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Differential Regulation of Uncoupling Protein-2 and Uncoupling Protein-3 Gene Expression in Brown Adipose Tissue during Development and Cold Exposure

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The expression of the two novel uncoupling proteins genes, UCP2 and UCP3, is differentially regulated during prenatal maturation of brown adipose tissue in the mouse. UCP2 gene is expressed early in prenatal development, when neither UCP1 nor UCP3 gene expression yet occurs. UCP3 mRNA is absent at any stage of fetal life; it appears suddenly at birth and reaches adult levels in a few hours. UCP2 mRNA increased after birth but more slowly than UCP3 and UCP1 mRNA. Short-time exposure of adult mice to cold caused a rise in UCP2 or UCP1 mRNA levels but not in that of UCP3. The postnatal rise in UCP2 gene expression appears to be a response to the thermic stress associated with birth, similarly to UCP1, whereas different biological signals may be responsible for the surge in UCP3 gene expression at birth.

A major mechanism for dissipation of metabolic energy as heat in mammalian cells is the uncoupling of the mitochondrial respiratory chain and oxidative phosphorylation systems. Despite considerable evidence that a proton leak across the mitochondrial inner membrane of different cell types is potentially involved (1), the brown fat uncoupling protein 1 (UCP1) was, until very recently, the only molecular mechanism known to mediate this uncoupling process. The fact that UCP1 is found only in the mitochondria of brown fat is consistent with the consideration of this tissue as the main site of non-shivering thermogenesis in rodents (2).

The recent discovery of two other UCP genes, UCP2 (3,4) and UCP3 (5,6), which encode mitochondrial uncoupling proteins similar to UCP1, has constituted a major new insight in the establishment of the molecular mechanisms of the uncoupled mitochondrial respiration in mammals. In both humans and rodents UCP2 is expressed in most tissues (3,4) whereas UCP3 gene expression is restricted to brown adipose tissue and muscle (5,6). Several lines of evidence suggest that the UCP2 and UCP3 gene have an important role in energy balance. Thus, the levels of UCP2 expression in different mice strains correlate negatively with susceptibility to diet-induced obesity (3). Mice lacking UCP1 are cold-intolerant, resistant to diet-induced obesity and show up-regulation of UCP2 expression in brown adipose tissue (7). Agonists of peroxisomal proliferator activated receptors, which influence adiposity, up-regulate UCP2 expression (8). On the other hand, UCP3 expression has been reported to be highly responsive to hormonal signals involved in the regulation of energy balance, such as thyroid hormones (9). Moreover, in humans the UCP2 and UCP3 genes are located close together in a region of chromosome 11 which is coincident with quantitative trait loci for obesity (3,10).

Brown fat is the only tissue in which the three UCP genes are co-expressed, and it provides a unique model to study their regulation. To date, nothing is known about developmental regulation of UCP2 and UCP3 expression in relation to tissue differentiation "in vivo". Moreover, their regulation according to the energy requirements elicited by cold exposure is also poorly known. We now report differential regulation of the expression of the three genes for uncoupling proteins during brown adipose tissue ontogeny and after cold exposure, thus suggesting a distinct role for these genes in brown fat thermogenesis and different regulatory mechanisms determining their expression.

MATERIALS AND METHODS

Female Swiss mice were mated with adult males and the day of pregnancy was determined by the presence of spermatozoa in vaginal

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smears. When prenatal mRNA expression was studied, Caesarian sections were performed on days 16,17,18 and 19 of pregnancy. When the postnatal period was studied, pups remained with their mother after spontaneous delivery and were killed before they started suckling (0 h), 15h, 48 h , 15 days or 21 days later. Fetuses, pups or young mice were killed by decapitation and interscapular brown adipose tissue was extracted and immediately frozen in liquid nitrogen. Samples from each litter of fetuses or pups were pooled. For studies using adults, two-month-old Swiss and C57BL/6J mice were kept under standard conditions of housing (12h light/dark cycles) feeding (A-03 type diet, Panlab, Spain) and environment temperature (21 + 1 °C). To determine the effects of cold exposure, mice were previously acclimated to thermoneutral environment temperature (28°C) for two weeks and then suddenly exposed to 4°C for 5 hours. The interscapular brown adipose tissue from each individual was extracted and frozen.

Total RNA from brown adipose tissue was extracted using a guanidine thiocyanate method (11). For Northern blot analysis, 10 μg of total RNA was denatured, electrophoresed on 1.5% formaldehyde-agarose gels and transferred to positively charged membranes (N+, Boehringer Mannheim, Germany). 0.2 μg of ethidium bromide was added to RNA samples in order to check equal loading of the gels and transfer efficiency. Prehybridization and hybridization were carried out at 55 °C using 0.25 M Na_2HPO_4 (pH 7.2), 1 mM EDTA, 20% SDS, 0.5% blocking reagent (Boehringer Mannheim, Germany) solution (12). Blots were hybridized using as probes the rat cDNA for UCP1 (13), the human cDNA for UCP3 (6) and a mouse UCP2 probe obtained after coupled reverse transcription and PCR amplification of mouse RNA using the primers 5′ TTAGAGAAGCTTGAC-CTTGG 3′ and 5′ CGTTCCAGGATCCCAAGCGG 3′, which correspond to positions 145 to 164 and 1148 to 1129 respectively of the mouse UCP2 cDNA (3) . Reactions were performed using 2 μg RNA from mouse gonadal white adipose tissue, 1 μM each primer, 0.2 μM each of the four deoxynucleotide triphosphates, 3 mM $MgSO_4$, 0.1 units/μl AMV reverse transcriptase, 0.1 units/μl *Thermus flavus* DNA polymerase and the AMV/*Tfl* buffer as provided by the supplier (Promega,USA). After 1h incubation at 48°C, 40 cycles were performed at 94°C, 30 s; 55°C, 2 min and 68°C, 1 min each. The resulting DNA product of approximately 1 kb was purified and cloned into a pGEM-T vector (Promega,USA). The DNA probes were labeled with (α-^{32}P)dCTP using the random oligonucleotide-primer method. Hybridization signals were quantified using a Molecular Image System GS-525 (BioRad).

RESULTS AND DISCUSSION

Fig 1 shows the pattern of mRNA expression for UCP1, UCP2 and UCP3 during early development of mouse brown adipose tissue. UCP1 gene expression started between days 17 and 18 of pregnancy, UCP1 mRNA levels increased abruptly before birth and a new rise occurred in the first hours post-partum. This profile is essentially parallel to our previous observations in brown adipose tissue development in the rat (14). UCP2 mRNA was expressed in brown fat as a single 1.7 kb mRNA species, as already reported (3,4) and it followed a completely different profile: substantial levels of UCP2 mRNA were already present in brown fat as early as day 16 of fetal life (around 30 % respect to the expression at birth), when UCP1 gene expression is absent. There was an increase of UCP2 mRNA abundance in late fetal life, followed by a new rise with a maximum on the second day after birth followed by a decline thereafter. This prenatal profile of expression is closely similar to that observed previously for mitochondrial or nuclear genes encoding components of the mitochondrial oxidative phosphorylation system (OXPHOS) (14,15) and, indeed, with the progressive mitochondrial biogenesis that takes place in brown adipose tissue during the perinatal period (16). These indicate that the regulation of UCP2 mRNA is mostly associated with this overall mitochodriogenesis in the tissue, and most probably under the control of the molecular mechanisms that coordinate regulation of OXPHOS gene expression during prenatal development. In the postnatal period, the time-course of the rise in UCP2 mRNA expression was clearly dissociated from the rise in UCP1 mRNA levels (maximum expression at 48h and at 15h, respectively), thus suggesting that mechanisms eliciting enhanced expression of UCP1 and UCP2 genes after birth are not likely to be identical.

The pattern of expression of the UCP3 gene during development was markedly different from that of the other UCP genes. UCP3 mRNA expression was negligible throughout the fetal period, and even long overexposure of films in Northern blot analyses yielded no signal at any time of fetal life studied. UCP3 gene expression began at birth and reached adult levels in a few hours (Fig 1). As already observed for human and rats (5, 10), the UCP3 probe detected two mRNA species of approximately 2.5 kb and 2.8 kb in mouse samples. It has been proposed that these two mRNAs encode two different forms of the UCP3 protein with potential differences in function (10) . The sudden appearance of UCP3 mRNA expression occurred in parallel for both forms of UCP3 mRNA (see Fig 1). This excludes a differential regulation for these two UCP3 mRNA species during brown fat development.

The profile of expression of UCP3 mRNAs is unusual. Expression of genes considered as molecular markers of terminal differentiation of brown adipose tissue, such as those for UCP1, fatty acid synthase or the glucose transporter GLUT4, begins before birth (14,17,18 and present data), much earlier than UCP3 . Thus the expression of the UCP3 gene is one of the features of brown fat switched on at the end of the maturation of the tissue. On the other hand, the dramatic postnatal rise in UCP3 gene expression, when neonatal mice face high thermogenic requirements, parallels that of UCP1 and suggests the participation of UCP3 in the thermogenic response of neonatal brown fat after birth. However, further data in cold exposed adult mice rule out a major role for thermic stress in eliciting enhanced expression of the UCP3 gene.

Adult Swiss mice exposed to cold for five hours showed a dramatic rise (more than ten fold) in UCP1 mRNA levels in brown fat (Fig 2B), as already reported (19). In the same situation UCP2 mRNA levels showed a more moderate (3-4 fold) increase and UCP3 mRNA levels were not significantly affected. The present find-

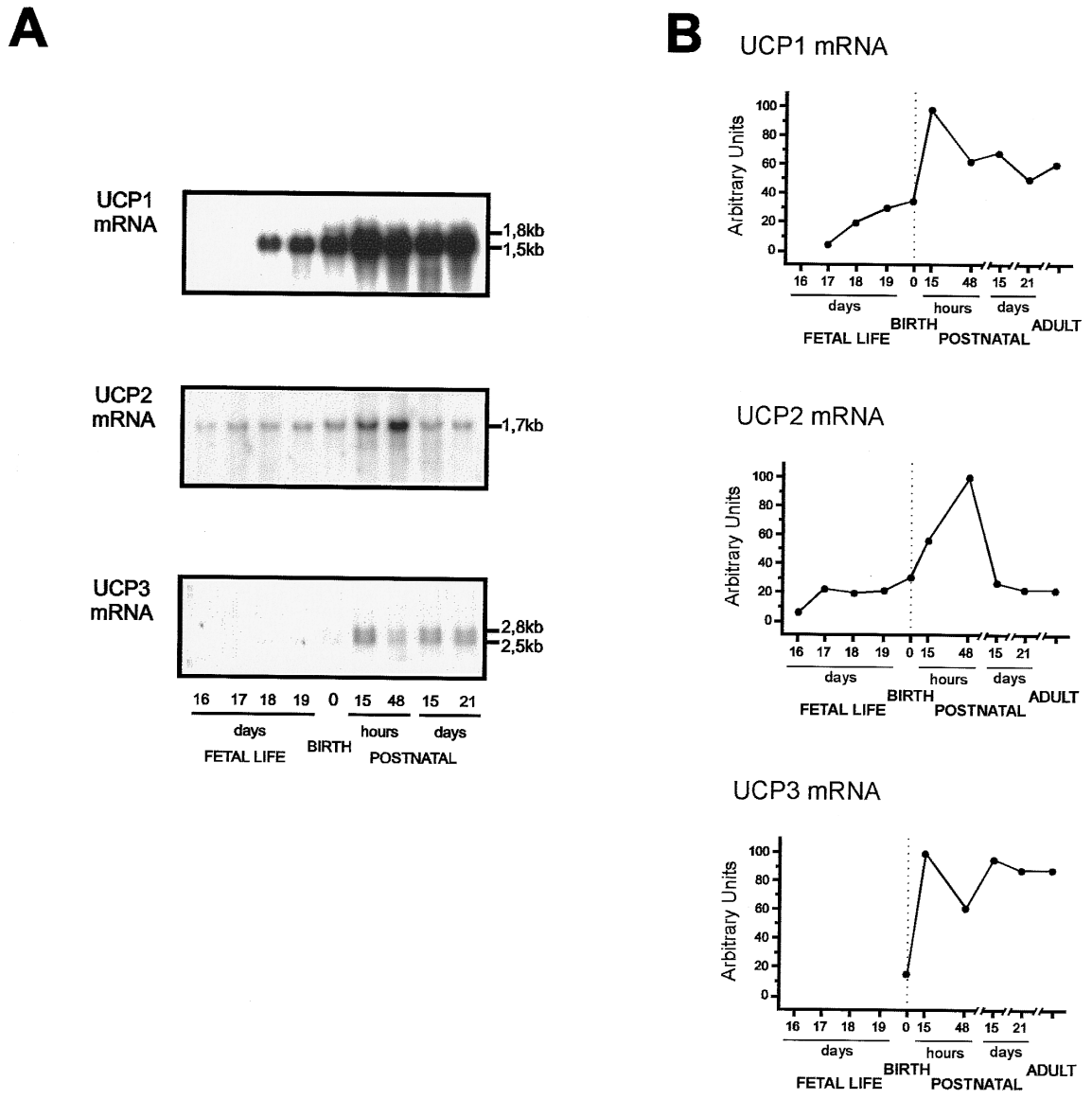

FIG. 1. Developmental regulation of expression of the mRNAs for UCP1, UCP2 and UCP3 in brown adipose tissue. A, Northern blot analysis of equal amounts or RNA (10 μg/lane) from interscapular brown adipose tissue of mice at the indicated stages of development. The sizes of the transcripts are depicted to the right. B, representation of the relative abundance of UCP1, UCP2 and UCP3 transcripts. Points represent means of the hybridization intensity signals of two samples differing less than 15% for each studied time. The 1.8 kb and 1.5 kb mRNAs in UCP1 and the 2.8 kb and 2.5 kb mRNAs for UCP3 were additively quantified as a single point. Data are expressed as percentage of the point of maximum expression, which was set to 100.

ings on UCP2 gene expression are consistent with the rise in UCP2 mRNA levels in brown fat previously reported in rats exposed to cold for 48h (20) and moreover indicate that UCP2 gene expression is highly sensitive even to short-time cold exposure.

Fleury et al. (3) reported marked changes in UCP2 mRNA levels in relation to the strain of mice, and the C57BL/6J strain was found to be highly sensitive to diet-induced obesity, in association with very low basal and diet-induced expression of UCP2 mRNA. Indeed, when brown adipose tissue from mice of the same age was studied, UCP2 mRNA levels were much lower in C57BL/6J mice than in Swiss (see Fig 2A). In contrast, basal UCP1 mRNA levels were not affected and UCP3 mRNA expression did not show the down-regulation observed for the UCP2 gene. However, C57BL/6J mice showed the same propensity to increase brown fat UCP2 mRNA levels when exposed to acute cold as Swiss mice whereas UCP3 mRNA levels were, again, no affected. These data indicate that UCP2 gene expression is up-regulated by cold exposure in brown adipose tissue regardless of the mouse strain studied and

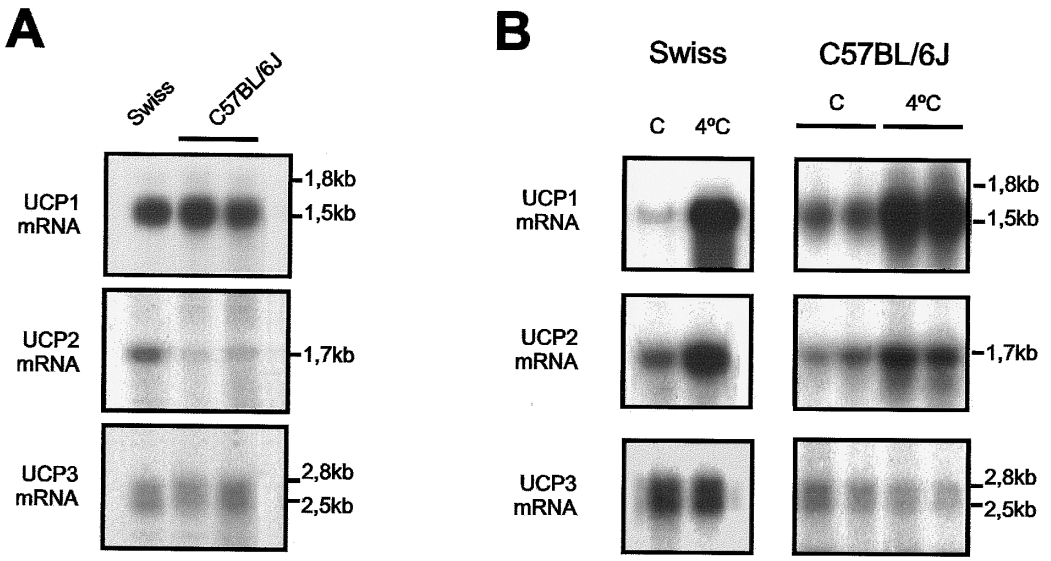

FIG. 2. Effects of cold exposure on the expression of the mRNAs for UCP1, UCP2 and UCP3 mRNAs in mouse brown adipose tissue. Northern blot analyses of RNA (10 μg/lane) from interscapular brown adipose tissue of adult mice. A, basal levels of UCP1 mRNA, UCP2 mRNA and UCP3 mRNA expression in the two indicated mouse strains. B, expression of UCP1 mRNA, UCP2 mRNA and UCP3 mRNA in mice from the indicated strain exposed for 5h to 4°C environment temperature (4°C) and controls (C). Figures shown are representative of two-three independent Northern blot analyses, in which the variation within the experimental groups was less than 15%.

that UCP3 gene did not show either strain-dependent or acute cold-induced regulation in brown fat. The recent report of enhanced UCP3 mRNA expression in brown adipose tissue after long-term exposure to cold (21) is not contradictory with present findings of lack of responsiveness of UCP3 expression to acute cold. Chronic cold exposure is known to produce hypertrophy and hyperplasia of brown adipose tissue due to enhanced proliferation and differentiation of precursor cells to brown adipocytes (22). Considering that UCP3 expression is characteristic of brown fat, all these changes elicited by long term cold exposure are likely to be detected as a higher UCP3 mRNA expression with respect to controls.

The increase in UCP2 mRNA elicited by acute cold suggests that the postnatal rise in UCP2 gene expression is most probably due to the thermal stress caused by the transition from an intrauterine to an extrauterine environment, similarly to what occurs for UCP1 (14). It also suggests also that UCP2 activity might participate in the adaptative thermogenesis elicited by cold stress in brown adipose tissue. A different situation is found for the UCP3 gene : the lack of response of UCP3 gene expression after cold stress in adult mice indicates that the sudden exposure of newborn mice to cold after birth is not the primary cause of UCP3 induction. Thus, molecular signals other than the cold-induced adrenergic stimulus are expected to mediate the appearance of UCP3 expression after birth. Although thyroid hormones have recently been reported to up-regulate UCP3 gene expression (9,21) several lines of evidence suggest that they are probably not mediators of the postnatal induction of UCP3 gene expression. Brown adipose tissue acquires the mature thyroid status before birth (23), when UCP3 gene is not expressed. Moreover, thyroid action in brown fat is mostly mediated by the endogenous generation of T_3 due to iodothyronine 5′-deiodinase, and only to a slight extent, by circulating T_3 (24). This enzyme activity is induced by cold exposure in adult rodents (25), when UCP3 gene expression is not stimulated. Conversely, postnatal cold stress did not elicit enhanced 5′-deiodinase activity (26) and this is the time of maximum induction of UCP3 mRNA levels. On the other hand, in contrast with UCP2, UCP3 gene expression in brown adipose tissue is sensitive to short-term changes in food intake in adult rodents (9). Thus, the initiation of suckling may be a signal for the induction of UCP3 gene after birth, acting through hormonal mechanisms as yet unidentified. In summary, further research will be needed to identify the molecular signals responsible for the induction of UCP3 gene expression at birth and to determine the extent to which this is ontogenically programmed or elicited by stimuli other than thermal stress, albeit associated with birth.

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