Mammalian adaptation to extrauterine environment: mitochondrial functional impairment caused by prematurity

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In this paper we report that, compared with term rat neonates, both mitochondrial content and function are diminished in liver of preterm neonates (delivered 24 h before full term) compromising cellular energy provision in the postnatal period. In addition, there is a parallel reduction in the content of mRNAs encoding mitochondrial proteins in preterm rats. Also, efficient oxidative phosphorylation is not attained in these pups until 3 h after birth. Although isolated liver mitochondria from preterm neonates show a two-fold increase in F_1 -ATPase β -subunit and cytochrome *c* oxidase activity 1 h after birth, the abnormal coupling efficiency between respiration and oxidative phosphorylation (ADP/O ratio) is due to maintenance of high H^+ -leakage

values in the inner mitochondrial membrane. Postnatal reduction of the H^+ leak occurs concomitantly with an increase in intra-mitochondrial adenine nucleotide concentration. Accumulation of adenine nucleotides in preterm and term liver mitochondria parallels the postnatal increase in total liver adenine nucleotides. Delayed postnatal induction of adenine biosynthesis most likely accounts for the lower adenine nucleotide pool in the liver of preterm neonates. The delayed postnatal accumulation of adenine nucleotides in mitochondria is thus responsible for the impairment in oxidative phosphorylation displayed by organelles of the preterm liver.

INTRODUCTION

Mammalian adaptation to the aerobic extrauterine environment involves profound hormonal, physiological and metabolic changes which need to be finely tuned to avoid neonatal morbidity and mortality. At the cellular level, successful adaptation to extrauterine life depends critically on the rapid switch in metabolic pathways relevant for energy provision, i.e. from fetal anaerobic glycolysis [1,2] to neonatal oxidative phosphorylation [3–5]. The latter requires both an appropriate number and functional activity of the mitochondria present in neonatal tissues in order to satisfy the energy requirements for maintenance of whole-body homeostasis. Mitochondrial biogenesis during rat liver development results in both (i) an increase in the number of mitochondria per cell (organelle proliferation), which is a continuous process that spans almost two-thirds of the suckling period (for a review see ref. [6]), and (ii) an increase in the functional capability of pre-existing mitochondria (organelle differentiation), which is a very rapid process that occurs during the first postnatal hour [5,7,8]. The postnatal onset of oxidative phosphorylation in liver mitochondria of term rat neonates is accomplished by the synergistic action of two main processes: (i) a preferential increase in the rate of synthesis of mitochondrial proteins [5,7–9] and (ii) an increase in intramitochondrial adenine nucleotide concentration [3–5,10]. The former is accomplished by prenatal accumulation of nuclear-encoded mitochondrial mRNAs [11–13], which are efficiently translated immediately after birth [13]. The latter, which is independent of protein synthesis [5,8], promotes coupling efficiency between respiration and oxidative phosphorylation [3–5,14–16].

Preterm birth is one of the main disorders responsible for infant death or disability [17]. The major diseases of the preterm

infant are secondary to immaturity of a variety of organ systems [17]. The cellular and molecular mechanisms responsible for the pathological state of preterm neonates are not well understood. In rat, preterm birth is also associated with an extremely high mortality rate (50%) during the first 3 h of life [8]. Preterm rat neonates exhibit during this time period impaired oxidative metabolism [8,18,19] and much higher glycolytic rates than full-term neonates [8,20], although their blood oxygen concentrations are normalized by 1 h after birth [21].

In the present study we have examined the onset of mitochondrial function in the liver of preterm rat neonates. The main objective was identification of the molecular and functional mechanisms which would explain the pathophysiology of oxidative metabolism in preterm mammals during the critical period of adaptation to extrauterine life. The results reveal that preterm birth interrupts the programme of mitochondrial biogenesis in fetal liver and impairs the function of pre-existing organelles in the postnatal period, thereby compromising cellular energy yield and homeostasis. Furthermore, liver mitochondria from preterm rats show a twofold increase in F_1 -ATPase β -subunit and cytochrome *c* oxidase activity during the first postnatal hour. However, the delayed postnatal induction of *de novo* adenine biosynthesis in the preterm liver compromises accumulation of mitochondrial adenine nucleotides. This is responsible for inefficient oxidative phosphorylation in the immediate postnatal period.

EXPERIMENTAL

Animals

Pregnant albino Wistar rats weighing 200 g were fed on standard laboratory chow and water *ad libitum*. Females were caged overnight with males, and conception was assumed next morning

Abbreviations used: TPP⁺, tetraphenylphosphonium; $\Delta\Psi$, membrane potential; J_{H^+} , proton current; 1 × SSC, 0.15 M NaCl plus 0.015 M sodium citrate.

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by the presence of spermatozoa in the vagina. Preterm (4.2 ± 0.1 g) and term (5.2 ± 0.1 g) fetuses were delivered on days 21 and 22 of gestation respectively by rapid hysterectomy of cervically dislocated pregnant rats. The neonates were maintained without feeding as previously described [18].

Liver homogenates and isolation of mitochondria

Fetal and neonatal rat liver mitochondria were prepared as described previously [5,7]. Briefly, the dissected livers were placed in ice-cold isotonic isolation medium (225 mM sucrose, 10 mM Tris/HCl, 1 mM EGTA, 0.2% BSA, pH 7.4) and homogenized with a Dounce homogenizer by three and two passes of A and B pestles respectively. Mitochondrial pellets for respiration studies were washed and resuspended (250 mM sucrose, 1 mM Tris/HCl, 0.3 mM EGTA, 0.2% BSA, pH 7.4) to obtain a final protein concentration of 20 mg/ml. A portion of each liver homogenate was freeze-thawed three times and kept at -70°C for determination of F_1 -ATPase β -subunit.

Mitochondrial respiration

Rates of oxygen consumption were measured polarographically in a thermostatically controlled (30°C) 2 ml chamber with the use of a Clark-type microelectrode. The respiration medium (pH 7.4) consisted of 225 mM sucrose, 10 mM KCl, 5 mM MgCl_2 , 10 mM phosphate buffer, 10 mM Tris/HCl and 0.05% BSA. Sodium succinate (8 mM) was used as respiratory substrate. To prevent respiration by endogenous site-I substrates, $2.5 \mu\text{M}$ rotenone was added.

Enzyme and protein assays

Cytochrome *c* oxidase activity was assayed at 30°C as previously described [5]. One unit of activity represents 1 μmol of product formed/min. Protein concentrations were determined by the Lowry method with crystalline BSA as standard.

Western-blot analysis of the β -subunit of the F_1 -ATPase complex

Liver and mitochondrial protein samples from preterm fetuses and neonates were fractionated in 12.5% polyacrylamide gels and transferred to poly(vinylidene difluoride) membranes [7]. Membranes were incubated with affinity-purified antibodies recognizing the β -subunit of the mitochondrial F_1 -ATPase complex as recently reported in detail [13]. Visualization of the immunoreactive β -subunit protein was achieved by incubation with 1:1500 dilution of a goat anti-rabbit IgG peroxidase conjugate (GAR/IgG (H+L)/PO) (Nordic Immunology, Tilburg, The Netherlands). The intensity of stain deposition on the membranes was determined by laser densitometric scanning and expressed as arbitrary units/ μg of protein.

Nucleic acid hybridization analysis

Total liver RNA was extracted from freeze-clamped fetal rat livers and separated by electrophoresis in formaldehyde/1.4% agarose gels [7,13]. Denatured RNAs were either stained with ethidium bromide or vacuum-blotted on to GeneScreen membranes (Du Pont-New England Nuclear) for hybridization

procedures. Membranes were incubated with [^{32}P]dCTP-labelled cDNA probes. The DNA probes used in this study were a 1577 bp cDNA for rat liver F_1 -ATPase β -subunit [22], and a 1676 bp cDNA for rat liver mitochondrial-encoded subunits 6 and 8 of the ATP synthase complex and subunit III of the cytochrome *c* oxidase and a 937 bp cDNA for rat liver mitochondrial 12 S rRNA [23], generously provided by Dr. P. L. Pedersen (The Johns Hopkins University, Baltimore, MD, U.S.A.) and Dr. P. Cantatore (University of Bari, Bari, Italy) respectively. A human β -actin cDNA clone (Clontech, Palo Alto, CA, U.S.A.) was also used. Hybridization conditions were as previously reported [7]. Membranes were subsequently washed according to the following protocol: (i) $6 \times \text{SSC}$ at room temperature for 10 min, (ii) $2 \times \text{SSC}$ containing 0.1% SDS at 65°C for 10 min and (iii) $0.1 \times \text{SSC}$ containing 0.1% SDS at 65°C for 10 min (where $1 \times \text{SSC} = 0.15 \text{ M NaCl}$ plus 0.015 M sodium citrate). For stripping labelled DNA probes, membranes were incubated in sterile water at 90 – 100°C for 20 min. Membranes were exposed to X-ray films and analysed by densitometric scanning.

Determination of current (J_{H^+})/voltage ($\Delta\psi$) relationship

Mitochondrial membrane potential ($\Delta\psi$) and matrix volume were measured as described [5]. $\Delta\psi$ was measured by monitoring the movements of tetraphenylphosphonium (TPP^+) across the mitochondrial membrane with a TPP^+ -selective electrode. The TPP^+ -selective electrode was calibrated with increasing TPP^+ concentrations (0.25 – $1 \mu\text{M}$). Non-specific binding of TPP^+ to mitochondrial membranes was measured in ($5 \mu\text{M}$) antimycin-inhibited mitochondria. Electrode measurements of $\Delta\psi$ were carried out simultaneously with mitochondrial respiration. The concentration of TPP^+ in the mitochondrial matrix was calculated from the amount of cation taken up by the mitochondria and from the matrix volume. Mitochondrial matrix volume was calculated as water – sucrose space by measuring the difference in distribution between $^3\text{H}_2\text{O}$ ($0.5 \mu\text{Ci/ml}$) and [$\text{U-}^{14}\text{C}$]sucrose ($0.1 \mu\text{Ci/ml}$). Proton currents (J_{H^+}) were estimated from the respiratory rates assuming a H^+/O stoichiometry of 6. Mitochondrial respiratory rates were titrated against $\Delta\psi$ by addition of the respiratory inhibitor malonate (0 – 25 mM) [15,16].

Determination of adenine nucleotide concentrations

Mitochondrial proteins (10 mg) were extracted for 5 min with 6% HClO_4 , and neutralized with 20% KOH. ATP + ADP were assayed after enzymic transformation of ADP into ATP, as previously reported [5]. Determination of adenine nucleotide concentrations in freeze-clamped liver samples has been previously reported in detail [24].

In vivo estimation of de novo rates of purine synthesis

Determination of *de novo* purine-biosynthesis rates was carried out by labelling the tetrahydrofolate pool with [^{14}C]formic acid [25,26]. After hydrolysis and isolation of the purine bases, the amount of ^{14}C radioactivity incorporated into total purines and adenine was quantified. Briefly, at the indicated time intervals after delivery, $2 \mu\text{Ci}$ of [^{14}C]formic acid was intraperitoneally injected into term and preterm newborn rats. In preliminary experiments, the initial rate of incorporation of [^{14}C]formic acid into liver purines was shown to be linear at least for the first 10 min after administration of the tracer. To reduce the effects of purine recapture, degradation and exportation on the estimated initial rate of purine biosynthesis, newborns were killed 5 min

Figure 1 Nuclear and mitochondrial mRNA stores encoding mitochondrial proteins in liver of preterm rat fetuses: developmental changes in F₁-ATPase β -subunit in liver homogenates and mitochondria

Pre-term (PT) and term (T) fetuses were delivered from pregnant rats on days 21 and 22 of gestation respectively. (a) After fractionation by electrophoresis of 40 μ g of total cellular RNA, the membrane was hybridized with [α -³²P]dCTP-labelled cDNA probes for mRNAs of rat liver F₁-ATPase β -subunit (β F1) and cytochrome *c* oxidase subunit III (COIII) + ATPase subunits 6–8 (ATP6–8). Below the autoradiograms a picture of a parallel ethidium bromide-stained gel is shown in which the 18 S and 28 S RNA bands demonstrate similar RNA loading in each track. Two different preparations assayed in the same gel are shown for each condition. (b) Autoradiograms showing the hybridization signals to labelled human β -actin and rat mitochondrial 12 S cDNA probes during intrauterine development of rat liver. Fetal RNA samples were obtained from fetuses on day 20 of gestation to illustrate more clearly that the accumulation of mRNA, encoded in either nuclear or mitochondrial genes, occurs at a time when other constitutive cytosolic mRNA and mitochondrial rRNA are less represented. (c) Quantification of F₁-ATPase β -subunit (□) and COIII + ATP6–8 (▨) mRNAs in the liver of preterm and term fetuses. The results shown are means $\pm$ S.E.M. from three or four different RNA preparations. **P* < 0.01, compared with pre-term results by Student's *t* test. (d) Isolated liver mitochondria were prepared from adult (A) and preterm rat neonates at 0, 1, 2 and 3 h after birth. Liver proteins (25 μ g) were fractionated by SDS/PAGE and probed with affinity-purified anti-F₁-ATPase β -subunit) antibodies. A representative immunoblot is shown. (e) Protein (100 μ g) from liver homogenates from adult (A) and preterm rat neonates at 0, 1, 2 and 3 h after birth were fractionated and probed with anti-F₁-ATPase β -subunit) antibodies as described above. A representative immunoblot is shown. (f) Quantification of the amount of F₁-ATPase β -subunit (arbitrary units/ μ g of protein) was expressed as percentage of that present in adult mitochondria (○) or liver homogenates (●) assayed in the same blot. The values shown are means $\pm$ S.E.M. of four or five different preparations. The discontinuous line indicates that the quantity of F₁-ATPase β -subunit assayed in liver homogenates of preterm fetuses (0 h value) is below reliable detection limits by this technique.

after receiving the tracer. Livers were removed and freeze-clamped in liquid nitrogen. Frozen tissue powders were extracted with 1.5 ml of 1 M HClO₄ at 100 °C for 1 h, after the addition of 0.2 μ Ci of [³H]ATP to each sample (used as an internal standard for purine recovery). Liver purines were precipitated at 4 °C in

basic medium (pH 11) with AgNO₃ (50 mM final concentration). Washed purine–Ag pelleted complexes were solubilized in 1 ml of 1 M HCl at 100 °C. The radioactivity recovered in [³H]adenine was in the 70–90% range. Adenine, guanine and hypoxanthine bases were separated by t.l.c. in cellulose plates contain-

Figure 2 Developmental changes in ADP/O ratio in mitochondria of preterm rat neonates: effect of ADP

(a) Oxygen-consumption rates were measured polarographically in isolated mitochondrial (1 mg/ml) from term (□) and preterm (▨) fetal livers using 8 mM sodium succinate as respiratory substrate. ADP/O ratios were calculated after successive ADP additions (+130 nmol). Values are means $\pm$ S.E.M. of four to six experiments. * $P < 0.005$, compared with the preceding ADP/O value by Student's *t* test. (b) ADP/O ratio in isolated liver mitochondria from preterm fetuses as a function of the amount of ADP added in a single pulse. Various amounts of ADP (43–268 nmol) were added to isolated mitochondria (1 mg/ml) and the resulting ADP/O ratio was calculated. The results are means $\pm$ S.E.M. The linear correlation between the ADP/O ratio and the amount of added ADP has the following parameters: *m* (slope) = 0.004; *b* (constant; ordinate intercept) = 0.16; $r = 0.959$; $P < 0.001$. (c) Developmental changes in the ADP/O ratio in isolated liver mitochondria of preterm newborn rats. Liver mitochondria were isolated at various times after delivery. ADP/O ratios were calculated after addition of 130 nmol (○) or 286 nmol (●) of ADP. The results shown are means $\pm$ S.E.M. of four experiments.

ing u.v. indicator using butanol/methanol/water/25% NH_3 (60:20:20:1, by vol.). After chromatography, adenine spots were scraped off and extracted in 1 M HCl at 60 °C. The amount of adenine present in the samples was determined by measuring the A_{260} using a molar absorption coefficient for adenine of $14.86 \text{ mM}^{-1} \cdot \text{cm}^{-1}$. The ^{14}C and ^3H radioactivity present in adenine was determined by liquid-scintillation counting.

RESULTS

Premature birth in rat by 1 day could result in altered mitochondrial function in the postnatal period if the tissue pattern of mitochondrial biogenesis (proliferation) is abruptly interrupted. Altered mitochondrial function at this stage of development could be related to abnormal differentiation of pre-existing organelles [4–13]. To distinguish between these two possibilities, mitochondrial $\text{F}_1\text{-ATPase } \beta$ -subunit was assayed in both liver homogenates and isolated mitochondria. These parameters were used as indexes of liver mitochondrial abundance (proliferation) and extent of organelle differentiation respectively [6–8]. In addition, as nuclear-encoded mitochondrial mRNAs accumulate in liver during fetal development [11–13], we determined their content in preterm fetuses.

Steady-state levels of $\text{F}_1\text{-ATPase } \beta$ -subunit mRNA (encoded in the nuclear genome) and that of cytochrome *c* oxidase subunit III + ATPase subunits 6–8 (encoded in the mitochondrial genome) in the liver of pre-term fetuses were found to be half of those present in liver of term fetuses (Figures 1a and 1c). However, this decrease in mRNA level did not affect postnatal differentiation of mitochondria in the liver of preterm neonates. In fact, both the relative amount of $\text{F}_1\text{-ATPase } \beta$ -subunit (Figures 1d and 1f) and the activity of the respiratory-chain enzyme cytochrome *c* oxidase (0.33 ± 0.03 and 0.56 ± 0.07 unit/mg of protein at 0 and 1 h respectively; $P < 0.0005$) in isolated mitochondria of preterm livers increased twofold during the first postnatal hour. These

findings suggest that mitochondrial differentiation in preterm neonatal liver is regulated by rapid postnatal changes in the translational efficiency of the mRNAs encoding mitochondrial proteins [5,7,13].

In contrast with the similarities observed for organelle differentiation in the liver of both neonatal groups, the relative mitochondrial abundance was found to be severely reduced in preterm neonates (Figure 1e). The β -subunit of the $\text{F}_1\text{-ATPase}$ complex in liver homogenates of preterm fetuses was decreased to 15% compared with that of adult liver (Figures 1e and 1f). The amount of $\text{F}_1\text{-ATPase } \beta$ -subunit in liver increased twofold during the first postnatal hour (Figures 1e and 1f), reflecting the differentiation process of the organelle (Figures 1d and 1f). However, the relative $\text{F}_1\text{-ATPase } \beta$ -subunit content in the liver of preterm neonates was significantly reduced (50%) compared with that in liver of age-matched full-term neonates [13]. These findings illustrate that premature birth could impair the oxidative capacity of the liver in the early postnatal period because of reduced mitochondrial abundance. This could be ascribed to a similar decrease in liver content of the mRNAs (Figures 1a and 1c) needed for proliferation of the organelle at this stage of development.

Functional activity of pre-existing mitochondria in the preterm fetal liver is also impaired during the first few hours after birth. In fact, efficient coupling between rates of respiration and oxidative phosphorylation (assessed by the ADP/O ratio) in isolated liver mitochondria from preterm animals is not attained until the third postnatal hour (Figure 2c, open circles), a result that is in contrast with the rapid postnatal onset (1 h after birth) of oxidative phosphorylation in mitochondria of term neonates [5,15]. Interestingly, the degree of coupling between respiration and oxidative phosphorylation in preterm fetal liver mitochondrial preparations depends on the amount of exogenously added ADP (Figure 2). In contrast, this abnormal behaviour of the ADP/O ratio was never observed in liver mitochondria

Figure 3 H^+ -leakage values of the mitochondrial inner membrane of preterm rat neonates

(a) Liver mitochondria (1 mg/ml) from 0, 1, 2 and 3 h-old preterm neonates were incubated at 30 °C in a standard oxygen electrode buffer with 2.5 μ M rotenone and 8 mM sodium succinate for the simultaneous determination of State-4 respiratory rates and membrane potentials ($\Delta\Psi$). Mitochondrial respiratory rates were titrated against $\Delta\Psi$ by the addition of the respiratory inhibitor malonate (0–25 mM). Proton fluxes (J_{H^+}) were calculated from respiratory rates assuming a constant H^+/O stoichiometry of 6. Represented values at each time period are from three to seven different mitochondrial preparations. (b) Developmental changes in H^+ leakage (nmol of H^+ /min per mg of protein per mV) were calculated. The parameters of the linear regression correlation between J_{H^+} and $\Delta\Psi$ are: $m = 0.69$, $b = -89$, $r = 0.945$, $P < 0.001$ for 0 h; $m = 1.01$, $b = -150$, $r = 0.936$, $P < 0.0001$ for 1 h; $m = 1.02$, $b = -164$, $r = 0.993$, $P < 0.001$ for 2 h; $m = 0.30$, $b = -50$, $r = 0.920$, $P < 0.01$ for 3 h. (c) Linear correlation between ADP/O ratios and H^+ -leakage values. Represented values are those from Figures 2(c) and 3(b) of this work and previously reported values for adult and full-term neonates in ref. [15]. The parameters of the linear correlation are: $m = -0.798$, $b = 1.79$, $r = 0.747$, $P < 0.05$.

isolated from term neonates (Figure 2a, open bars). An improvement in ADP/O ratio in mitochondrial preparations from preterm fetal liver was observed after either successive additions of the nucleotide to the same mitochondrial preparation (Figure 2a, hatched bars) or a single nucleotide pulse (Figure 2b). Further, the nucleotide effect on coupling efficiency of liver mitochondria isolated from preterm neonates was manifest up to the second postnatal hour (Figure 2c, closed circles). These

results indicate that energy conservation in liver mitochondria from preterm fetuses and neonates is limited by the amount of ADP.

We also investigated the reasons for the inefficient coupling between respiration and oxidative phosphorylation in isolated liver mitochondria from preterm neonates. In energy-transducing membranes H^+ leakage is responsible for heat dissipation of the H^+ electrochemical gradient and thus for inefficient oxidative phosphorylation [27,28]. Analysis of developmental changes in H^+ conductance and H^+ leakage in mitochondria from preterm neonatal livers during adaptation to extrauterine environment revealed an anomalous ohmic current/voltage relationship (Figure 3a) and higher H^+ -leakage values (Figure 3b) than that found in mitochondria of full-term neonates and adults (~ 0.2 nmol of H^+ /min per mg of protein per mV) [15]. The characteristic biphasic behaviour of H^+ conductance (Figure 3a, 3 h) and values for H^+ leakage (Figure 3b, 3 h) were not attained until 3 h after delivery. The high H^+ leakage in preterm mitochondrial membranes during the first 2 h probably accounts for the lower efficiency of coupling between respiration and phosphorylation (Figure 2c).

Determination of adenine nucleotide concentration in preterm rat liver mitochondria during postnatal development revealed a significant decrease compared with the concentrations recorded in mitochondria from adult rats (Figure 4a) and mitochondria from term neonates [5,16]. ATP + ADP concentrations in mitochondria of term neonates 0, 1 and 2 h after birth amounted to 44, 53 and 80 % respectively of the concentrations recorded in adult mitochondria [5,16]. Moreover, a significant increase in intramitochondrial nucleotides was observed (Figure 4a) only at the time of a decrease in H^+ leakage (3 h after birth) (Figures 3a and 3b). Interestingly, it was found that the concentration of mitochondrial nucleotides that affected H^+ leakage and H^+ conductance in mitochondria from preterm animals was about 30 % of normal adult values (Figure 4a) [16]. These findings indicate that the change in phosphorylation in liver mitochondria of preterm neonates (Figures 2 and 3) was due to a low intramitochondrial adenine nucleotide content (Figure 4a). This could be overcome, at least *in vitro*, by increasing the supply of ADP to the organelle (Figure 2).

We then examined factors that could be rate limiting for the postnatal increase in intramitochondrial ATP + ADP concentrations in liver of preterm neonates. Net influx of adenine nucleotides into mitochondria is driven by the ATP gradient across the mitochondrial membrane [29,30] and is mediated by the carboxyatractyloside-insensitive ATP-Mg/P_i antiporter ([31,32]; for a recent review of this carrier see ref. [33]). At all time points studied, ATP and total adenine nucleotide concentrations in liver of preterm neonates were significantly lower than those recorded in liver of term neonates (Figure 4b). In addition, the postnatal increase in mitochondrial nucleotides (Figure 4a, and see ref. [6] for term) coincided with an increase in liver ATP concentration (Figure 4b, left panel) both in preterm (3 h postnatal) and full-term (1 h postnatal) neonates. However, in both neonatal groups, the changes in liver ATP concentration occurred simultaneously with an increase in total adenine nucleotide pool (Figure 4b, right panel). This observation suggests that the postnatal increase in ATP concentration observed in term and preterm livers actually results from the onset of efficient oxidative phosphorylation (see Figure 2c for preterm and refs. [5,15] for term neonates).

Immediately after birth many hepatic ATP-consuming processes are stimulated. Differential developmental changes in rates of purine biosynthesis/degradation could affect the increase in adenine nucleotide pool of neonatal liver (Figure 4b). In this regard, *in vivo* estimation of the rates of *de novo* adenine

Figure 4 Developmental changes in intramitochondrial adenine nucleotide concentrations and *in vivo* rates of adenine biosynthesis in the liver of preterm neonates

(a) Intramitochondrial ATP + ADP concentrations in liver of preterm rats during postnatal development. The values shown are expressed as percentage of the values present in isolated

biosynthesis in the liver of term neonates showed a profound increase immediately after birth (Figure 4c). The rate of adenine biosynthesis in liver of preterm neonates was found to be much lower and there was a 1–2 h delay in induction compared with rates found in livers of age-matched term neonates (Figure 4c). These findings strongly suggest that the lower rates of adenine biosynthesis (Figure 4c) are probably responsible for the lower adenine nucleotide content observed in liver of preterm neonates (Figure 4b, right panel). Further, a delayed induction of purine synthesis in liver of preterm neonates limits the accumulation of nucleotides in the mitochondria.

DISCUSSION

Biogenesis of mitochondria in mammalian cells has been mainly described at the transcriptional level [34–36]. In agreement with these findings, the proliferative phase of fetal liver mitochondria during the last day of gestation (Figures 1e and 1f and ref. [13]) is paralleled by similar changes in steady-state mRNA levels of mitochondrial transcripts encoded by both genomes (Figures 1a–1c). Recent findings indicate that regulation of organelle biogenesis in the liver of newborn rats is exerted at the translational level [13]. Also, regulation at both the post-transcriptional and post-translational levels has been suggested to be involved in biogenesis of mitochondria in other mammalian cellular types [37]. This further illustrates the complexity of the mechanisms involved in mammalian mitochondrial biogenesis.

A lower mitochondrial content of the preterm liver (Figures 1e and 1f) is one of the causes of the altered oxidative metabolism observed in these neonates after birth [8,18,19]. Impaired phosphorylation of pre-existing mitochondria during the first postnatal hour (Figures 2 and 3) also compromises adaptation of preterm neonates to extrauterine life [8]. We have shown that delayed induction and lower postnatal rates of adenine biosynthesis in liver of preterm neonates (Figure 4c) account for the lower concentrations of adenine nucleotides (Figure 4b). The lower availability of these effector molecules during postnatal development delays the net accumulation of adenine nucleotides in mitochondria of preterm livers until the third postnatal hour (Figure 4a). Therefore this delayed accumulation of adenine nucleotides in mitochondria is responsible for the postnatal delay in switching from a state of low to a state of high energy conservation in liver mitochondria of preterm neonates (Figures 2c and 3).

At this point we would like to emphasize a finding of this study that partially reconciles various results reported in the literature with regard to the postnatal onset of mitochondrial function after birth [3–10,13–16,38–40]. It has been demonstrated that the specific activity of various respiratory enzymes [3,5], as well as the activities and amounts of the F_1F_0 -ATPase [5,7,13] and pyruvate dehydrogenase complexes [9,41], increase twofold during the first postnatal hour in liver homogenates and isolated liver mitochondria from full-term neonates. Further, inhibition of protein synthesis in full-term neonates at the time of birth prevents development of efficient oxidative phosphorylation, although the postnatal increase in intramitochondrial adenine nucleotides is

adult liver mitochondria. ATP and ATP + ADP concentrations in adult mitochondria are 1.9 ± 0.2 and 4.4 ± 0.5 nmol/mg of protein respectively. The results shown are means $\pm$ S.E.M. of four experiments. * $P < 0.01$, compared with the preceding time period by Student's *t* test. (b) ATP and total adenine nucleotide concentrations in liver of term (●) and preterm (▲) neonates during postnatal development. The results shown are means $\pm$ S.E.M. of seven to ten samples. (c) *In vivo* rates of *de novo* adenine biosynthesis in liver of term (○) and preterm (△) neonates. The results shown are means $\pm$ S.E.M. for eight different samples.

not affected [5,8]. In line with these findings, we show in this study that, during the first postnatal hour, both the specific activity of cytochrome *c* oxidase and the amount of F_1 -ATPase β -subunit increase twofold in liver mitochondria of preterm neonates (Figure 1). This indicates that synthesis of mitochondrial proteins is also required for postnatal differentiation of liver mitochondria in preterm animals. On the other hand, it appears that the efficiency of oxidative phosphorylation in mitochondria from preterm fetal livers is not limited by the amount of ATP synthase, but rather by nucleotide availability, because addition of ADP *in vitro* to isolated mitochondria is enough to promote efficient coupling between respiration and oxidative phosphorylation (Figure 2). This finding is in agreement with other reports [3,4,10,14,38], but is in contrast with previous findings and the finding reported here for mitochondria of full-term neonates (Figure 2a) [5,8,39].

It is difficult to explain the molecular mechanism that causes such dissimilar behaviour in the control of coupling efficiencies between mitochondria of term and preterm fetuses. There are, however, specific features in the two populations of mitochondria: (i) the relative amount of F_1 -ATPase β -subunit is similar in mitochondria of term [5,7] and preterm (Figure 1f) fetuses; (ii) adenine nucleotide concentrations in preterm mitochondria are lower than those in term mitochondria (Figure 4a, and see refs. [5,16]); (iii) the respiratory chain of preterm mitochondria is less active than that of term mitochondria (assessed by both rates of oxygen consumption and the activity of cytochrome *c* oxidase). Estimations of the ratio of cytochrome *c* oxidase activity/ F_1 -ATPase β -subunit in liver mitochondria revealed values of 0.9 and 0.6 for term and preterm mitochondria respectively. It is therefore tempting to suggest that the provision of adenine nucleotides is able to promote efficient coupling between respiration and oxidative phosphorylation in preterm mitochondria. The nucleotides act by changing the conformation of the adenine nucleotide translocase, the limiting factor of oxidative phosphorylation responsible for maintaining high H^+ -leakage values [16] (for further details see the Discussion sections in refs. [15 and 16]). In contrast, in mitochondria from term fetuses, the relative activity of the respiratory chain exceeds that of the ATP synthase. Therefore it is reasonable to suggest that phosphorylation is limited by the amount of the latter complex rather than by adenine nucleotide concentration.

It has been reported that injection of adrenaline, glucagon or cyclic AMP into fetal rats *in utero* triggers both efficient energy conservation and accumulation of adenine nucleotides in liver mitochondria [10]. In contrast, administration of glucose at the time of birth [42,43] or *in utero* administration of the β -blocker propranolol [10] prevents rapid postnatal development of efficient energy conservation in liver mitochondria. As adenine nucleotides accumulation in liver mitochondria (Figure 4a) [5] correlates with the nucleotide availability (Figure 4b), it is suggested that these treatments affect the rates of adenine biosynthesis in the neonatal liver. The altered hormonal profile of the preterm rat neonate (i.e. maintenance of a high insulin/glucagon ratio after birth [44–46]) may contribute to the postnatal delay in accumulation of intramitochondrial adenine nucleotides and hence inefficient oxidative phosphorylation of pre-existing mitochondria during adaptation to extrauterine life. On the other hand, the maintenance of high H^+ -leakage values in liver mitochondria of preterm neonates during early postnatal development (Figure 3) does not necessarily represent a pathological situation when homeostasis of the whole organism is considered. In fact, inefficient oxidative phosphorylation is the main mechanism for providing heat by mitochondrial respiration in brown adipose tissue of mammalian newborns [47]. In this regard, it is possible

that maintenance of high H^+ -leakage values during first 2 h after birth in liver mitochondria from preterm neonates may provide a thermogenic safeguard mechanism [16,48], until thermogenesis develops in brown-adipose-tissue mitochondria.

Premature birth, by interfering with the fetal proliferative phase of mitochondria, results in an organism with an obvious disability at the cellular level to adapt to extrauterine life. Thyroid hormones have been shown to control biogenesis of mitochondria during fetal and early postnatal development of the rat [7]. The mechanism of thyroid hormone regulation of mitochondrial biogenesis is found at the DNA level and involves promotion of transcriptional activation of nuclear-encoded mitochondrial genes ([49] and references therein). We therefore suggest that thyroid hormone treatment of preterm infants at the time of birth could result in a therapeutic improvement in mitochondrial function by accelerating biogenesis of the organelle.

We show that liver mitochondrial function in preterm rat neonates is impaired during the critical period of mammalian adaptation to the extrauterine environment. This impediment is mainly due to a reduction ($\sim 50\%$) in mitochondrial content because birth occurs before hepatic organelle proliferation has been completed. During this time period, pre-existing mitochondria exhibit a severe impairment in phosphorylation because H^+ leakage is threefold higher than normal as a result of a delayed increase in the mitochondrial content of adenine nucleotides. The postnatal delay in onset of purine biosynthesis, which provides the nucleotides needed for accumulation in the mitochondria, accounts for this latter alteration. These findings have obvious implications for achieving improvements in mitochondrial function in premature neonates during the first few hours of postnatal life.

We thank Professor J. Satrústegui (C.B.M.-U.A.M.), Professor M. S. Patel (S.U.N.Y. at Buffalo, U.S.A.) and Professor I. V. Sandoval (C.B.M.-U.A.M.) for critical reading of the paper. Dr P. L. Pedersen and Dr. P. Cantatore are acknowledged for supply of cDNA probes. A. Franco and D. Jelenic are thanked for technical and secretarial assistance respectively. The work was supported by grants from Comunidad de Madrid (CAM 92/039) and Dirección General de Investigación Científica y Técnica (PB91-0032), Spain. We acknowledge an institutional grant from Fundación Ramón Areces.

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