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Uncoupling protein in embryonic brown adipose tissue – existence of nonthermogenic and thermogenic mitochondria

Josef Houštěk, Jan Kopecký, Zdeněk Rychter and Tomáš Soukup

Institute of Physiology, Czechoslovak Academy of Sciences, Prague (Czechoslovakia)

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Embryonic development of mouse and rat brown adipose tissue was characterized by electron microscopy and by quantifying the mitochondrial oxidative, phosphorylating and thermogenic capacities immunochemically, using antibodies against cytochrome oxidase, F_1-ATPase and uncoupling protein, respectively. Mitochondria and cytochrome oxidase were detected from the 15–16th day of pregnancy and their amounts continuously increased toward birth. F_1-ATPase was also found on the 15th day but it reached a maximum level already on the 19th day when the uncoupling protein appeared and rapidly increased during further maturation of brown adipose tissue. It thus appears that mitochondria in early prenatal brown adipose tissue lack completely uncoupling protein and are nonthermogenic. They transform into typical thermogenic mitochondria abruptly only 2 days before birth.

Introduction

Thermogenesis in brown adipose tissue results from physiological H^+-shortcircuiting of mitochondrial membrane [1], due to the unique, tissue-specific and regulatable H^+ channel, the so-called uncoupling protein [2]. In contrast to other tissues, where the redox energy is mostly used for ATP synthesis, in brown adipose tissue mitochondria the oxidation of substrates is highly exothermic being naturally uncoupled from phosphorylation [1].

While the thermogenic function [1] of brown adipose tissue during postnatal development and during adaptation to changing thermal environment and diet has been extensively studied and is well documented (for references, see Refs. 3 and 4), little is still known about the energetics of brown adipose tissue during prenatal development [5], when the thermal homeostasis of the fetus is fully secured by the mother organism. We studied here embryonic mouse and rat brown adipose tissue using both electron microscopy and immunochemistry which enabled us to assess the profile and changes of mitochondrial energy conversion. The results show that typical thermogenic, physiologically competent, mitochondria appear both in mouse and rat only two days before birth.

A part of this work was already published as a symposium abstract [6].

Materials and Methods

Interscapular brown adipose tissue was obtained from fetal mice (Balb C) and rats (Wistar I pav) at indicated times after mating. For electron

Abbreviations: SDS, sodium dodecyl sulfate; F_1, catalytic part of mitochondrial H^+-translocating ATP synthase.

Correspondence: J. Houštěk, Institute of Physiology, Czechoslovak Academy of Sciences, Vídeňská 1083, 142 20 Prague, Czechoslovakia.

microscopy, the tissue was fixed in 1% paraformaldehyde and 1% glutaraldehyde in 0.12 M phosphate buffer (pH 7.3), and then postfixed in 2% osmium tetroxide, dehydrated and embedded in Durcupan (Fluka). Sections cut on a Reichert OMU 3 ultramicrotome were stained with 1% uranylacetate and 0.1% lead citrate and then examined in JEM 100 B electrone microscope.

For detection of the mitochondrial antigens, brown adipose tissue was homogenized in 0.25 M sucrose, 10 mM Tris-HCl, 1 mM EDTA (pH 7.4). Aliquots (50 μg protein) of pooled samples from 9–12 animals of each age group were analyzed by SDS-polyacrylamide gel electrophoresis [7] on slabs of 14–25% linear gradient of polyacrylamide. For determination of apparent molecular weight the following standards (Bio-Rad) were used: phosphorylase B, 92.5 kDa; bovine serum albumin, 66.2 kDa; ovalbumine, 45 kDa; carbonic anhydrase, 31 kDa; soybean trypsin inhibitor, 21.5 kDa; and lysozyme, 14.4 kDa. After electrotransfer [8] to nitrocellulose (NCM; pore size, 0.45 μm; Schleicher-Schuell) proteins were immunodecorated [9] using specific antisera raised in rabbits [10,11] against the hamster uncoupling protein isolated as in Ref. 2, rat heart cytochrome oxidase [11] and bovine heart F_1-ATPase (F_1 [10]). Immune complexes were detected by peroxidase-labelled second antibody ([9], pig anti-rabbit, SWAR-Px; from USOL, Prague) and the peroxidase reaction was performed with 9-aminoethylcarbazole as substrate. Immunodecorated nitrocellulose sheets were scanned in reflected light (600 nm; Shimadzu TLC Chromatoscanner CS-930). In some cases also isolated mitochondria from rat liver [12] and hamster brown adipose tissue [13] were used.

Protein was measured by the method of Lowry et al. [14] with bovine serum albumin as standard.

Results and Discussion

The differentiation of mouse and rat brown adipose tissue was followed from the 15–16th day of fetal development, which was the first time when interscapular brown adipose tissue could be macroscopically identified and dissected. As shown in Fig. 1, the amount (wet weight) of interscapular brown adipose tissue in mouse increases between

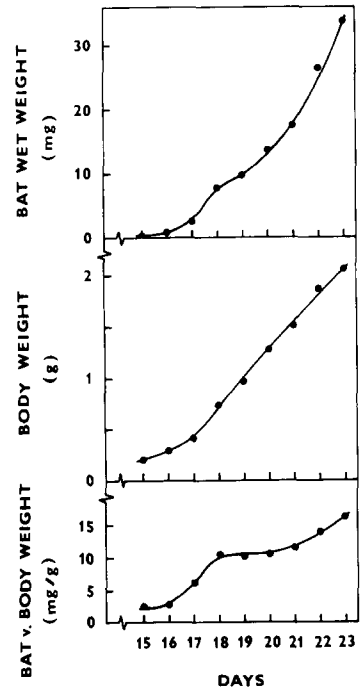

Fig. 1. Growth and development of interscapular brown adipose tissue (BAT) in the embryonic mouse. The data are the mean value from 10–12 animals in each age group (days after mating).

the 15th and 21th day (birth) after mating from 0.5 to 17.5 mg, i.e., more than 30 times. During this period the relative contribution of interscapular brown adipose tissue to the total body weight increases from 2.7 to 11.9 mg per g, with the most pronounced change occurring between the 15th and 18th day. Analogous pattern of embryonic development was found in the rat.

As shown in Fig. 2, similarly as described in the rat [15], the adipocytes from 16-day-old mouse fetuses had a polygonal shape and contained first few differentiating mitochondria and small lipid depots. During successive differentiation and maturation (Fig. 2) the size of adipocytes, the number and size of mitochondria and of lipid inclusions quickly increased, the most remarkable change occurring between the 17th and 18th day (Fig. 2B and C).

The structural complexity of mitochondria developed in parallel (Fig. 2). The typical brown adipose tissue mitochondria were, when longitudinally sectioned, ovoid in shape with parallel,

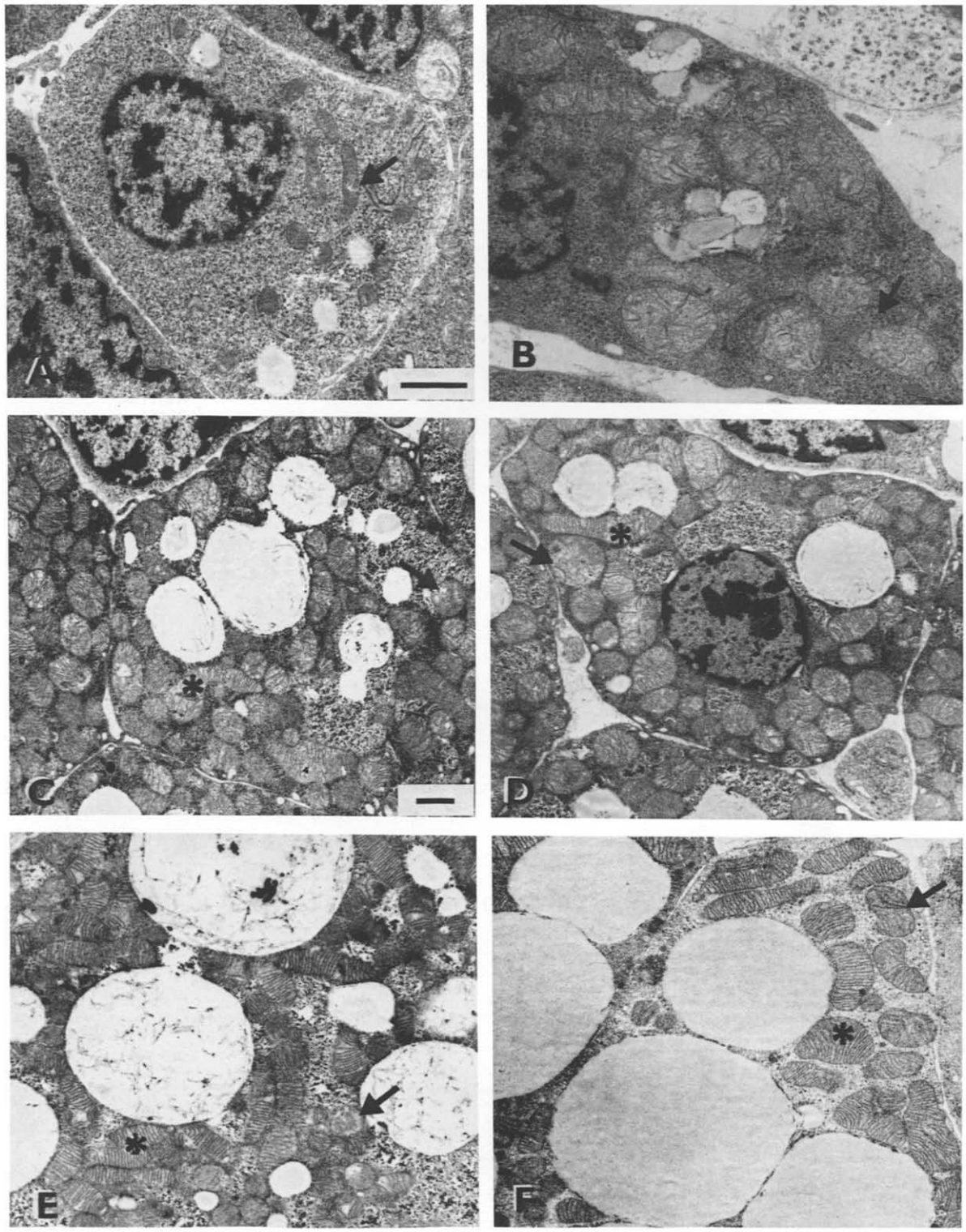

Fig. 2. Electron microscopy of mouse embryonic brown adipose tissue (days after mating: A, 16; B, 17; C, 18; D, 19; E, 20; F, 21). Typical mature mitochondria (with numerous parallel cristae) are indicated by asterisks and differentiating mitochondria (only few and irregularly arranged cristae) by arrowheads. Magnification was $1.1 \cdot 10^4$ times (A, B) or $6 \cdot 10^3$ times (C–F). Bars indicate 1 μm.

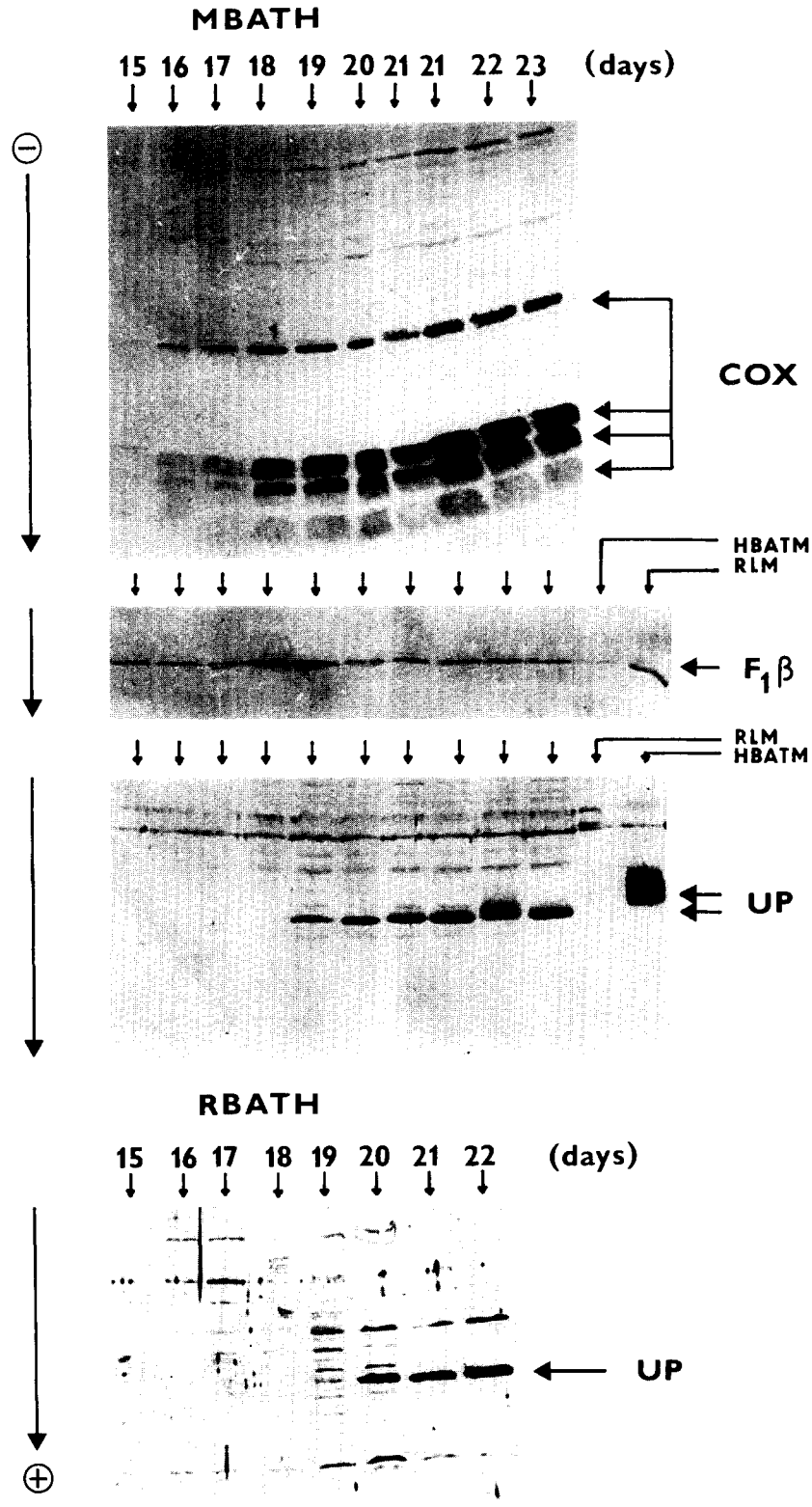

Fig. 3. Immunochemical detection of cytochrome oxidase (COX) subunits (arrows from top to bottom indicate subunits II (27 kDa), IV (17 kDa), V (12 kDa) and VI (9 kDa)), β-subunit of F_1-ATPase ($F_1\beta$; 48 kDa) and uncoupling protein (UP; 32 kDa) in homogenates of brown adipose tissue (50 μg protein aliquots) from mouse (MBATH) and rat (RBATH) of indicated age after mating. Where indicated, mitochondria (10 μg protein aliquots) from adult hamster brown adipose tissue (HBATM) or rat liver (RLM) were analyzed.

densely packed cristae, while the differentiating mitochondria contained no cristae or only very few. The 'mature' mitochondria (more than three parallel cristae), which were practically absent on the 16th day, represented 50–60% on the 19th day. Nevertheless, even postnatally, 10–20% of the mitochondria contained randomly and loosely packed cristae (in each group $n = 100$).

During the early stage of embryonic development the miligram quantities of brown adipose tissue (see Fig. 1) are too low to isolate mitochondria and measure the activity of mitochondrial enzymes and transport systems directly. Therefore, to assess the functional equipment of brown adipose tissue by the key enzymes of mitochondrial energy conversion, an immunochemical approach was used. Homogenates of embryonic interscapular brown adipose tissue were assayed for the content of cytochrome oxidase, F_1-ATPase (F_1) and uncoupling protein, using specific antibodies. Although mitochondrial antigens of different origin were used for immunization, all the antisera cross-reacted well with both mouse and rat brown adipose tissue antigens (Fig. 3). The only difference was found with uncoupling protein where the electrophoretic mobility of mouse uncoupling protein was somewhat higher than that of hamster uncoupling protein, indicating that mouse uncoupling protein differs by about 0.5–1.0 kDa from hamster uncoupling protein, similarly to rat [16] and human [17] uncoupling protein.

Both Fig. 3 and quantification of immunoblotting experiments in Fig. 4 indicate that the three enzymes develop differently during embryogenesis. Cytochrome oxidase was found already on the 15–16th day and, in agreement with morphological data (see Fig. 2), its amount then continuously increased, resulting in almost a 16-fold increase between the 16th and 21th day. The proportions between individual subunits of cytochrome oxidase, however, did not change during development. F_1 was detected mainly as the β-subunit antigen, which exhibits the highest degree of homology among F_1 subunits from various organisms [18]. F_1 was also found on the 15–16th day, but in contrast to cytochrome oxidase, its amount increased between the 16th and the 21st day only 2-fold (Figs. 3 and 4) and the maximum level was

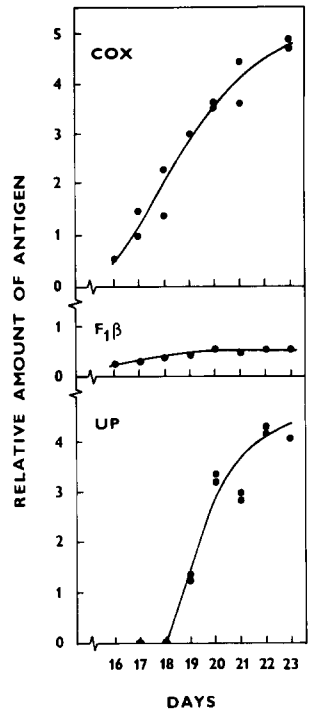

Fig. 4. Quantitative evaluation of cytochrome oxidase (COX), β-subunit of F_1-ATPase ($F_1\beta$) and uncoupling protein (UP) in mouse embryonic brown adipose tissue. Densitometry of immunoblots was performed as described in Materials and Methods.

reached already around the 19th day. Analogous results were obtained using rat brown adipose tissue (not shown). In contrast to both cytochrome oxidase and F_1, uncoupling protein was first detected in mouse on the 19th day and in rat on the 19–20th day, respectively. Its amount rapidly increased in the following days with the rate of increase that was the highest of all antigens tested.

Appearance of the three enzymes reflecting oxidative (cytochrome oxidase), phosphorylating (F_1) and thermogenic (uncoupling protein) capacities of the tissue at different times of embryogenesis, as well as their strikingly different quantitative development, indicate a clear asynchrony in the regulation of their synthesis. While the synthesis of cytochrome oxidase, and to a lesser extent of F_1, paralleled the changes in the number of mitochondria observed in the first part of the period examined, uncoupling protein appeared in both species suddenly only two days before birth, when the F_1 content leveled off. It is noteworthy,

however, that the marked increase of the total number of mitochondria per cell thus preceded by one day the appearance of uncoupling protein.

The observed developmental changes agree with the specific ratio between cytochrome oxidase and ATPase in postnatal brown adipose tissue, which is one order of magnitude higher than in other 'nonthermogenic' tissues, due to a physiologically low content of ATPase in brown adipose tissue mitochondria [19,20]. The abrupt beginning of uncoupling protein synthesis in mouse and rat is also in good agreement with the rapid increase of mRNA for uncoupling protein found in rabbits shortly before birth [21] while in bovine brown adipose tissue, uncoupling protein occurs already at the beginning of the last third of intra-uterine life [22].

The regulation of uncoupling protein synthesis involves both the α_1 and β-adrenergic receptors [23]. Accordingly, functional adrenergic receptors appear in rat brown adipose tissue 1–2 days before birth [24]. Similarly, sympathetic nerve endings appear in brown adipose tissue late in neonatal development [25]. However, the control of brown adipose tissue differentiation is likely to be a more complex event [5], where participation of, e.g., thyroid hormones may be expected [26]. In adult rat intracellular production of triiodothyronine by 5′-deiodinase of thyroxine was shown to be required for optimal thermogenic function of brown adipose tissue [27] and for cold-induced synthesis of uncoupling protein [28]. Importantly, recent studies show high activity of 5′-deiodinase in rat brown adipose tissue two days before birth [29] and dependence of prenatal expression of uncoupling protein gene on thyroid hormones [30].

In conclusion, the immunochemically estimated amounts of mitochondrial antigens which are related to the capacities of the key enzymes of mitochondrial energy conversion in mouse and rat brown adipose tissue during embryogenesis indicate the following.

(1) The basic pattern of brown adipose tissue energetics dramatically changes during embryogenesis and mitochondria before the 19th day are unable to produce heat by dissipating the electrochemical proton gradient via uncoupling protein. They do not differ from mitochondria of other phosphorylating tissue and have to be considered as 'nonthermogenic'.

(2) Transformation of brown adipose tissue energetics into the thermogenic mode occurs two days before birth by switching on the synthesis of uncoupling protein and by decreasing ATPase synthesis. Mitochondria thus quickly develop the properties found in thermogenically active brown adipose tissue after birth and can be considered as 'thermogenic'.

As the rapid qualitative changes of energy conversion in brown adipose tissue are accompanied by a significant increase of the total number and structural complexity of mitochondria, the question arises whether mitochondria existing in brown adipose tissue before the 19th day remain unchanged on subsequent, while the newly appearing mitochondria become thermogenic. In fact, two types of mitochondria [31] were found in tissue cultures of brown adipose tissue, occurring during cell differentiation which could represent the nonthermogenic and thermogenic mitochondria described above. Alternatively, changes in the equipment of mitochondrial membranes could include all the mitochondria present, their reconstruction being made possible by balanced and differentiated synthesis and degradation of individual mitochondrial enzymes.

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