Sequential changes in the expression of mitochondrial protein mRNA during the development of brown adipose tissue in bovine and ovine species

Sudden occurrence of uncoupling protein mRNA during embryogenesis and its disappearance after birth

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Samples of adipose tissue were obtained from different sites in bovine and ovine fetuses and newborns. RNA was isolated and analysed using bovine cDNA and ovine genomic probe for uncoupling protein (UCP), cDNA for subunits III and IV of cytochrome *c* oxidase and cDNA for ADP/ATP carrier. UCP mRNA was characterized for the first time in foetal bovine and ovine adipose tissue. It appeared later than mRNA of cytochrome *c* oxidase subunit III, and increased dramatically at birth (10-fold). ADP/ATP carrier mRNA was expressed at a lower level but also increased 10-fold at birth. It was demonstrated that UCP mRNA reached its highest level at birth in all bovine adipose tissues studied, except subcutaneous tissue. It disappeared quickly afterwards, being no longer detectable two days after birth. Similar variations were observed in newborn lambs. ADP/ATP carrier mRNA showed the same pattern of expression as UCP mRNA; although it was still lightly expressed two days after birth, it disappeared soon afterwards. Only mRNAs for cytochrome *c* oxidase subunits III and IV remained at the same level during the first postnatal week. On the basis of these data and of observations reported in the literature a sequence of events for the development of brown adipose cells *in vivo* is proposed. Soon after birth the perirenal adipose tissue of ruminants, which still contains mitochondria of typical brown adipose tissue morphology and high levels of cytochrome *c* oxidase mRNA, lacks UCP mRNA. Can it still be considered as brown fat? Ruminant species appear to be attractive models to study both the differentiation of brown adipose tissue and its possible conversion to white fat in large animals.

INTRODUCTION

Most studies on brown adipose tissue function have been done in rodents. In these animals brown adipose tissue obviously plays a significant role in metabolic thermogenesis; a failure in its thermogenic function could contribute to the increase of white adipose tissue mass (see reviews by Girardier, 1983; Himms-Hagen, 1984; Nicholls & Locke, 1984; Cannon & Nedergaard, 1985; Rothwell & Stock, 1985). In larger animals the biological significance of brown adipose tissue is not yet established and is a matter of dispute. For a long time it was accepted that these mammals had no significant amount of functional brown adipose tissue. However, around 1970, several authors described brown adipose tissue in infant and adult humans (reviewed by Lean *et al.*, 1986a), newborn goats (Thompson & McEwan Jenkinson, 1970), newborn lambs (Gemmell *et al.*, 1972; Cannon *et al.*, 1977), newborn calves (Alexander *et al.*, 1975) and monkeys (Chaffee *et al.*, 1970). More generally, brown adipose tissue has been described in most mammals (Smith & Horwitz, 1969; Rowlett *et al.*, 1971).

However, until recently, all these observations were based on histology of adipose tissue samples. Cannon *et al.* (1977) have also biochemically characterized brown adipose tissue in newborn lamb.

Since the uncoupling protein (UCP) has been shown to be not only a key enzyme in the thermogenic process, but also a highly specific component of brown adipocytes, its detection is considered to be an excellent criterion for the unequivocal characterization of such cells. UCP has been detected in foetal and adult monkeys (Strielemann *et al.*, 1985; Swick *et al.*, 1986), in dogs (Holloway *et al.*, 1985) and also in humans (Ricquier *et al.*, 1982; Bouillaud *et al.*, 1983; Lean & James, 1983; Cunningham *et al.*, 1985; Lean *et al.*, 1986a,b).

More recently, typical brown adipose tissue containing the UCP was characterized in bovine fetus and newborn calf (Casteilla *et al.*, 1987). In this study the UCP was detected using a GDP-binding titration, photo-affinity labelling with 8-azido-ATP and immunoblotting. This work led to the conclusion that young lambs and calves were highly interesting animals in which to study the possible conversion of brown adipose tissue into white

Abbreviation used: UCP, uncoupling protein.

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adipose tissue. The study of brown adipose tissue development in fetuses and young bovines and ovines is reported here. This work was made possible by the use of a cDNA for the bovine UCP and a genomic probe for ovine UCP that we recently isolated and sequenced (L. Casteilla, F. Bouillaud, C. Forest & D. Ricquier, unpublished work). We also used different probes coding for ADP/ATP carrier (pAAC9, Rasmussen & Wohlrab, 1986), and subunits III and IV of cytochrome *c* oxidase (COX III, Fink *et al.*, 1987; COX IV, Lomax, *et al.*, 1984).

EXPERIMENTAL

Materials

[α - 32 P]Deoxycytidine triphosphate was obtained from Amersham Corp. Deoxyribonuclease I, DNA polymerase I, Klenow fragments and restriction endonucleases were obtained from Appligene, Illkirch, France; Bethesda Research Laboratories or Promega Biotech, Madison, Wisconsin. Oligo(dT)-cellulose was obtained from Boehringer Mannheim and nylon membranes (Hybond N) were from Amersham Corp.

Animals and tissue sampling

Bovine (Friesian) fetuses were obtained after slaughter of dairy cows. All Friesian calves after birth were males. The ovine breed was Ile de France $\times$ Romanov $\times$ Limousin.

Subcutaneous, perirenal, pericardiac, peritoneal, retroperitoneal, mesenteric and intermuscular tissues were collected for calves, and subcutaneous, perirenal, pericardiac, intermuscular and peritoneal tissues for lambs. The tissues were dissected, frozen immediately in liquid N₂ and stored at -80°C until RNA extraction.

RNA extraction and Northern-blot analysis

Frozen tissues were powdered in liquid N₂ and RNA was obtained from a sample by phenol extraction according to Brawerman (1973). The lysis buffer was 10 mM-sodium acetate/5 M-NaCl/0.5% SDS/50 mM-aurintricarboxylic acid. Poly(A⁺) RNA was purified using chromatography on oligo(dT)-cellulose, as described by Aviv & Leder (1972). RNA samples were electrophoresed in agarose gels containing formaldehyde and then transferred on to nylon membranes. cDNA probes were 32 P-labelled using either nick-translation or the random priming method. Hybridization of RNA was done at 42°C in the presence of 50% formamide (v/v) as in Ricquier *et al.* (1986). The blots were washed [2 $\times$ 30 min at 65°C in 2 $\times$ SSC/0.1% SDS and then 2 $\times$ 10 min at 65°C in 0.1 $\times$ SSC/0.1% SDS (1 $\times$ SSC = 3 M-NaCl, 0.3 M-sodium citrate)]. Autoradiographs were scanned for densitometric analysis using an LKB spectrophotometer.

UCP probes

Molecular probes specific for bovine and ovine UCP were used. These probes were isolated and sequenced in the laboratory (L. Casteilla, F. Bouillaud, C. Forest and D. Ricquier, unpublished work). The bovine UCP probe is a 1.4 kb cDNA covering the coding region of UCP mRNA except for nucleotides corresponding to the first 14 N-terminal amino acids. This bovine UCP cDNA was isolated from a cDNA library constructed from mRNA purified from newborn calf brown adipose tissue.

The ovine UCP probe is a 0.7 kb genomic fragment which contains exons III and IV. This ovine UCP genomic fragment was isolated from a partial ovine genomic library. Clones corresponding to bovine and ovine UCP probes were detected by hybridization to rat UCP cDNA and human genomic fragment. Moreover, the identity of bovine and ovine probes was confirmed through sequencing.

RESULTS

Prenatal expression of UCP mRNA in perirenal adipose tissue

In bovine fetuses the largest single depot of adipose tissue was located around the kidneys. It represented about 4–5% of the foetal weight. As reported by Casteilla *et al.* (1987) the appearance of this tissue was somewhat different from that of typical rodent brown adipose tissue.

Northern-blot analysis was performed with 10 μg of RNA extracted from perirenal adipose tissue of bovine fetuses at different stages of gestation (Table 1). Hybridizations were performed with cDNA for bovine UCP, and with two probes detecting mRNA coding for mitochondrial proteins: subunit III of cytochrome *c* oxidase coded for by the mitochondrial genome (Fink *et al.*, 1987) and ADP/ATP carrier coded for by the nuclear genome (Rasmussen & Wohlrab, 1986) (Fig. 1a). The two molecular probes for subunit III of cytochrome *c* oxidase and UCP each detected one major band of mRNA at 0.5 and 1.9 kb respectively, and a minor band at higher molecular masses. These small bands may correspond to incompletely-processed mRNA. The

Table 1. Characteristics of bovine fetuses and tissue samples

Values reported for gestational age were deduced from the date of dairy cow insemination. In this species, the length of gestation is about 9 months. Birth weight was estimated as about 42 kg for FFPN breed (Robelin *et al.*, 1984). nd, not determined.

Foetus	Presumed gestational age (days)	Sex	Weight	
			(kg)	(% of birth wt.)
a	81	F	0.11	0.25
b	86	M	0.16	0.39
c	89	F	0.15	0.36
d ₁	103	nd	0.38	0.90
e	115	F	0.73	1.74
f	157	F	3.86	9.2
g	174	F	6.20	14.8
h	177	M	8.32	19.8
i	184	F	7.50	17.9
j	193	F	9.92	23.6
k	211	F	16.1	38.3
l	213	M	19.0	45.2
m	234	F	22.4	53.3
m ^o	234	M	24.6	58.6
n	244	M	27.9	66.5
o	250	F	26.1	62.2
o ⁺	250	F	28.9	68.7
p	259	M	40.8	97.2
q	266	F	45.1	107.0
q ^o	266	F	40.0	95.2

Fig. 1. Emergence and evolution of UCP mRNA during foetal life in bovine species, comparison with other mitochondrial markers

RNA was obtained as described in the Experimental section from perirenal adipose tissue of several foetuses identified in Table 1. (a) Northern blots were performed with 10 μ g of total RNA and hybridized successively with the different probes: uncoupling protein (UCP), cytochrome *c* oxidase subunit III (COX III) and ADP/ATP carrier (AAC). Letters above the Figure refer to the identification of the foetuses (see Table 1). Subscripts ₁ and ₂ correspond respectively to the pericardiac and perirenal tissues of the same animal. Numbers below the Figure refer to days of pregnancy. (b) Quantification of the Northern blots in (a). The results are expressed in % of the value at birth for the different mRNAs: UCP (●); COX III (□); AAC (○).

molecular probe for the ADP/ATP carrier revealed two different mRNAs (1.2 and 1.5 kb) according to Rasmussen & Wohlrab (1986). Quantification of the Northern blot of Fig. 1(a) is presented in Fig. 1(b).

As revealed by hybridization with bovine UCP cDNA, the mRNA encoding for UCP was not detectable before day 211 of pregnancy (Fig. 1a, line k). The difference observed between lines k and l, although these samples were from foetuses of the same age (211 and 213 days respectively), could be explained by some disparity of

development related to the sex of the animal (k was female; l was male). In a previous study (L. Casteilla, F. Bouillaud, C. Forest & D. Ricquier, unpublished work) we detected UCP in bovine foetuses at day 190 of gestation. This apparent discrepancy could be due to individual variability.

UCP mRNA level remained unchanged until day 259 when it rose suddenly. A 10-fold increase was observed at birth (Figs. 1a and 1b).

To make sure that UCP mRNA was not expressed

Fig. 2. Rapid fall of UCP mRNA compared with other mitochondrial marker mRNA in different adipose tissues of calves

RNA was extracted from subcutaneous (lanes 1); intermuscular (lanes 2); mesenteric (lanes 3); peritoneal (lanes 4); pericardiac (lanes 5); and perirenal (lanes 6) adipose tissues. RNA (10 μ g per line) was deposited except for line 7, which always corresponds to 5 μ g of RNA from perirenal fat of newborn calf. The age of the animals is indicated above the autoradiographs. For abbreviations see legend of Fig. 1.

before day 210 of gestation, Northern-blot analysis was performed with poly(A⁺) RNA extracted from 200 μ g of total RNA to increase the sensitivity of the method. No UCP mRNA could be detected in poly(A⁺) RNA from foetal perirenal tissue at 177, 184 and 193 days of gestation (results not shown).

Cytochrome *c* oxidase subunit III mRNA appeared much earlier than UCP mRNA, being already detected at day 80 of gestation (Figs. 1*a* and 1*b*). Its level rose at mid-gestation, to reach a peak at day 184, followed by a

rapid fall of 50 %. The level of this mRNA then remained unchanged until birth, when it increased 2-fold. ADP/ATP carrier mRNA was present in foetal adipose tissue at day 211. Its level remained constant until day 266, and then increased suddenly, as sharply as UCP mRNA, at birth (about 10-fold).

To achieve this study of the expression of UCP mRNA during foetal life in ruminants, several ovine foetuses were killed just before birth (–2 days) and samples of their adipose tissue were isolated. A Northern-blot

Fig. 3. Kinetics of disappearance of UCP mRNA in ovine species during postnatal life

The samples correspond to different adipose tissues of newborn, 1-day-old and 6-day-old lambs. RNA was isolated from pericardiac (lane 1); peritoneal (lanes 2); intermuscular (lanes 3) and perirenal (lanes 4) adipose tissues.

analysis revealed that UCP mRNA was present in the perirenal adipose tissue, and also in the pericardiac depot, although at a lower level (results not shown).

Postnatal expression of UCP mRNA

In the newborn calf several adipose depots are present: subcutaneous, intermuscular, mesenteric, peritoneal, pericardiac and perirenal. Samples were isolated from all these tissues and RNA was extracted as described in the Experimental section.

Northern-blot analysis was performed with 10 μ g of RNA from the different adipose depots of a newborn calf and of animals at 3, 5.5, 8, 15 and 38 days after birth. The same three molecular probes as before were hybridized to the blots; a fourth probe was added coding for subunit IV of cytochrome *c* oxidase (Lomax *et al.*, 1984). This subunit, contrary to subunit III, is transcribed from a nuclear DNA. Cytochrome *c* oxidase subunit IV cDNA revealed a single band on Northern blots.

UCP mRNA is present at birth in all but subcutaneous bovine adipose tissues (Fig. 2). It is highly expressed in perirenal tissue (PR). Its level is also high in pericardiac and intramuscular depots (about 50% of PR), lower in peritoneal (15% of PR), and hardly detectable, though present, in mesenteric tissue. The extraction yield of

mesenteric RNA per weight of wet tissue was very high, suggesting that adipose cells were contaminated with fibroblasts, which are much richer in RNA but deprived of UCP mRNA; this could explain the very low level of this mRNA in mesenteric adipose tissue.

At birth the other mRNAs studied presented the same distribution as UCP mRNA, as shown in Fig. 2. It is interesting to note that the two mRNAs coding for cytochrome *c* oxidase subunits are expressed at different levels: subunit III mRNA is much more abundant than subunit IV mRNA. The level of ADP/ATP carrier mRNA is particularly high in pericardiac adipose tissue, which may be due to some contamination with cardiac muscular tissue.

Unexpectedly UCP mRNA was no longer detectable two days after birth in any of the calf adipose tissues. ADP/ATP carrier mRNA was still present, although at a much lower level than at birth. Only mRNAs for cytochrome *c* oxidase subunits were still expressed at the same level as before (Fig. 2).

A newborn lamb and two lambs aged 1 and 6 days respectively were also used in this study. Northern-blot analysis of RNA extracted from different adipose tissues is shown in Fig. 3. Hybridization was with a genomic probe for ovine UCP (L. Casteilla, F. Bouillaud, C. Forest & D. Ricquier, unpublished work). As for bovine UCP, ovine UCP mRNA was highly expressed at birth in peritoneal, intermuscular and perirenal depots, but could hardly be detected 6 days later.

It was important to measure if the total RNA deposited on Northern blots contained the same quantity of poly(A⁺), regardless of the tissue concerned and the age of the animal. Therefore, we made a dot-blot analysis of total RNA samples from the different adipose tissues. This blot was hybridized with a [³²P]poly(dT) probe. The signals obtained confirmed that there was no variation among the different samples.

To detect mRNA more accurately, bovine poly(A⁺) RNA was isolated and analysed on Northern blot. Quantification was made by scanning the autoradiographs. The results are shown in Fig. 4. It should be

Fig. 4. UCP mRNA detection in calves during the first week after birth

The animals were slaughtered in autumn (☆) or in winter (★). Poly(A⁺) RNA corresponds to 200 μ g of RNA isolated from perirenal (PR), pericardiac (PC), intermuscular (IM) and peritoneal (PER) adipose tissues. Quantification was made through scanning of a Northern blot. The results are expressed in % of the value obtained with 2 μ g of total RNA from perirenal tissue at birth.

pointed out that the first bar of the diagram corresponds to 2 μ g of total RNA, while the other bars correspond to poly(A⁺) RNA extracted from 200 μ g of total RNA. Some UCP mRNA was detected at 2 days of age in bovine perirenal, pericardiac and intermuscular adipose tissues. At 8 days UCP mRNA was barely detectable in any of the tissues studied.

Unexpectedly, UCP mRNA was present at a rather high level in perirenal and pericardiac adipose tissues of a 15-day-old calf and in the perirenal depot of a 38-day-old calf. These results could be explained by the fact that these two animals were slaughtered in winter.

DISCUSSION

Differentiation of brown adipose tissue *in vivo*: occurrence of UCP mRNA and other mitochondrial markers

Despite the importance of brown adipose tissue in the regulation of thermogenesis and energy expenditure in both newborn and adult mammals, studies on the functional ontogenesis of this tissue were not undertaken until recently because of a lack of appropriate tools.

Most of the investigations on large animals have used ovine species and have dealt primarily with quantitative and ultrastructural development (Gemmell & Alexander, 1978). No direct proof in favour of the existence of brown adipose tissue in ruminants during foetal life was reported until recently, when biochemical approaches were made by different groups (Klein *et al.*, 1983; Wu *et al.*, 1986; Casteilla *et al.*, 1987).

To achieve further progress in these studies we chose a molecular approach. We isolated molecular probes for bovine and ovine UCP and used them in association with existing probes for other mitochondrial proteins. The present work represents the first characterization of UCP mRNA and its ontogenic changes in brown adipose tissue during foetal life in bovine and ovine species.

The results obtained with cytochrome *c* oxidase subunit III cDNA showed that the gene coding for this protein was transcribed early in perirenal tissue of bovine foetuses (first trimester of gestation). This corresponds with the appearance of differentiated adipose tissue, as reported by Broad & Davies (1980) for ovine species, and with the histological studies made in ovine species by Gemmell & Alexander (1978). The peak in level of subunit III mRNA observed in the second half of gestation could be related to the new mitochondriogenesis described by the latter authors.

UCP mRNA occurred later, and suddenly, at the beginning of the last trimester of gestation. Thus from this date on, the perirenal adipose tissue can be identified as brown fat in bovine foetus. This ability to develop a capacity for thermogenesis *in utero* is very surprising. At this stage of gestation nerves are not visible between the adipose cells (Gemmell & Alexander, 1978). Moreover, a chemical sympathectomy by treatment of the foetuses with 6-hydroxydopamine does not prevent the appearance of histological characteristics of brown fat (Alexander & Stevens, 1980).

On the basis of these results and of observations made in rodents (Nechad & Olson, 1983; Freeman & Patel, 1984; Nnodim & Lever, 1985; Houstek *et al.*, 1986) we propose the following sequence of events in the development of brown adipose cells *in vivo*: proliferation

of mitochondria of typical 'brown adipose tissue' morphology; expression of UCP mRNA; innervation of adipose cells.

An intact sympathetic innervation would not be necessary for the normal development of brown adipose tissue *in utero*, as was pointed out by Alexander & Stevens (1980) and confirmed by Nechad & Olson (1983). Noradrenaline would not be necessary for UCP gene induction, but would modulate its level of transcription (Ricquier *et al.*, 1986) and the rate of mitochondriogenesis (Nechad *et al.*, 1987).

As reported by Wu *et al.* (1986), thyroxine 5'-deiodinase increases progressively in foetal-lamb brown adipose tissue during the last trimester of gestation. This enzyme is indeed a marker of brown adipose tissue and is considered by some authors (Bianco & Silva, 1987) as necessary for the transcription of the UCP gene. It would therefore be interesting to know at which stage of the development of bovine foetal brown adipose tissue thyroxine 5'-deiodinase activity appears.

Importance of brown adipose tissue at birth and its postnatal changes

Teleologically, in precocial animals, such as calf and lamb, one would expect that the capacity for non-shivering thermogenesis should peak at birth to cope with the potentially lethal situation of exposure to maximal heat loss at birth.

According to our results, this seems to be the case. The expression of UCP mRNA was specifically highly stimulated in bovine and ovine newborns, and all the adipose depots were affected, except the subcutaneous one. The ratio of UCP mRNA/cytochrome *c* oxidase subunit III mRNA (calculated from scanning the autoradiographs) was highest in perirenal adipose tissue, indicating a peculiar enrichment in UCP of this depot, which is the earliest and the most specific brown adipose tissue in bovine and ovine species.

In ruminants brown adipose tissue undergoes morphological changes during postnatal life and is progressively transformed into white adipocytes (Gemmell *et al.*, 1972; Alexander *et al.*, 1975; Cannon *et al.*, 1977). These morphological observations were confirmed by biochemical results (Casteilla *et al.*, 1987; Vatnick *et al.*, 1987), and by the present work (Fig. 2). There was a rapid fall of UCP mRNA after birth in the different adipose tissues; it was usually no longer expressed 1 week later, a time when the apparent number of adipocytes is maintained at a constant level in bovine species (Robelin, 1985). ADP/ATP carrier mRNA followed a similar, though slower, disappearance. The levels of mRNAs coding for cytochrome *c* oxidase subunits III and IV remained unchanged during this early postnatal period, but decreased slowly afterwards (results not shown). It is noteworthy that mRNA levels for subunits III and IV varied in parallel, although these two subunits are coded for by two different genomes, mitochondrial and nuclear respectively.

It should be pointed out that soon after birth the perirenal adipose tissue of ruminants, which still contains adipocytes with typical mitochondria (Gemmell *et al.*, 1972; Cannon *et al.*, 1977; Vatnick *et al.*, 1987) and high levels of cytochrome *c* oxidase mRNA, lacks UCP mRNA. Can it still be considered as brown fat?

It is also noteworthy that, when the young animals were slaughtered in winter, UCP mRNA could still be

detected 15 and 38 days after birth. This could be explained by the cold environment, which either delayed the disappearance of brown fat characteristics (Gemmell *et al.*, 1972) or induced a further stimulation of UCP mRNA.

In conclusion, ruminant species seem attractive models for the study of the differentiation of brown adipose tissue, as long as there is no '*in vitro*' model. The rapid transformation of brown adipose tissue after birth in these species reinforces the hypothesis of a conversion from brown fat to white fat, and young ruminants are highly interesting models in which to study this possible conversion in large animals.

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