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# RAPID POSTNATAL CHANGES IN $F_1$-ATPase PROTEINS AND IN THE UNCOUPLING PROTEIN IN BROWN ADIPOSE TISSUE MITOCHONDRIA OF THE NEWBORN RAT

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**Summary:** Changes in $F_1$-ATPase and UCP protein contents and in the activity of respiratory complexes I, II and IV of brown adipose tissue mitochondria are reported during the first 0-6 hours of life in the rat. Mitochondrial UCP/$F_1$-ATPase protein ratio is used to define the onset of thermogenic differentiation of brown adipose tissue mitochondria. It is concluded that mitochondrial differentiation occurs soon after birth and that the process is accelerated by hypothermic conditions. 

Postnatal development of mitochondrial functions is a key regulatory process for the adaptation to extrauterine life of the newborn mammal. The main physiological function of brown adipose tissue (BAT) in newborns is the generation of heat in a process called "non-shivering thermogenesis" (1,2). The specialized function of this tissue depends on a mitochondrial inner membrane protein of 32 KDa called the "uncoupling protein" (UCP) (1,3) or "thermogenine" (2,4). The role of the 32 KDa protein in heat production is to provide a short-circuit for the proton electrochemical gradient generated by the respiratory chain (1). This unique protein (measured as GDP-binding activity) of BAT mitochondria has been shown to increase during development in several species, in cold-adapted mammals and in several diet-induced states (for review see 5), most likely by increasing the rate of transcription of its gene (3).

In this report we have examined, using immunological methods, the postnatal increase of BAT mitochondria $F_1$-ATPase and

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Abbreviations used: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; e.l.i.s.a., enzyme-linked immunosorbent assay; BAT, Brown adipose tissue; UCP, Uncoupling protein.

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UCP proteins, in an effort to establish the timing of induction of the thermogenic capacity in BAT mitochondria. Further, since stimulation of brown adipocytes by sympathetic nerve endings is involved in postnatal development and adaptation to the cold of BAT (1,2,5), we have also investigated the effect of neonatal hypothermia (22ºC) in the early postnatal development of BAT mitochondria. The results indicate that differentiation of BAT mitochondria takes place immediately after birth depending on processes related with the synthesis of inner mitochondrial membrane proteins, and suggest that noradrenaline may be one among the mitochondriogenic signals for the tissues of the newborn rat.

## EXPERIMENTAL

Term newborns (5.2 ± 0.1 g) were obtained by rapid hysterectomy from cervically dislocated timed-pregnant rats (6) and rapidly placed in a humidicrib at either 37ºC (euthermic) or 22ºC (hypothermic) (7). Brown adipose tissue mitochondria were isolated (8) from the dissected intraescapular BAT from 0,2 and 6 postnatal h-old newborns and from adults. The protein concentration and mitochondrial NADH-dehydrogenase, succinate dehydrogenase and cytochrome c oxidase activities were assayed as reported previously (6). One unit of activity represents 1 $\mu$mol of product formed/min. The postnatal changes in $F_1$-ATPase protein content observed in BAT mitochondria were quantitated by e.l.i.s.a., using purified rat liver mitochondrial $F_1$-ATPase as standard and rabbit anti-[rat liver mitochondrial $F_1$-ATPase] serum (6). The e.l.i.s.a. standard curves showed linear regression correlation coefficients of $r=0.9817$, $p<0.001$.

BAT mitochondria UCP was estimated in Western blots (6), by using a sheep anti-[rat UCP] antibody generously provided by Dr. Ricquier (9). The antibodies bound to the filter were visualized by using a rabbit anti-sheep IgG peroxidase conjugate [RAS/IgG (H+L)/PO]. The intensity of the 32 KDa band was calculated in a Chromoscan densitometer and expressed as percent of adult values. An adult standard curve of 50-300$\mu$g of BAT mitochondria was simultaneously run in each gel. The linear regression correlation coefficient of this type of standard curve ($n=4$) was $r = 0.9583$, $p < 0.05$.

## RESULTS

Postnatal development of respiratory enzymes in BAT mitochondria of euthermic neonates takes place during the first 2-6 postnatal h (Table 1), a time in which the activities of complexes I and IV double those found at 0 h. Interestingly, complex II showed no changes throughout the time period studied (Table 1). However, when neonates were placed in a hypothermic condition (22ºC), the postnatal surge in both complexes I and IV was accelerated, showing a significant increase in activity at the second postnatal h (Table 1). A further stimulatory effect of

**Table 1.** Effect of postnatal hypothermia on the development of respiratory chain enzyme activities of brown adipose tissue mitochondria.

| Postnatal age (h) | Complex I (mU/mg) | | Complex II (mU/mg) | | Complex IV (U/mg) | |
|---|---|---|---|---|---|---|
| | euthermic | hypothermic | euthermic | hypothermic | euthermic | hypothermic |
| 0 | 18±3 | | 95±9 | | 1.1±0.1 | |
| 2 | 21±3 | $28\pm2^{*}$ | 98±8 | 96±8 | 1.1±0.1 | $2.0\pm0.1^{***,xxx}$ |
| 6 | $57\pm5^{tt}$ | $87\pm1^{*,x}$ | 97±15 | $141\pm13^{**,xx}$ | $2.1\pm0.2^{t}$ | 2.9±0.3 |

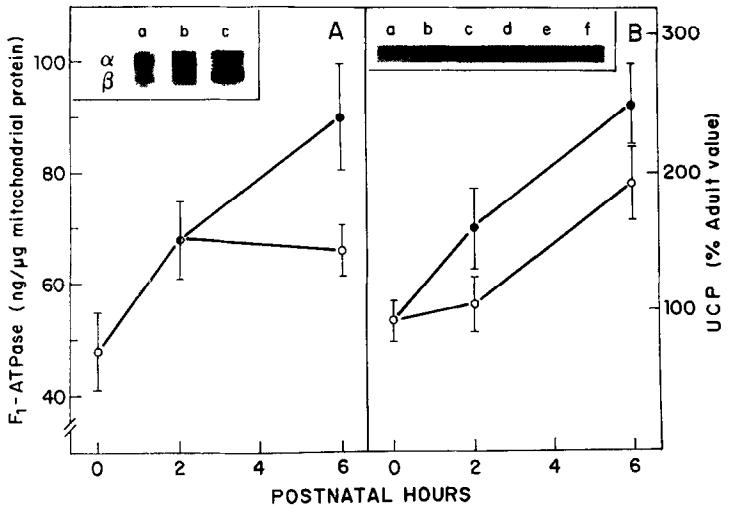
Term fetuses were delivered by rapid hysterectomy and were either placed in a humidicrib at 37ºC (euthermic) or 22ºC (hypothermic). At 0, 2 and 6 h after delivery they were sacrified and their BAT mitochondria isolated as described in the experimental section. The results shown are means ± SEM of 4-8 different preparations. t, $p<0.01$ and tt, $p<0.0005$ when comparing continouos euthermic time period samples; *, $p<0.05$; **, $p<0.025$ and ***, $p<0.005$ when comparing continouos hypothermic time period samples; x, $p<0.05$; xx, $p<0.025$ and xxx, $p<0.0025$ when comparing euthermic versus hypothermic age-matched samples by students t test.

hypothermia was observed in the activity of the three respiratory enzyme complexes at the sixth postnatal h (Table 1).

Western-blot analysis of BAT mitochondrial proteins using the rabbit-anti [rat liver mitochondrial $F_1$-ATPase] serum recognized the two major subunits, α and β, of the BAT $F_1$-ATPase complex Figure 1A (insert), with a similar migration profile as that shown by the liver complex (6). Figure 1A also shows that the amount of $F_1$-ATPase proteins in BAT mitochondria increased by about 50% ($p<0.05$) during the first 2 h of life in both euthermic and hypothermic neonates. At the sixth postnatal h and as shown above in Table 1 for the respiratory enzyme activities, neonatal hypothermic conditions promoted a further stimulatory effect ($p<0.05$) on the amount of $F_1$-ATPase found in BAT mitochondria (Fig. 1A).

The amount of UCP in BAT mitochondria of euthermic newborn rats showed a developmental profile (Fig. 1B) similar to that of the activities of complexes I and IV of the respiratory chain (Table 1). Although no changes in the relative amount of UCP could be observed during the first 2 h, a significant increase ($p<0.025$) in this protein was measured during the 2-6 h period. Similarly, when the neonates were placed at 22ºC (hypothermia), the amount of UCP showed significant postnatal increases at 2 and 6 h post partum ($p<0.05$ and $p<0.0025$, respectively).

Figure 2 represents the postnatal preferential enrichment of UCP protein versus $F_1$-ATPase proteins in mitochondria from hypothermic neonates. The mitochondrial UCP/$F_1$-ATPase protein ratio could be used as an index of BAT mitochondrial differentiation; hence, hypothermic conditions significantly



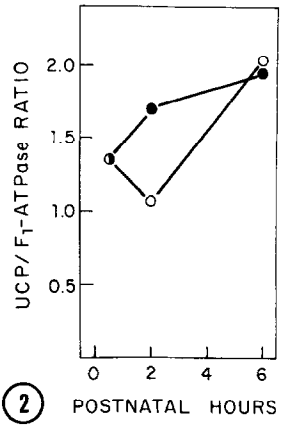




**Figure 1:** Development of BAT mitochondria $F_1$-ATPase and "uncoupling protein". Term fetuses were delivered and were either placed at 37ºC (euthermic) (o) or 22ºC (hypothermic) (●). At 0,2 and 6 h after delivery they were sacrified and their BAT mitochondria isolated as described in the experimental section. A: The amount of $F_1$-ATPase was quantitated by e.l.i.s.a. using pure rat liver mitochondria $F_1$-ATPase as standard and rabbit anti-[rat liver mitochondria $F_1$-ATPase] antibodies (6). The results shown are means ±S.E.M. of 4-10 different mitochondrial preparations assayed in duplicate. Insert: Rat liver (a) and BAT (b) mitochondria α and β $F_1$-ATPase proteins. 64μg of pure rat liver $F_1$-ATPase is also shown (c). 150μg of mitochondrial proteins were fractioned in 12% SDS-PAGE and transferred to PVDF membranes (Millipore, USA). They were probed with a 1:260 dilution of anti-$F_1$-ATPase serum, followed by $^{125}$I-labelled protein A and exposed for autoradiography. B: BAT mitochondrial proteins were fractioned in 9% SDS-PAGE and transferred to PVDF membranes. They were probed with a 1:100 dilution of sheep anti-rat UCP serum, followed by rabbit anti-sheep IgG peroxidase conjugate. The intensity of the 32KDa band was calculated and interpolated in the standard curve runned in each gel. The results are means ± S.E.M. of 5 different mitochondrial preparations. Insert: Western-blot of 150μg of mitochondrial proteins fractioned from BAT mitochondria of 0 h euthermic-(a); 2h euthermic-(b); 2h hypothermic-(c); 6h euthermic-(d); 6h hypothermic-(e) neonates and from adults-(f).

**Figure 2:** UCP/$F_1$-ATPase protein ratio in developing BAT mitochondria of the rat. This figure has been constructed using the protein content of UCP and $F_1$-ATPase proteins reported in Figures 2 and 1, respectively. The amount of UCP protein present in BAT mitochondria of neonatal rats was calculated from the values shown in Fig. 2 using previous radioimmunoassay estimates of the protein in BAT mitochondria of adult rats kept at 22ºC (69±9μg/mg mitochondrial protein) (10). UCP/$F_1$-ATPase ratio in neonates kept at 37ºC (o) and 22ºC (●) in the immediate postnatal period.

accelerated the process of mitochondrial differentiation showing a rapid postnatal onset after birth, while in BAT mitochondria of neonates held at thermoneutrality there was at least a 2h delay in its onset (Fig. 2). However, at the sixth postnatal h this

ratio was the same in both groups of neonates (Fig. 2), although the amounts of UCP and $F_1$-ATPase proteins present in BAT mitochondria of euthermic neonates were significantly lower (Fig. 1). The finding that at the sixth postnatal h the UCP/$F_1$-ATPase protein ratio was the same in both groups of neonates also holds when the amount of any of the two proteins is expressed relative to the activity of complexes I, II and IV of the respiratory chain (data not shown).

## DISCUSSION

The maintenance of newborn rats at two different temperatures, thermal neutrality (37ºC) and nest temperature (22ºC), a condition known to promote hypothermia and to enhance brown fat lipolysis in the immediate postnatal period of the newborn (7), have helped to define the time and conditions of natural onset of the thermogenic process and a putative mitochondriogenic signal for BAT. The results presented show for the first time, a rapid postnatal increase in UCP and $F_1$-ATPase (Fig. 1) proteins in BAT mitochondria of the newborn rat. The increase in UCP protein in the BAT of several mammalian species has been extensively documented (for review see 5), and has been used as a thermogenic index for defining the immature (hamster), altricial (rat) and precocial (guinea pig) patterns of development in mammals (4,11). By using the UCP/$F_1$-ATPase protein ratio as an index of thermogenic function, our results (Fig. 2) indicate that the thermogenic function of BAT mitochondria is triggered immediately after birth, especially when neonates were housed at 22ºC. It is important to point out that the UCP/$F_1$-ATPase protein ratio is a better index for estimating the thermogenic function of BAT mitochondria than the expression of UCP content alone (2,4,11,12), since increases in total GDP-binding activity of the tissue or its activity per mg mitochondrial protein, could mask concurrent enrichments of other proteins of the BAT mitochondria, as observed for the $F_1$-ATPase proteins (Fig. 1A) and for the activities of respiratory complexes (Table 1).

As occurs in newborn rat liver mitochondria (6), BAT mitochondria of both euthermic and hypothermic neonates significantly increase their $F_1$-ATPase content rapidly after birth (Fig. 1A), suggesting a common biological signal for the trigger of mitochondrial differentiation in both tissues. It has recently been demonstrated that UCP mRNA levels in BAT of developing rats reached its highest tissue content during the

first 6-10 h after birth (3). Unfortunately, no time-points between birth and the 6-10 h period were included nor indication of at which temperature the neonates were housed after delivery. UCP synthesis is known to be under noradrenergic control, in fact, the UCP gene is acutely (within 15 min) regulated at the level of transcription after activation of the plasma membrane β-adrenoreceptors of the brown adipocyte (3,13,14). Thus, it is reasonable to suggest that the rapid postnatal increase in UCP protein found in BAT mitochondria of neonates held at 22ºC is due to a rapid (first postnatal h) increase in the rates of transcription of the UCP gene. Stimulation of sympathetic nervous System activity of BAT is thought to be the major physiological regulator of the response of this tissue in animals exposed to a cold environment (3,13,14 and for reviews see 1,2 and 5), suggesting that the concurrent postnatal increase in $F_1$-ATPase proteins (Fig. 1A) and in the activity of the respiratory complexes (Table 1) observed in hypothermic neonates is a general mitochondriogenic response that shares the same physiological signal and probably the same mechanism of induction proposed for the UCP protein.

BAT mitochondrial differentiation in euthermic neonates shows at least a 2h delay on its postnatal onset (Figs. 1 and 2 and Table 1). This finding may reflect the mitochondriogenic response of the brown adipocyte to the same physiological signal triggered by circulating plasma noradrenaline (or catecholamines). Postnatal increases in these hormones have been reported to occur after the second postnatal h in euthermic newborn rat neonates (15, for review see 16).

In conclusion, the results reported herein show that postnatal mitochondrial differentiation in BAT occurs rapidly after birth. Furthermore, the UCP/$F_1$-ATPase protein ratio used as an index of the thermogenic mitochondria shows an immediate developmental pattern for the BAT of the newborn rat.

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