Uncoupling protein-3 gene expression in skeletal muscle during development is regulated by nutritional factors that alter circulating non-esterified fatty acids

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Abstract Uncoupling protein-3 gene expression in skeletal muscle is up-regulated during postnatal development of mice. A high-carbohydrate diet at weaning induces a decrease in uncoupling protein-3 mRNA levels that does not occur when mice were weaned onto a high-fat diet. Uncoupling protein-3 mRNA levels do not increase in response to fasting in young pups. Only after day 15 of life, when fasting increases serum non-esterified fatty acids, uncoupling protein-3 mRNA is up-regulated by starvation. Over-nutrition or under-nutrition during lactation increases or decreases, respectively, uncoupling protein-3 mRNA expression in skeletal muscle. Regulation of uncoupling protein-3 gene expression in skeletal muscle during development is mediated by ontogenic and nutritional factors determining changes in circulating non-esterified fatty acids.

Key words: Skeletal muscle; Uncoupling protein-3; Development; Gene expression

1. Introduction

Uncoupling protein-2 (UCP2) and uncoupling protein-3 (UCP3) are two recently cloned genes with a high sequence homology with the brown adipose tissue uncoupling protein-1 [1–4]. They uncouple oxidative phosphorylation when expressed in yeast and may be involved in regulatory thermogenesis. Mitochondrial proton leaks occur not only in brown fat but also in other mammalian tissues [5] and the more widespread expression of the UCP2 and UCP3 genes suggests that they may regulate body energy expenditure. The UCP2 gene is expressed ubiquitously, especially in macrophages, spleen and white adipose tissues [1,2,6]. The UCP3 gene is preferentially expressed in thermogenic tissues, brown fat and skeletal muscle in rodents and skeletal muscle in humans [3,4]. Short-time cold exposure induces UCP3 gene expression in skeletal muscle [7] which is consistent with its involvement in non-shivering thermogenesis. However, UCP3 mRNA levels are also up-regulated in starvation, a situation of reduced muscle thermogenesis [8]. Non-esterified fatty acids have recently been shown to induce UCP3 gene expression in muscle [9]. Both cold exposure and fasting raise circulating non-esterified fatty acid levels and fatty acid concentrations in skeletal muscle increase after cold stress [10].

During postnatal development, the skeletal muscle metabolism changes dramatically. The mitochondrial biogenesis develops progressively after birth and gene expression for components of metabolic pathways of carbohydrate and the lipid metabolism are regulated according to the changing nutritional situations, especially in relation to the intake of milk as a fuel supply and to weaning [11]. The UCP2 gene is expressed in fetal skeletal muscle whereas the UCP3 gene is first expressed soon after birth, in response to suckling and lipid intake [12]. This induction may be mediated by activation of peroxisome-proliferator activated receptor α [12]. Here, we examine the changes in the expression of the UCP3 and UCP2 genes in skeletal muscle during postnatal development in the mouse. Changes in circulating non-esterified fatty acids, attributable to ontogenic and nutritional factors, appear to mediate developmental regulation of UCP3 gene expression.

2. Materials and methods

Female Swiss mice were mated with adult males and after birth, litter sizes were adjusted to 10 pups, unless otherwise indicated. For developmental studies, pups were studied immediately after birth, before suckling began, and on days 1, 2, 6, 15 and 21 after birth. Lactating mothers were maintained on a standard, high-carbohydrate diet composed of (% of total gross energy) 72 carbohydrate, 6 fat, 22 protein (B.K. Universal, Spain). For determining the effects of diet at weaning, a high-fat diet composed of (% of total gross energy) 36 carbohydrate, 42 fat, 22 protein (Harlan Teklad, USA) was used. Regular chow was replaced by the high-fat diet on day 15 of lactation and pups were studied on day 21. When the effects of fasting were analyzed, pups on days 1, 6, 15 or 21 were separated from their mothers and studied 8, 16 or 24 h thereafter. The effects of different times of fasting were determined in pups from the same litter. For studies on the effects of the over-nutrition or under-nutrition caused by differences in litter size, litters were adjusted at birth to four pups or to 16 pups, respectively. Pups were killed by decapitation and blood was collected for serum preparation. The whole muscle from both legs was dissected from skin and bone and frozen in liquid nitrogen.

RNA from muscle was extracted using a modified guanidine thiocyanate method [13]. For Northern blot analysis, 20 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 gels and the transfer efficiency. Pre-hybridization 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 [14]. Blots were hybridized using as probes the human cDNA for UCP3 [4] and a UCP2 probe obtained after coupled reverse transcription and PCR amplification of RNA from mouse [15]. The DNA probes were labelled with [α-^{32}P]dCTP using the random oligonucleotide-primer method. Hybridization signals were quantified using Molecular Image System GS-525 (Bio-Rad). Serum non-esterified fatty acid levels were quantified using a colorimetric acyl-CoA synthetase and acyl-CoA oxidase-based method (Wako Chemicals, USA).

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Abbreviations: UCP2, uncoupling protein-2; UCP3, uncoupling protein-3

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3. Results

3.1. UCP3 mRNA and UCP2 mRNA expression in skeletal muscle of developing mice: effects of a high-fat or high-carbohydrate diet on weaning

Northern blot analysis of UCP2 mRNA in skeletal muscle of developing mice showed the presence of a single transcript of 1.7 kb whereas UCP3 mRNA was expressed as two species of approximately 2.5 kb and 2.8 kb (see Fig. 1), as described previously [12]. The changes in UCP3 mRNA levels during development or those caused by changes in food intake described in the present study always occurred in parallel for the two forms of UCP3 mRNA. Fig. 2 shows the profile of changes in UCP3 mRNA and UCP2 mRNA levels in skeletal muscle of developing mice. The patterns of expression were different for each gene. UCP3 mRNA expression was almost undetectable at birth, increased suddenly during the first day of life and increased progressively until a maximum at day 15, the peak of suckling activity of mouse pups. This is consistent with previous reports [12]. UCP2 mRNA was expressed at birth and it also rose in the first days of life but the maximal levels of expression were achieved on day 2 and there was a decline thereafter. On day 21, when milk (a high-fat diet) was replaced by regular chow (a high-carbohydrate diet), UCP3 mRNA expression and UCP2 mRNA expression declined. When the diet was changed on day 15 of lactation from regular high-carbohydrate chow to a high-fat diet, the UCP3 mRNA decline was suppressed but the UCP2 mRNA decrease was not (see Fig. 2). Levels of serum non-esterified fatty acids during development and in pups weaned onto high-carbohydrate or high-fat diets are shown in Fig. 2 (bottom). The profiles of changes in UCP3 mRNA levels in skeletal muscle and in serum non-esterified fatty acid concentration were closely parallel.

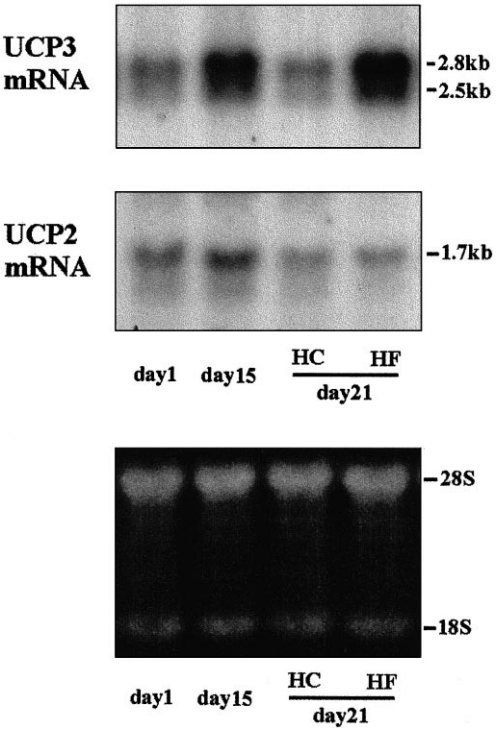

Fig. 1. Northern blot analysis of equal amounts of RNA (20 μg/lane) from skeletal muscle of 1, 15 and 21 day old pups. Effects of a high-fat (HF) or high-carbohydrate (HC) diet from day 15 to day 21. The top and middle panels show UCP3 mRNA and UCP2 mRNA, respectively. The sizes of the transcripts are shown on the right. The bottom panel shows ethidium bromide staining of the gels demonstrating equal loading.

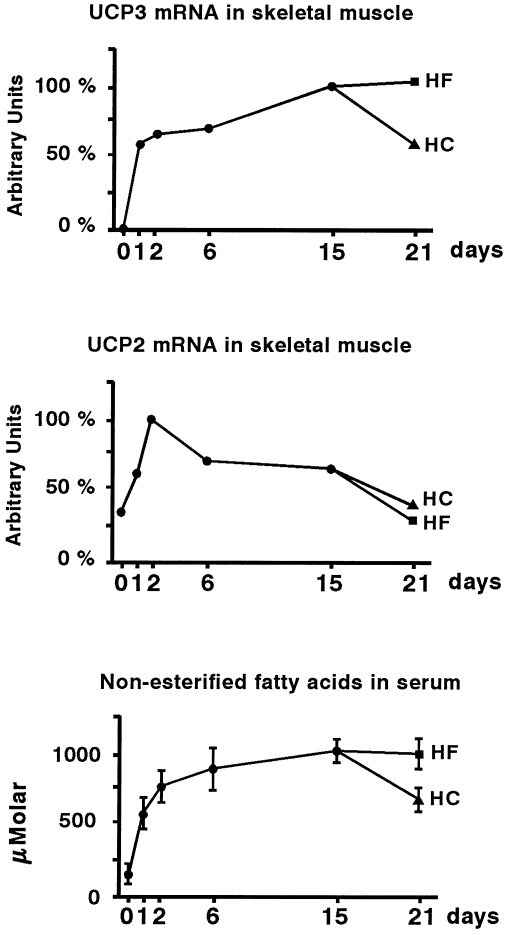

Fig. 2. Changes in UCP3 mRNA and UCP2 mRNA expression in skeletal muscle and serum non-esterified fatty acid concentrations during mice postnatal development. Effects of a high-carbohydrate (HC) or high-fat (HF) diet at weaning. In the top and middle panels, points are means of signals obtained in Northern blot analyses of 2–3 RNA samples obtained from pups of independent litters. Data are expressed as percentages relative to the mean value at the developmental time of maximum levels of expression which was set to 100. In the bottom panel, points are means ± S.E.M. of serum non-esterified fatty acid concentrations of at least four samples from independent litters, each one being a pool of the sera from pups of the same litter.

3.2. Effects of fasting on UCP3 and UCP2 mRNA expression in skeletal muscle of mice at different stages of development

Fig. 3 shows the effects of fasting on UCP3 mRNA and UCP2 mRNA levels in 1, 6, 15 or 21 day old mice. Fasting of 15 or 21 day old pups caused a marked time-dependent increase in UCP3 mRNA levels that reached a maximum after 16 h. In contrast, UCP3 mRNA abundance decreased slightly in skeletal muscle of 1 day and 6 day old pups. Starvation did not cause major changes in UCP2 mRNA levels and only a slight increase was observed in 21 day old pups. There were no changes in serum non-esterified fatty acids in 1 and 6 day old pups. However, fasting caused a rise in circulating non-

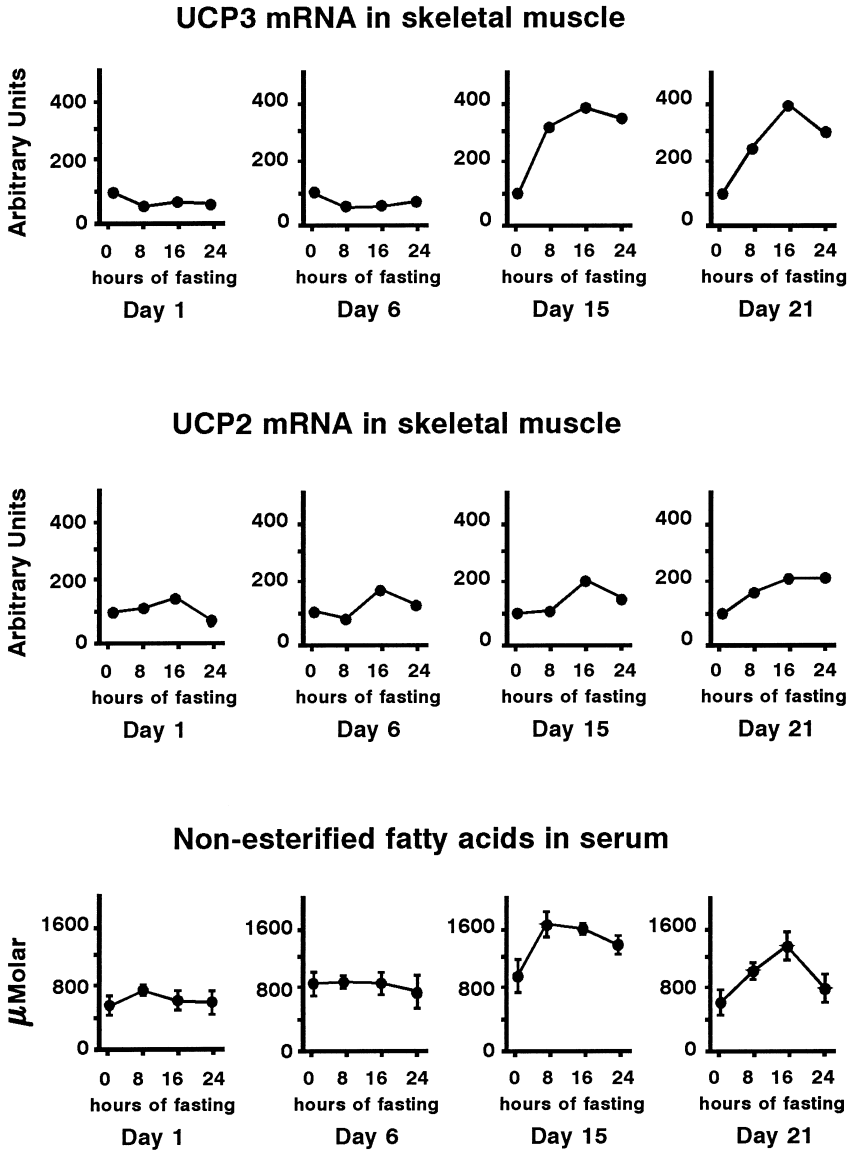

Fig. 3. Effects of fasting on UCP3 mRNA and UCP2 mRNA expression in skeletal muscle and on serum non-esterified fatty acid concentrations in 1, 6, 15 and 21 day old mice. In the top and middle panels, points are means of densitometric signals obtained in Northern blot analyses of 2–3 RNA samples obtained from pups of independent litters. Data are expressed as percentage induction with respect to the fed controls (time 0). In the bottom panel, points are means ± S.E.M. of serum non-esterified fatty acid concentrations of at least four samples from pups of independent litters.

esterified fatty acids in 15 and 21 day old pups. The curves of changes in non-esterified fatty acid levels and skeletal muscle UCP3 mRNA expression in response to fasting at different ages were similar.

3.3. *Effects of under-nutrition or over-nutrition caused by changes in litter size on the expression of UCP3 mRNA and UCP2 mRNA in developing mice*

Nutritional deprivation of mice pups was induced by in-

Table 1
Effects of litter size on the body weight, UCP3 mRNA and UCP2 mRNA levels in skeletal muscle and serum non-esterified fatty acid levels in 15 day old mice

	Litter size (number of pups)		
	4	10	16
Body weight (g)	11.4 ± 0.3*	8.6 ± 0.3	6.0 ± 0.2*
UCP3 mRNA (arbitrary units)	190 ± 22*	100 ± 15	52 ± 7*
UCP2 mRNA (arbitrary units)	111 ± 14	100 ± 18	92 ± 19
Serum non-esterified fatty acids (µM)	1 510 ± 180*	972 ± 64	730 ± 69*

UCP3 mRNA and UCP2 mRNA levels are expressed as percentage changes with respect to the ten pups/litter group that was adjusted to 100. Results are shown as means ± S.E.M. of at least three independent experiments using pooled samples of at least two pups for each experimental point.
*Statistically significant difference ($P \leq 0.05$) compared with the 10 pups/litter group.

creasing the litter size to 16 whereas over-nutrition was induced by reducing the number of pups at birth to only four, according to previously reported studies [16]. On day 15, the body weight of pups from large litters was significantly lower than pups from regular litters whereas pups from small litters had a higher body weight (see Table 1). This confirms the impact of the litter size on the individual amounts of milk taken by pups. Over-nourished pups had higher UCP3 mRNA levels in muscle than regularly fed pups whereas the opposite occurred for chronically underfed pups (Table 1). Again, serum non-esterified fatty acid levels parallelled these changes. UCP2 mRNA expression in skeletal muscle was unaffected by the litter size.

4. Discussion

The expression of UCP3 and UCP2 genes is differentially regulated in skeletal muscle during development. The UCP3 mRNA levels follow a progressive pattern of increase after birth that reaches a maximum at day 15 of lactation. This is coincident with the time of maximum intake of milk. In contrast, UCP2 mRNA follows a different pattern of changes with a maximum in early lactation (day 2 after birth). Spontaneous weaning, beginning around day 15 of life, is associated with a progressive substitution of a high-fat diet (milk) by a high-carbohydrate (regular chow) diet. This process results in a decline in muscle UCP3 gene expression and our present data demonstrate that weaning onto a high-fat diet suppresses this decline whereas UCP2 mRNA is unaffected by the type of diet at weaning. The progressive induction of UCP3 mRNA after birth and its regulation due to diet in the weaning period follow a close parallelism with changes in the levels of circulating non-esterified fatty acids. Fatty acids have been proposed to be major regulators of UCP3 mRNA in skeletal muscle of adult rodents [9] and we have recently reported that neonatal pups are already sensitive to this action of fatty acids [12]. Thus, nutritional changes related to the intake of lipids and most probably their consequences in circulating non-esterified fatty acids mediate changes in UCP3 gene expression during development.

We have previously demonstrated that the initiation of suckling and particularly lipid intake is essential for the onset of UCP3 mRNA expression after birth [12]. The low expression of UCP3 mRNA in neonates that were not allowed to suckle contrasted with the behavior of adult rodents, which show up-regulation in response to fasting [9]. Our present results indicate that UCP3 mRNA is up-regulated by fasting only at the age when fasting is able to cause a raise in circulating non-esterified fatty acids. Young pups (before day 6) are unable to raise serum non-esterified fatty acids in response to fasting, surely due to the fact that white fat develops after birth in rodents and very low amounts of white adipose tissue are still present at this period of development [17]. As a consequence, these pups are unable to up-regulate UCP3 gene expression in muscle. After day 15 of life, when circulating non-esterified fatty acids increase due to fasting, UCP3 mRNA in muscle is up-regulated. Thus, the development of white adipose tissue and the ability of fasting to promote lipolysis appears to be what determines the up-regulation of the UCP3 gene in skeletal muscle in response to starvation. Other factors claimed to affect UCP3 mRNA expression, such as leptin or thyroid hormones [8] which follow a progressive profile of induction after birth [18,19], that would be compatible with their involvement in the postnatal UCP3 mRNA induction. However, both leptin and thyroid hormones are down-regulated in adult mice in response to fasting [20,21] and are therefore unlikely mediators of UCP3 mRNA expression in skeletal muscle during this period. Thus, present findings point to interorgan signalling between UCP3 gene expression in skeletal muscle and the development and lipolytic activity of white adipose tissue and support the major regulatory role of non-esterified fatty acids in UCP3 gene expression. In contrast, UCP2 mRNA expression in skeletal muscle is poorly responsive to fasting at any developmental period.

Present data also show that long-term changes in caloric intake affect the expression of UCP3 mRNA but not of UCP2 mRNA in skeletal muscle. An increased litter size, a well-established model of nutritional deprivation before weaning [16], resulted in a reduced UCP3 mRNA expression. This behavior is similar to that found in adult rodents exposed to chronic caloric under-nutrition [22] and contrasts with the up-regulation of UCP3 mRNA due to fasting. Again, the levels of non-esterified fatty acids, which in contrast with suddenly starved mice are low in nutritionally deprived pups, parallel the changes in UCP3 mRNA. Over-nutrition (higher intake of milk) caused by a very low litter size resulted in enhanced UCP3 mRNA expression in skeletal muscle associated also with a mild rise in serum non-esterified fatty acids.

In summary, present results indicate that developmental regulation of UCP3 gene expression in skeletal muscle is mediated by nutritional determinants (starvation, amount of food intake, carbohydrate versus a fat-enriched diet) and closely parallels the consequences that changes in food intake have on the levels of circulating non-esterified fatty acids. In this last sense, the development of white adipose tissue has a major consequence on the regulation of UCP3 gene expression in muscle. In contrast, UCP2 gene expression in skeletal muscle is developmentally regulated but this regulation is insensitive to nutritional changes. In some of the physiological and nutritional situations studied here, there is a correlation between thermogenic activity and UCP3 mRNA expression, as for example in the early postnatal period, when high thermogenesis occurs [23], or in nutritionally deprived pups, which show reduced non-shivering thermogenesis [16]. In these situations, fatty acids would play multiple roles in thermogenesis, they are the most calorigenic oxidation substrates, they promote mitochondrial uncoupling through UCPs and other mitochondrial anion carriers [5] and they regulate UCP3 mRNA synthesis in skeletal muscle. Other situations such as fasting did not show this correlation between thermogenesis and UCP3 gene expression. In the context of the current debate on the physiological role of UCP3 in skeletal muscle, our present findings support the notion that, together with the involvement of UCP3 in thermogenesis, UCP3 gene expression is regulated according to the fatty acid availability to skeletal muscle.

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