition of efficient energy transducing mitochondria, which is accomplished within the first hour of extrauterine life [1–3]. Postnatal differentiation of mitochondria is brought about by the synergistic action of two main processes such as the enhancement of the synthesis of protein components of mitochondrial complexes [3] and an increase in the intramitochondrial adenine nucleotide concentrations [1].

Flow cytometry measurements of isolated mitochondria has become a useful tool for the study of these organelles [4–8]. In recent years, there has been increasing interest in rhodamine-123 (rh-123), which selectively accumulates in the mitochondria of living cells by a mechanism that depends on both the cell and the mitochondrial membrane potential [9–12]. Although the binding sites of rh-123 to the mitochondria are not yet fully established, it has been suggested that the F_0F_1–ATPase complex is the primary biochemical target of rh-123 because the inhibition of mitochondrial bioenergetic function by rh-123 suggests a specific association of the dye with the complex [11,13]. Likewise, flow cytometry analysis of isolated liver mitochondria stained with rh-123 has revealed the occurrence of two mitochondrial populations with different intensities of fluorescence, i.e. a high fluorescence population (HFP) and a low fluorescence population (LFP) [6]. The percentages of these populations change as postnatal development progresses and with the increase in mitochondrial respiratory efficiency [7,8].

The aim of present work was to study the role played by protein synthesis and by ATP in postnatal changes in mitochondrial fluorescence populations in order to analyze whether these changes correlate with those observed in mitochondrial function immediately after birth.

2. Materials and methods

2.1. Chemicals

Cycloheximide, streptomycin and rhodamine-123 were purchased from Sigma Chemical Co. (St Louis, MO, USA). Percoll was obtained from Pharmacia (Uppsala, Sweden). Standard analytical grade laboratory reagents were from Merck (Darmstad, Germany) or Sigma.

2.2. Animals

Pregnant albino Wistar rats were fed *ad libitum* on a stock laboratory diet (carbohydrates 49.8%, protein 23.5%, fat 3.7%, minerals 5.5% and added vitamins and amino acids). Fetuses were delivered on 21.5 days of gestation (21.7 days for full gestation) by rapid hysterectomy after cervical dislocation of the mother. To inhibit cytosolic or mitochondrial protein synthesis, newborns were injected immediately after birth with 0.9% NaCl containing 10 mg of cycloheximide/kg body weight or 100 mg streptomycin/kg body weight, respectively [3]. Litter mates were injected with the same volume (50 μl) of the vehicle. Newborns were kept in an incubator at 37°C for 1 h with a continuous stream of water-saturated air.

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Abbreviations: rh-123, rhodamine-123; RCI, respiratory control index; SSC, side (90°) scatter; IGF, intensity of green fluorescence; LFP, low fluorescence population; HFP, high fluorescence population.

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2.3. Mitochondria isolation

Newborns were killed by decapitation at the time indicated (0 h or 1 h after delivery) and their livers were homogenized in 4-fold (w/v) of isolation medium containing 250 mM sucrose, 1 mM EDTA and 10 mM HEPES, pH 7.4. Female virgin rats were used to isolate adult liver mitochondria. The whole mitochondrial fraction was isolated from liver homogenates by a discontinuous Percoll gradient technique [6,7].

2.4. Respiratory measurements

Respiratory measurements were carried out in respiration medium containing: 225 mM sucrose, 10 mM succinate, 10 mM KCl, 5 mM $MgCl_2$, 10 mM KH_2PO_4, 1 mM EDTA and 10 mM Tris (pH 7.4). 200 nmol of ADP were added to the respirometer (Oxymeter, Gilson Medical Electronics, France) cell (2 ml) to measure the respiratory rate in state 3 and the ADP/O ratio.

2.5. Rhodamine-123 staining and flow cytometry analysis

The isolated mitochondrial fraction was preincubated in isolation medium in the absence or the presence of ATP (500 nmol of ATP per mg of mitochondrial protein measured by the Lowry [14] method). Mitochondria were stained by incubation with rh-123 (10 μg/ml) at 37°C for 15 min in the dark. The unbound dye was removed by centrifugation and the pellet washed twice with isolation medium [6]. All measurements of mitochondrial fluorescence and 90° angle light scatter (SSC) were made for at least 10,000 events/test with the FACStar flow cytometer using the Consort 30 software program (Becton/Dickinson, San José, Ca, USA). Data on mitochondrial fluorescence and side light scatter were obtained using a 5-W argon ion laser tuned at 488 nm and 250 mW and rh-123 green fluorescence was measured through a 530 ± 15 nm band pass filter placed in front of the green photomultiplier tube using a four decade log mode scale. Graphics were plotted by means of the Lysys 1.0 software (Becton/Dickinson). In order to compare the values of mean intensities of green fluorescence, acquisition of the data was performed with the same laser alignment.

2.6. Statistical analysis

Statistical analysis of paired data were carried out using Student's *t*-test.

3. Results

3.1. Changes in respiratory parameters of liver mitochondria during early development

Liver mitochondria RCI increased twofold during the first hour of extrauterine life, attaining values found in the adult. This enhancement was mainly due to the increase in the rate of respiration in state 3, although a slight decrease in the rate of respiration in state 4 was also observed under the same circumstances (Table 1). The mitochondrial ADP/O ratio sharply increased to adult values during the first hr after birth (Table 1).

Preincubation with ATP significantly ($P < 0.001$) increased the rate of respiration in state 3 and subsequently the RCI of term fetus mitochondria but the rate of respiration in state 4 and the ADP/O ratio were not changed under these circumstances (Table 1). On the other hand, preincubation with ATP did not affect the respiratory parameters in early newborn or adult mitochondria (Table 1).

3.2. Changes in the proportion of mitochondrial fluorescence populations during the first hour after birth

Figure 1 shows the flow cytometry analysis of the rh-123-stained isolated liver mitochondria from term fetuses and from early (1 h) newborn rats. In agreement with our previous results [7,8], liver mitochondria showed two different fluorescence peaks, i.e. a major low fluorescence population (LFP) and a minor high fluorescence population (HFP). Both populations were also observable after preincubation with ATP or after administration to the newborns of protein synthesis inhibitors (Fig. 1).

At birth (term fetus), the LFP accounted for about 71% of the total mitochondrial fraction, increasing to 82% in early neonates (1 h or extrauterine life). In term fetal mitochondria (0 h) the HFP accounted for the remaining 29%, decreasing to 18% 1 h after birth (Table 2). The mean intensity of green fluorescence (IGF) of both mitochondrial populations did not change significantly during the first hour of extrauterine life (Table 2).

3.3. Effect of ATP on mitochondria fluorescence populations

In term fetuses (0 h), preincubation of mitochondria

Table 1
Effect of ATP on the respiratory parameters of newborn and adult rat liver mitochondria

	Preincubation with ATP	State 3	State 4	RCI	ACP/O
0 h (Term fetus)	−	55.0 ± 2.7	25.4 ± 1.8	2.2 ± 0.1	1.3 ± 0.1
	+	87.7 ± 4.4†††	31.0 ± 2.1	2.9 ± 0.2†††	1.4 ± 0.1
1 h (Early newborn)	−	80.5 ± 1.8***	18.9 ± 0.6*	4.2 ± 0.1***	1.7 ± 0.1***
	+	93.9 ± 5.8†	23.7 ± 1.2	4.0 ± 0.2	1.5 ± 0.1†
Adult	−	82.2 ± 5.2***	20.3 ± 0.8*	4.1 ± 0.1***	1.8 ± 0.1
	+	92.2 ± 8.4	24.0 ± 1.2	3.9 ± 0.2	1.6 ± 0.1

Results are means ± S.E.M. of 4–10 different experiments. The respiratory rate in state 3 and in state 4 are expressed as nmol O_2/min per mg of mitochondrial protein. Results statistically different from term fetus (0 h) values are expressed as $^{*}P < 0.05$; $^{***}P < 0.001$. Results statistically different from age-matched values without preincubation with ATP are expressed as $^{\dagger}P < 0.05$, $^{\dagger\dagger\dagger}P < 0.001$.

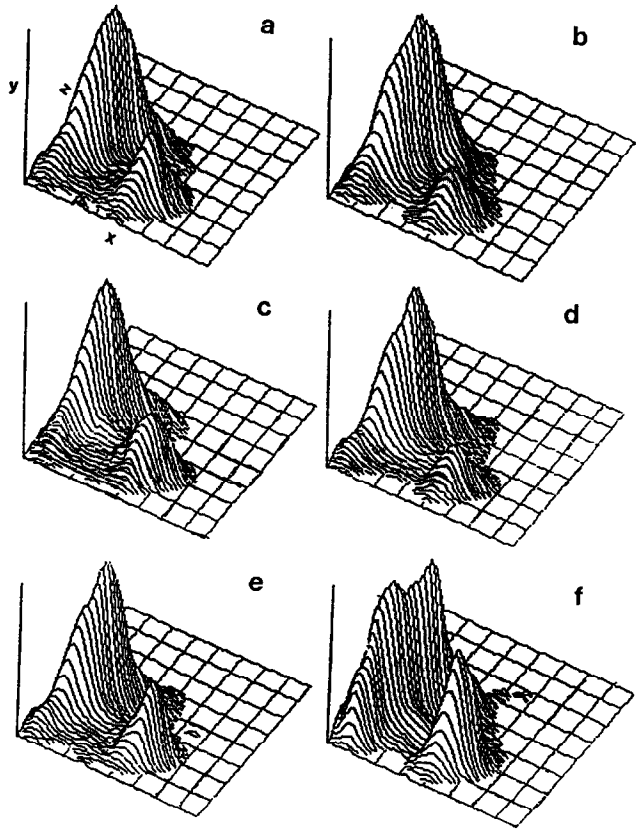

Fig. 1. Flow cytometry analysis of rhodamine-123-stained mitochondria from rat liver during the early postnatal period. (a) Term fetus (0 h) mitochondria incubated with isolation medium. (b) Term fetus (0 h) mitochondria incubated with isolation medium plus ATP. (c) Newborn (1 h) mitochondria incubated with isolation medium. (d) Newborn mitochondria incubated with isolation medium plus ATP. (e) Mitochondria from streptomycin-treated newborns. (f) Mitochondria from cycloheximide-treated newborns. x-axis: green fluorescence (log scale); y-axis: number of events; z-axis: 90° angle light scatter (side scatter: SSC, linear scale).

with ATP significantly ($P < 0.001$) increased the percentage of the LFP, that of the HFP subsequently decreasing (Table 2). However, in the early newborns (1 h) the LFP/HFP ratio was slightly increased ($P < 0.05$) after incubation with ATP (Table 2). It should be noted that preincubation with ATP raised the LFP/HFP ratio of fetal mitochondria to values observed in untreated early newborns. In fact, the effect of ATP on mitochondrial fluorescence populations was markedly higher immediately after birth than 1 h later.

The mean IGF of the LFP was increased by the presence of ATP by about 30% and 20% in term fetal and early newborn mitochondria, respectively. However, ATP did not significantly change the mean IGF of the HFP (Table 2).

3.4. *Effect of the inhibition of protein synthesis on mitochondrial fluorescence populations*

Since protein synthesis is presumably responsible for postnatal mitochondrial differentiation [3], we were prompted to investigate the effect of the inhibition of protein synthesis on postnatal changes in mitochondrial populations. Thus, cycloheximide or streptomycin as cytosolic or mitochondrial protein synthesis inhibitors, respectively, were administered to the newborns immediately after birth and flow cytometry analysis of rhodamine-123-stained mitochondrial populations was carried out 1 h later.

The administration of cycloheximide decreased by about 1.5-fold the percentage of LFP, that of the HFP increasing the same proportion as compared with age-matched (1 h) control newborns (Table 3). It is noteworthy that cycloheximide-treated newborns showed LFP/HFP ratios (Table 3) even lower than those observed in term fetuses (0 h), indicating that cycloheximide not only prevented but also reversed the changes in the percentages of mitochondrial populations caused by postnatal development (Table 2). Treatment with streptomycin decreased the LFP/HFP ratio (Table 3) to values similar to those found in liver mitochondria immediately after birth (Table 2). The mean IGF of both the LFP and the HFP were not changed by treatment with cycloheximide or streptomycin (Table 3).

Table 2
Effect of ATP on the percentage and mean intensity of green fluorescence (IGF) of rh-123-stained mitochondrial populations of rat liver during the early postnatal period

		0 h	1 h	0 h + ATP	1 h + ATP
LFP	Mean IGF	31 (100)	32 (98)	40 (121)	39 (119)
	Percentage	71 ± 2.4	82 ± 1.9**	85 ± 0.5†††	88 ± 1.5†
HFP	Mean IGF	890 (68)	898 (65)	920 (75)	928 (72)
	Percentage	29 ± 2.4	18 ± 1.9**	15 ± 0.5†††	12 ± 1.5†

LFP and HFP, low and high-fluorescence mitochondrial populations, respectively. Mean IGF (intensity of green fluorescence) is given in arbitrary units with the variation coefficients in brackets. The values of the mean IGF depicted in this table were acquired in the same experimental session and determined using the Consort 30 program. Percentage values are means ± S.E.M. of 4–10 different experiments. Results statistically different from newborn (0 h) values are expressed as $^{**}P < 0.01$. Results statistically different from age-matched values without preincubation with ATP are expressed as $^{\dagger}P < 0.05$; $^{\dagger\dagger\dagger}P < 0.001$.

Table 3
Effect of the inhibition of protein synthesis on the percentage and mean intensity of green fluorescence (IGF) of rh-123-stained mitochondrial populations of rat liver during the early postnatal period.

		1 h	+ Cyclo-heximide	+ Strepto-mycin
LFP	Mean IGF	21 (172)	18 (168)	20 (172)
	Percentage	82 ± 1.9	58 ± 3.0###	70 ± 2.5##
HFP	Mean IGF	227 (74)	235 (70)	230 (70)
	Percentage	18 ± 1.9	42 ± 3.0###	30 ± 2.5##

To inhibit cytosolic or mitochondrial protein synthesis, newborns were injected immediately after birth with 50 μl of 0.9% NaCl ('1 h') or 50 μl of 0.9% NaCl containing 10 mg of cycloheximide/kg body weight or 100 mg streptomycin/kg body weight, respectively. Newborns were kept in an incubator at 37°C for 1 h with a continuous stream of water-saturated air. LFP and HFP, low and high-fluorescence mitochondrial populations, respectively. Mean IGF (intensity of green fluorescence) is given in arbitrary units with the variation coefficients in brackets The values of the mean IGF depicted in this table were acquired in the same experimental session and determined using the Consort 30 program. The percentage results are means ± S.E.M. of 3–8 different experiments. Results statistically different from 1 h values are expressed as ##P <0.01; ###P < 0.001 (Student's t-test).

4. Discussion

The acquisition of fully developed mitochondria after birth is an important homeostatic mechanism that enables newborn mammals to succesfully adapt to extrauterine life in which oxygen is freely available. Thus, liver mitochondria attain the functional capacity of adult life within the first hour after birth as shown by the sharp increase in RCI [1,15] and the enhancement in the synthesis of mitochondrial oxidative machinery components [3,16]. In agreement with this our results show (Table 1) that the RCI increased 2-fold within the first hour of extrauterine life, reaching values very similar to those of adult mitochondria. The rapid postnatal enhancement of the RCI and of the ADP/O ratio to adult values suggests that liver mitochondria attain the oxidative phosphorylation capacity of the adult shortly after birth, in agreement with what has been described previously [1,3,15,16]. Under the same circumstances, the percentages of mitochondria fluorescence populations changed markedly during the first hour of extrauterine life (Table 2). Thus, the percentage of the HFP sharply decreased while that of the LFP increased (Table 2), suggesting that the HFP is an immature form of mitochondria that is converted into the mature one, the LFP [7,8], which is the major mitochondrial form in the adult rat liver [6]. In addition, the postnatal increase in the LPF/HFP ratio observed in our experiments (Table 2) may be related to the postnatal improvement in mitochondrial function (Table 1) as one part of the adaption of liver mitochondria to extrauterine life [1,16].

Pollak et al. [1,17] postulated that the acquisition of energy-transducing mitochondria after birth would be mediated by mitochondrial enrichment in ATP. In fact, a rapid enhancement in the intramitochondrial adenine nucleotide content has been observed shortly after birth concurrently with the increase in RCI [1,2,18,19]. In addition, it has been reported [20–23] that incubation with ATP increases the RCI in fetal mitochondria. In our experiments (Table 1), the increase in the RCI caused by ATP was mainly due to the increase in the rate of respiration in state 3 rather than a decrease in state 4 (Table 1). This suggests that the effect of ATP is exerted on the oxidative phosphorylation system rather than on the permeability of the inner mitochondrial membrane. In fact, it has been suggested that ATP promotes the assembly of F_0F_1–ATPase components, increasing mitochondrial capacity for ATP synthesis [22,23]. Consistent with this, the presence of ATP markedly changed the mitochondrial LFP/HFP ratio during the earlier postnatal period. Thus, the incubation of term fetus liver mitochondria with ATP decreased the percentage of the HFP, that of the LFP increasing, reaching the values observed in early newborns (Table 2). These results suggest that the putative conversion of the HFP into the LFP (Table 2) that occurred postnatally may be due to the increase in the intramitochondrial adenine nucleotide content that coincides with postnatal maturation of the mitochondrial function [1,3]. If so, the conversion of the HFP into the LFP that occurred within the first hour of extrauterine life (Tabel 2) may be a consequence of F_0F_1–ATPase assembly caused by the increase in intramitochondrial ATP levels [1,22,23]. Actually, the effect on the LFP/HFP ratio (Table 2) was 1.5-fold higher at birth than 1 h later, a fact consistent with the idea that the sensitivity of mitochondrial populations changes to ATP rapidly decreases with age. This is in agreement with the observation of Pollak [1] that the effect of ATP on the RCI faded soon after birth the mitochondria of early newborns and of adult rat liver (Table 1) being insensitive to ATP [1].

On the other hand, some evidence supports the hypothesis that protein synthesis is the limiting factor in the acquisition of mitochondrial function maturity that occurs immediately after birth [3,16]. Actually, there is an important increase in cytochrome and respiratory complex contents in rat liver mitochondria during the first hour after delivery [3,16,24] concurrent with the enhancement in the respiratory parameters observed under these circumstances (Table 1). Accordingly, the induction of the synthesis of mitochondrial proteins is triggered immediately after birth [16], coinciding with the rapid postnatal enhancement in the LFP/HFP ratio observed in our experiments (Table 2). Indeed, protein synthesis is involved in the postnatal changes in mitochondrial populations because the administration of cycloheximide or streptomycin, -inhibitors of cytoplasmic

or mitochondrial protein synthesis, respectively- at birth clearly altered the postnatal changes in the mitochondrial LFP/HFP ratio (Tables 2 and 3). These results suggest that the conversion of the HFP into the LFP depends on the synthesis of mitochondrial proteins coded either by nuclear or by mitochondrial DNA. Since the changes in LFP/HFP ratio are probably associated with rh-123 binding to the F_0F_1–ATPase complex [6,7,11,13], it may be suggested that proteins involved in LFP-HFP interconversion are subunits of this enzyme. This is in agreement with our previous suggestion that the HFP is transformed into the LFP by the incorporation of some components of F_0F_1–ATPase, resulting in an increase in mitochondrial efficiency [7]. In the present work our results suggest that this transformation is coordinated between cytosolic and mitochondrial protein synthesis because both cycloheximide and streptomycin administration affected postnatal changes in the LFP/HFP ratio (Table 3). Thus, streptomycin inhibited postnatal conversion of the HFP into the LFP (Table 3), suggesting that one or both of the two F_0F_1–ATPase subunits coded by mitochondrial DNA [25] would be involved in this conversion. However, cytoplasmic synthesis is also responsible for the observed changes because cycloheximide not only inhibited but also reversed the postnatal transformation of the HFP into the LFP (Table 3). Actually, most F_0F_1–ATPase subunits are synthesized in the cytosol, and are later imported into mitochondria [25]. In this sense, it has been reported that the β-subunits of F_1-ATPase are actively synthesized immediately after birth their synthesis being inhibited by cycloheximide but not by streptomycin [3]. Consequently, it may be suggested that β-subunit synthesis is involved in the transformation of the HFP into LFP. However, it is intriguing that cycloheximide not only blocked but even reversed the transformation of the HFP into the LFP (Table 3). It might be speculated that both forms of mitochondria are in equilibrium, HFP being displaced to LFP by the presence of a cytosolically-synthesized protein. When the synthesis of this protein is inhibited by cycloheximide, LFP-HFP interconversion is displaced to the HFP (Table 3). Nevertheless, the incorporation of this or another putative protein into mitochondria depends on the compulsory synthesis of some proteins coded by mitochondrial DNA apparently necessary for assembling the protein(s) of cytosolic origin.

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