Differential regulation of expression of genes encoding uncoupling proteins 2 and 3 in brown adipose tissue during lactation in mice

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Thermogenic activity in brown adipose tissue (BAT) decreases during lactation; the down-regulation of the gene encoding uncoupling protein 1 (UCP1) is involved in this process. Our studies show that UCP2 mRNA expression does not change during the breeding cycle in mice. In contrast, UCP3 mRNA is down-regulated in lactation but it recovers after weaning, in parallel with UCP1 mRNA. This leads to a decrease in the content of UCP3 in BAT mitochondria during lactation. Lowering the energy-sparing necessities of lactating dams by decreasing litter size or feeding with a high-fat diet prevented the down-regulation of UCP1 mRNA and UCP3 mRNA. In most cases this resulted in a less marked decrease in UCP1 and UCP3 protein in BAT mitochondria owing to lactation. Fasting for 24 h caused a different response in UCP1 and UCP3 mRNA expression: it decreased UCP1 mRNA levels but had no effect on UCP3 mRNA abundance in virgin mice; it even increased UCP3 mRNA expression in lactating dams. These changes did not lead to modifications in UCP1 or UCP3 protein abundance. Whereas acute treatment with peroxisome-proliferator-activated receptor (PPAR)α and PPARγ agonists increased UCP1 mRNA levels only in lactating dams, UCP3 mRNA expression was induced by both kinds of PPAR activator in lactating dams and by PPARα agonists in virgin mice. It is concluded that modifications of UCP2 mRNA levels are not part of the physiological adaptations taking place in BAT during lactation. In contrast, the down-regulation of UCP3 mRNA expression and mitochondrial UCP3 content is consistent with a role for the gene encoding UCP3 in the decrease in metabolic fuel oxidation and thermogenesis in BAT during lactation.

Key words: fibrate, mitochondria, obesity, peroxisome-proliferator-activated receptor, troglitazone.

INTRODUCTION

Brown adipose tissue (BAT) is a site of non-shivering thermogenesis in rodents. It provides heat in response to a cold environment in small mammals as well as in the newborns of most mammalian species, including humans [1]. Rothwell and Stock [2] proposed that BAT might also be responsible for diet-induced thermogenesis in rodents. The recent development of transgenic mice with ablation of BAT confirmed this, as these mice were cold-intolerant and tended to develop obesity [3].

The thermogenic function of BAT has been attributed to the presence of the uncoupling protein 1 (UCP1), a member of the mitochondrial inner-membrane carrier family. UCP1 uncouples oxidative phosphorylation and is present only in brown adipocyte mitochondria [4]. In 1997 the genes encoding two novel uncoupling proteins, UCP2 and UCP3, were identified. UCP2 and UCP3 have a high sequence identity with UCP1 and they uncouple oxidative phosphorylation when expressed in yeast. UCP2 is almost ubiquitous, whereas UCP3 is present only in skeletal muscle and BAT [5–8]. The physiological role of UCP2 and UCP3 in energy expenditure is unclear and, for instance, UCP3 gene expression in skeletal muscle is regulated in accordance with fatty acid availability rather than with thermogenic requirements [9,10]. In contrast, transgenic mice bearing a targeted disruption of the gene encoding UCP1 were, as expected, cold-intolerant; however, they showed no signs of impairment in diet-induced thermogenesis. UCP2 mRNA expression was up-regulated, whereas UCP3 mRNA was unaltered in BAT of these mice [11,12]. It was proposed that the loss of UCP1 might have been compensated for by UCP2 up-regulation [11], although further research questioned this possibility [12]. In contrast, mice with targeted disruption of the UCP3 gene did not show major metabolic abnormalities [13,14] but the overexpression of UCP3 in muscle causes decreased fat accumulation despite enhanced food intake [15]. UCP2 and UCP3 genes are subject to a complex hormonal regulation in brown adipocytes, especially by leptin, retinoids, thyroid hormones or β_3-adrenergic agonists (reviewed in [16]). Activators of peroxisome-proliferator-activated receptor-γ (PPARγ), such as thiazolidinediones or 15-deoxy-prostaglandin J_2 induce the expression of UCP2 mRNA in cultured brown adipocytes [17,18], whereas chronic treatments of rodents with thiazolidinediones induced the expression of both UCP2 and UCP3 mRNA in BAT [19].

Lactation is associated with a decrease in adaptive thermogenesis in rodents as well as in women under unfavourable nutritional conditions [20,21]. This adaptation has been considered an energy-sparing mechanism to facilitate the use of energy for milk production, although it has also been interpreted as an indirect consequence of the extra heat produced during milk synthesis [22]. Lactation is a unique physiological situation in which hyperphagia develops in association with decreased energy expenditure, which is usually observed in experimental models of obesity or in obese humans [23]. Moreover, the regulation of energy metabolism in lactating women has recently been a matter of debate in the context of the advisable dietary treatment of overweight lactating women [24,25]. In rodents, the

Abbreviations used: BAT, brown adipose tissue; PPAR, peroxisome-proliferator-activated receptor; UCP, uncoupling protein; WY-14,643, pirixinic acid.

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thermogenic function of BAT decreases during lactation as shown by tissue hypotrophy, a decrease in mitochondrial biogenesis and an impaired expression of the gene encoding UCP1 [26–28]. However, it is not known whether the expression of other genes encoding UCPs in BAT is altered during the breeding cycle. Here we report a study on the changes in expression of genes encoding UCP2 and UCP3 in BAT during late pregnancy, lactation and weaning. We also measured the effects on the genes encoding UCPs of direct (starvation or high-fat diet) or indirect (changes in litter size) nutritional manipulations during the breeding cycle. Finally we examined how lactation affects the responsiveness of the genes encoding UCPs to PPAR activators.

MATERIALS AND METHODS

Adult female Swiss mice were maintained in standard conditions of light (12 h light/12 h dark cycle) and temperature (21 ± 1 °C) and were fed with a standard diet composed of 72 % carbohydrate, 6 % fat and 22 % protein (based on percentages of gross energy) (B. K. Universal, Barcelona, Spain), unless indicated otherwise. They were mated and the day of pregnancy was determined by the presence of vaginal plugs. Litter sizes were adjusted at birth to 10 pups except when the effects of exceptionally small (4 pups) or large (18 pups) litters were determined. Mice were studied under the following conditions: late-pregnant (day 19), lactating (days 1, 7 and 15), 24 h after abrupt weaning (pups removed after 15 days of suckling), after spontaneous weaning (on day 30 of lactation) and virgin controls. Virgin mice, pregnant dams and dams after abrupt weaning were caged in pairs and lactating dams were caged singly. The effects of fasting were determined after 24 h of suppression of food in 19-day pregnant mice, 15-day lactating dams and virgin mice. The effects of a high-fat diet during lactation were assessed by the replacement, after parturition, of regular chow with a diet composed of 36 % carbohydrate, 42 % fat and 22 % protein (percentages of gross energy) (Harlan Teklad, Madison, WI, U.S.A.) and dams were studied on day 15 of lactation. Virgin mice were treated during the equivalent period (15 days) with the same high-fat diet. When indicated, mice received a single intraperitoneal injection of bezafibrate (Sigma, St Louis, MO, U.S.A.) (100 μg/g body weight), pirixinic acid (WY-14,643; Cayman Chemicals, Ann Arbor, MI, U.S.A.) (50 μg/g body weight) or troglitazone (Glaxo Wellcome, Stevenage, Herts., U.K.) (100 μg/g body weight) in 50 % (v/v) DMSO in saline solution. Controls were given equivalent volumes of vehicle and mice were studied 6 h after injections. Direct observation and weighing of litters before and after the injections did not reveal major changes in lactational performance due to the treatments. Mice were killed by decapitation; interscapular BAT was dissected out and immediately frozen in liquid nitrogen.

RNA from BAT was prepared with an affinity-column-based method (RNeasy; Qiagen GmbH, Hilden, Germany). RNA (20 μg) was denatured, subjected to electrophoresis on 0.9 % formaldehyde/1.5 % (w/v) agarose gels and transferred to positively charged membranes (N^+; Boehringer Mannheim, Mannheim, Germany). Ethidium bromide (0.2 μg) was added to RNA samples to check for equal loading of gels and efficiency of transfer. Prehybridization and hybridization were performed at 55 °C with 0.25 M Na_2HPO_4 (pH 7.2)/1 mM EDTA/20 % (w/v) SDS/0.5 % blocking reagent (Boehringer Mannheim) solution. Blots were hybridized with the cDNA species for rat UCP1, UCP2 or UCP3 as probes, as reported previously [29]. The DNA probes were labelled with [α-^{32}P]dCTP by a random oligonucleotide-primer method. Hybridization signals were quantified with Molecular Image System GS-525 (Bio-Rad, Richmond, VA, U.S.A.).

Mitochondrial preparations were obtained from interscapular BAT. Samples of mitochondrial protein were mixed with equal volumes of 2 × SDS loading buffer, incubated at 90 °C for 5 min and subjected to SDS/PAGE [12 % (w/v) gel]. Proteins were transferred to PVDF membranes; immunological detection was performed with a rabbit affinity-purified UCP3 antiserum (Alpha Diagnostic, San Antonio, TX, U.S.A.) that had previously been established to detect UCP3 protein in mouse samples [33]. It was used at 4 μg/ml dilution; detection was achieved by the enhanced chemiluminescence detection system (ECL®; Amersham, Little Chalfont, Bucks., U.K.). As a positive control we used L6E9 cells transduced with an adenoviral vector driving the expression of the UCP3 cDNA (C. García, F. Villarroya and A. M. Gómez-Foix, unpublished work). Blots were stripped and probed with a rabbit antiserum against mouse UCP1, provided by Dr E. Rial, and with a mouse antibody against rat cytochrome *c* (BD PharMingen, Heidelberg, Germany). The sizes of the proteins detected were estimated by using protein molecular mass standards (Bio-Rad). Quantification of autoradiographs and enhanced chemiluminescence signals was performed by scanning densitometry.

Statistical analysis was performed by Student's *t* test.

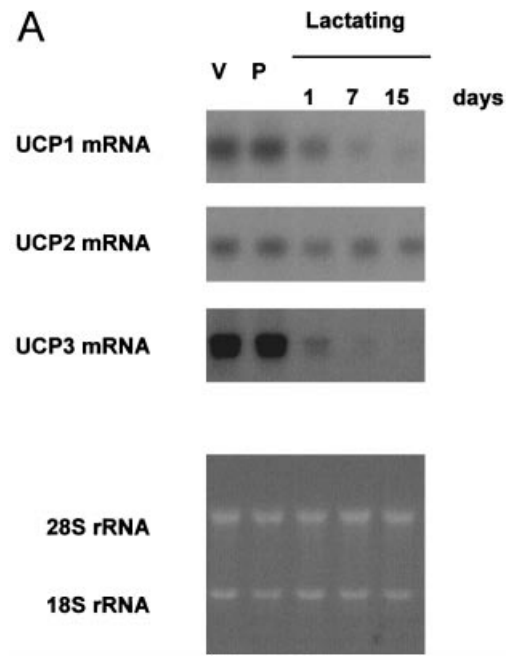
RESULTS

Changes in mRNA levels of UCPs in BAT from late-pregnant mice, lactating mice and dams after weaning

Figure 1 shows the changes in expression of the three mRNA species of UCPs in late-pregnant mice and in dams at different stages of lactation [just after parturition (day 1), at early lactation (day 7), at mid-lactation (day 15) and after spontaneous (day 30) weaning]. UCP1 mRNA levels were not modified in late-pregnant mice; they declined progressively during lactation and reached a minimum in mid-lactation. After spontaneous weaning, dams showed UCP1 mRNA levels similar to those in virgin controls. UCP2 mRNA levels were not significantly different from virgin controls in pregnant or lactating mice. UCP3 mRNA expression was unaltered in late-pregnant mice but declined only 1 day after parturition and was barely detectable in dams at days 7 or 15 of lactation. After spontaneous weaning, dams showed UCP3 mRNA levels similar to those in controls. The effects of abrupt weaning of 15-day lactating dams were studied and results indicated that the down-regulation of expression of UCP1 mRNA and UCP3 mRNA in BAT was completely reversed 24 h after the withdrawal of pups from lactating mothers (106 ± 14 % for UCP1 mRNA and 118 ± 28 % for UCP3 mRNA, compared with virgin controls; means ± S.E.M.).

Changes in the mitochondrial content of UCP1 and UCP3 in BAT from late-pregnant mice, lactating mice and dams after weaning

Immunoblot analysis of mitochondrial protein from virgin control rats with specific antibodies led to the detection of single bands of 34 and 32 kDa for UCP1 and UCP3 respectively, in accord with previous findings [30]. Analysis of mitochondrial preparations for L6E9 cells transduced with an adenoviral vector driving the expression of the UCP3 cDNA (see the Materials and methods section) indicated the appearance of a strong 32 kDa band when the anti-UCP3 antibody was used and no signal was obtained with the anti-UCP1 antibody. Conversely, a faint but detectable band was obtained with the anti-UCP1 antibody in mitochondria from fetal BAT, whereas no evident band was detected in this sample with the anti-UCP3 antibody. This is in



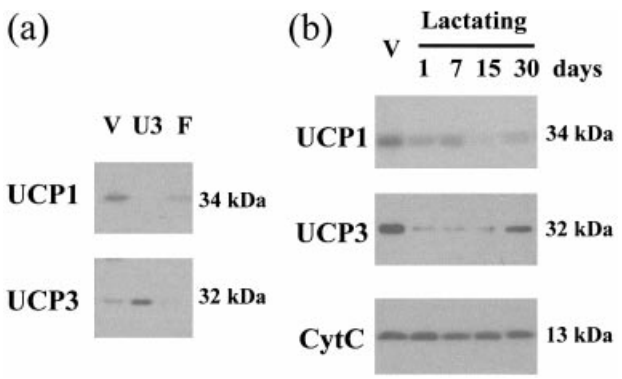


Figure 2 Immunoblot analysis of changes in the content of UCP1 and UCP3 proteins in mitochondria from BAT in lactating and spontaneously weaned mice

(**a**) Western blot analysis showing UCP1 and UCP3 protein detection in BAT mitochondrial preparations from virgin controls (4 μg, V), from L6E9 cells transduced with an adenoviral vector driving the expression of the UCP3 cDNA (2 μg, U3) and from mice fetuses at term (35 μg, F). (**b**) Example of immunoblot analysis of 15 μg mitochondrial protein from interscapular BAT with specific antibodies against UCP1, UCP3 and cytochrome *c*. V, virgin controls.

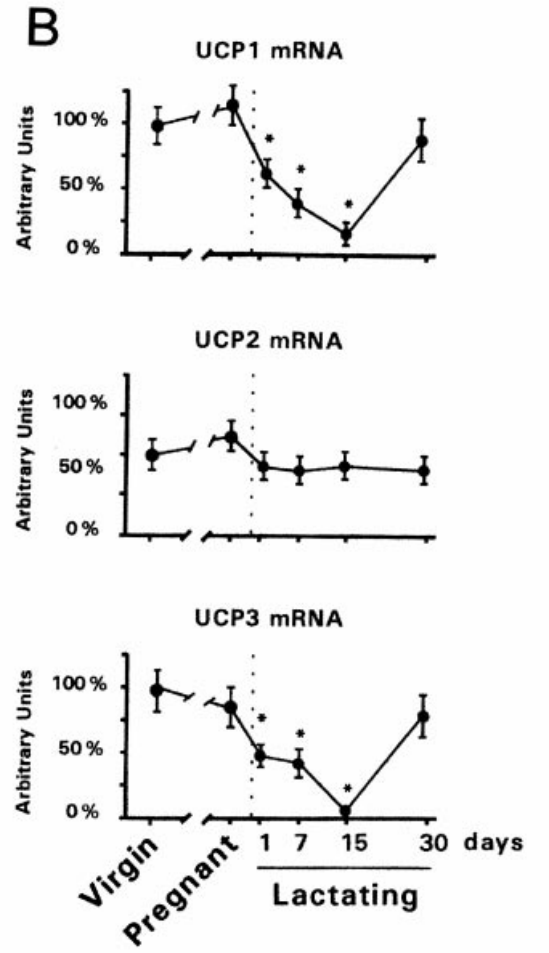


Figure 1 Changes in the expression of UCP1, UCP2 and UCP3 mRNAs in BAT in late-pregnant, lactating and spontaneously weaned mice

(**A**) Example of Northern blot analysis of RNA (20 μg per lane) from interscapular BAT probed for UCP1, UCP2 and UCP3 mRNA detection (top panels) and ethidium bromide staining of the gel showing equal loading of RNA samples (bottom panel). V, virgin controls; P, pregnant. (**B**) Densitometric analysis of changes in the relative abundances of UCP1, UCP2 and UCP3 mRNA species. Each point is the mean $\pm$ S.E.M. for three to five independent samples. *Statistically significant difference ($P \leqslant 0.05$) compared with the virgin controls.

agreement with the known expression of the UCP1 gene in late fetal life but the virtual absence of UCP3 mRNA from brown fat before birth [29]. These results indicate that the cross-reactivity of the anti-UCP1 and anti-UCP3 antibodies was negligible and that they could be used for specific assessment of the relative abundance of these proteins in BAT mitochondrial samples.

The content of UCP1 in BAT mitochondrial preparations was unchanged in late-pregnant mice in comparison with virgin controls (results not shown), whereas it decreased progressively from early lactation to mid-lactation ($38 \pm 6\%$, $19 \pm 4\%$ and $7 \pm 1\%$ in 1-day, 7-day and 15-day lactating mice respectively, compared with virgin controls; means $\pm$ S.E.M., $P \leqslant 0.05$, $n = 3$ or 4). On day 30 of lactation, UCP1 protein levels were only partly recovered ($33 \pm 6\%$ compared with virgin controls; $P \leqslant 0.05$, $n = 3$). Similarly, a marked decrease in UCP3 protein in mitochondria from BAT was present during lactation ($19 \pm 5\%$, $21 \pm 4\%$ and $18 \pm 4\%$ in 1-day, 7-day and 15-day lactating mice respectively; $P \leqslant 0.05$, $n = 3$ or 4). Spontaneously weaned dams (day 30 of lactation) had UCP3 levels in mitochondria similar to those in virgin mice ($87 \pm 14\%$ of virgin control values). Abrupt weaning (for 24 h) of mice in mid-lactation did not significantly modify the UCP1 and UCP3 mitochondrial protein levels. Changes in the content of UCP1 and UCP3 protein were specific, because cytochrome *c* was unaltered during the breeding cycle (Figure 2).

Effects of fasting on the mRNA levels of UCPs and the mitochondrial content of UCP1 and UCP2 in BAT from late-pregnant and lactating mice

The effects of 24 h of fasting on the mRNA expression of UCPs in BAT were determined in virgin, late-pregnant or mid-lactating

Table 1 Effects of fasting for 24 h on the abundance of UCP1, UCP2 and UCP3 mRNA species in BAT of virgin, late-pregnant and mid-lactating mice

Virgin, 19-day pregnant and 15-day lactating mice were fasted for 24 h and BAT was studied. RNA (20 μg) was analysed by Northern blot hybridization and results were quantified by densitometric scanning. Results are expressed as percentages of virgin control values and are means ± S.E.M. for three independent experiments. *Statistically significant difference ($P \leqslant 0.05$) compared with the corresponding values in fed mice.

Animals	Treatment	UCP1 mRNA (% of control)	UCP2 mRNA (% of control)	UCP3 mRNA (% of control)
Virgin	Fed	100 ± 21	100 ± 28	100 ± 18
	Fasted	47 ± 8*	210 ± 22*	91 ± 24
Pregnant	Fed	111 ± 19	124 ± 15	73 ± 18
	Fasted	51 ± 16*	196 ± 20*	117 ± 22
Lactating	Fed	7 ± 2	78 ± 19	6 ± 1
	Fasted	11 ± 3	230 ± 44*	39 ± 8*

Table 2 Effects of litter size on the abundance of UCP1, UCP2 and UCP3 mRNA species in BAT of 15-day lactating mice

Litter sizes were adjusted at birth to 4, 10 or 18 pups and BAT from 15-day lactating dams was studied. RNA (20 μg) was analysed by Northern blot hybridization and results were analysed by densitometric scanning. Results are expressed as percentages of virgin control values and are means ± S.E.M. for three independent experiments. *Statistically significant difference ($P \leqslant 0.05$) compared with virgin controls (see Table 1); †statistically significant difference ($P \leqslant 0.05$) compared with dams nursing normal-sized litters (10 pups).

Litter size (number of pups)	UCP1 mRNA (% of control)	UCP2 mRNA (% of control)	UCP3 mRNA (% of control)
4	76 ± 12†	82 ± 22	59 ± 7*†
10	7 ± 1*	76 ± 1	19 ± 4*
18	11 ± 4*	41 ± 5*†	26 ± 8*

mice (Table 1). Expression of UCP1 mRNA decreased in fasted virgin mice and the same response was found in late-pregnant mice. However, in 15-day lactating mice, which showed very low levels of UCP1 mRNA, fasting did not cause a further decrease. Fasting up-regulated UCP2 mRNA expression in BAT by approx. 2–3 fold in virgin, pregnant and lactating mice. UCP3 mRNA expression was unaltered by fasting in BAT from virgin or pregnant mice. However, it was significantly up-regulated when lactating mice were fasted, although not to the levels found in virgin controls.

No significant changes were observed in the content of UCP1 and UCP3 in BAT mitochondria owing to fasting of virgin, late-pregnant or mid-lactating mice for 24 h (results not shown).

Effects of litter size on the mRNA levels of UCPs and the content of UCP1 and UCP3 proteins in BAT from lactating mice

Dams nursing litters of different sizes showed different levels of UCP1 mRNA expression in BAT. Down-regulation of UCP1 mRNA levels owing to lactation was prevented in mice nursing only four pups (Table 2). Dams nursing 18 pups showed the same UCP1 mRNA levels as those nursing ten pups. UCP2 mRNA was altered only in dams nursing large litters; UCP2 mRNA expression in dams nursing 18 pups was approximately half of that in virgin controls. UCP3 mRNA expression in dams behaved similarly to that of UCP1 mRNA: the decrease due to lactation was partly prevented when litters were small and equally low when dams nursed normal-sized or large litters. When the content of UCP1 protein in BAT mitochondria was determined under these experimental situations, the only significant change observed was that dams nursing small litters showed a less marked decrease (54 ± 15 % compared with virgins; $P \leqslant 0.05$, $n = 3$) than dams nursing normal-sized litters. For UCP3 protein, no significant changes due to litter size were observed.

Table 3 Effects of a high-fat diet on the abundances of UCP1, UCP2 and UCP3 mRNA species in BAT of virgin and lactating mice

Lactating mice were fed after parturition with a high-fat or a high-carbohydrate diet and studied on day 15 of lactation. Virgin mice were fed for an equivalent period (15 days) with the two different diets. RNA (20 μg) was analysed by Northern blot hybridization and results were quantified by densitometric scanning. Results are expressed as percentages of virgin control values and are means ± S.E.M. for four independent experiments. *Statistically significant difference ($P \leqslant 0.05$) between groups treated with the two different diets.

Animals	Diet	UCP1 mRNA (% of control)	UCP2 mRNA (% of control)	UCP3 mRNA (% of control)
Virgin	High-carbohydrate	100 ± 24	100 ± 16	100 ± 21
	High-fat	85 ± 19	117 ± 11	166 ± 33
Lactating	High-carbohydrate	11 ± 3	109 ± 21	13 ± 2
	High-fat	89 ± 11*	155 ± 31	45 ± 14*

Effects of a high-fat diet on the mRNA levels of UCPs and the content of UCP1 and UCP3 proteins in BAT from virgin or lactating mice

Mice were treated with a high-fat diet from delivery to day 15 of lactation and the effects on the mRNA expression of UCPs were determined. A parallel study in virgin mice fed with the high-fat diet for the same duration (15 days) was undertaken. This diet resulted in an average increase in energy intake of 22.1 % in virgin mice and 22.5 % in lactating dams. UCP1 mRNA was unaffected by the high-fat diet in virgin mice but was up-regulated in lactating mothers (Table 3). The down-regulation of UCP1 mRNA due to lactation was prevented almost completely by the high-fat diet. UCP2 mRNA was not affected significantly by the diet. UCP3 mRNA was not affected significantly by the diet in virgin mice, whereas the high-fat diet caused an increase in UCP3 mRNA expression in lactating mice compared with dams fed with the control diet. Figure 3(a) shows a representative Northern blot analysis of these changes.

Neither the UCP1 nor UCP3 content in BAT mitochondria was significantly modified by the high-fat diet in virgin mice (Figure 3b). However, the relative abundances of both proteins were increased in lactating mice fed with the high-fat diet in comparison with dams fed with the high-carbohydrate diet (4.2 ± 0.7-fold and 4.9 ± 0.7-fold increases for UCP1 and UCP3 proteins respectively; $P \leqslant 0.05$, $n = 3$).

Effects of bezafibrate, WY-14643 or troglitazone on the mRNA levels of UCPs in BAT from virgin or lactating mice

Table 4 shows the effects of single injections of bezafibrate, a preferential PPARα activator [31], WY-14,643, a specific PPARα activator [32], and troglitazone, a PPARγ activator [33], on the expression of UCP mRNA species in BAT. None of these compounds affected UCP1 mRNA or UCP2 mRNA levels in virgin mice, although the two PPARα activators increased UCP3 mRNA. In contrast, all three agonists increased the expression of UCP1, UCP2 and UCP3 mRNA species in lactating dams, although to differing extents: bezafibrate, WY-14,643 and troglitazone caused a 3–5-fold induction of UCP1 mRNA and a 2-fold induction of UCP2 mRNA, whereas UCP3 mRNA was

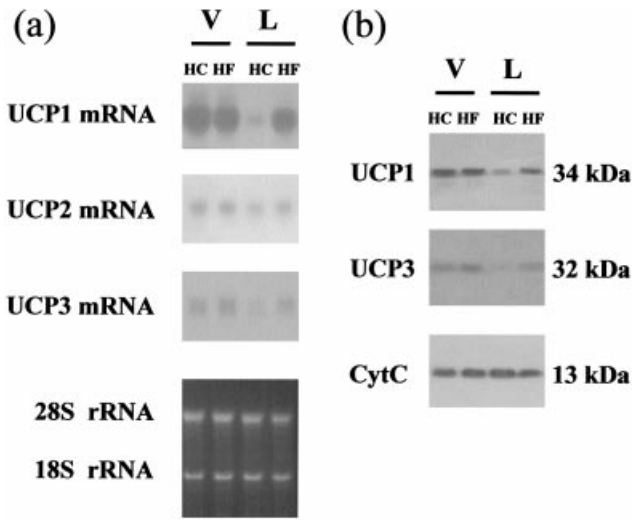


Figure 3 Representative blots of levels of UCP mRNA species and the content of UCP1 and UCP3 protein in BAT from virgin and lactating mice fed with a high-fat diet

(**a**) Example of Northern blot analysis of RNA (20 μg per lane) from interscapular BAT probed for UCP1, UCP2 and UCP3 mRNA detection (top panels) and ethidium bromide staining of the gel showing equal loading of RNA samples (bottom panel). (**b**) Example of immunoblot analysis of 15 μg of mitochondrial protein from interscapular BAT with specific antibodies against UCP1, UCP3 and cytochrome *c*. V, virgin controls; L, 15-day lactating dams; HC, mice fed with the high-carbohydrate diet; HF, mice fed with the high-fat diet.

Table 4 Effects of bezafibrate, WY-14,643 and troglitazone on the abundances of UCP1, UCP2 and UCP3 mRNA species in BAT of virgin and lactating mice

Virgin or 15-day lactating mice were injected intraperitoneally with bezafibrate (100 μg/g body weight), WY-14,643 (50 μg/g body weight), troglitazone (100 μg/g body weight) or vehicle solution; BAT was studied. RNA (20 μg) was analysed by Northern blot hybridization and results were analysed by densitometric scanning. Results are expressed as percentages of vehicle-treated virgin mice and are means $\pm$ S.E.M. for three independent experiments. *Statistically significant difference ($P \leqslant 0.05$) between treated groups and vehicle controls in virgin or lactating mice.

Animals	Treatment	UCP1 mRNA (% of control)	UCP2 mRNA (% of control)	UCP3 mRNA (% of control)
Virgin	Vehicle	100 $\pm$ 11	100 $\pm$ 18	100 $\pm$ 14
	Bezafibrate	85 $\pm$ 18	118 $\pm$ 21	177 $\pm$ 20*
	WY-14,643	120 $\pm$ 13	106 $\pm$ 17	210 $\pm$ 31*
	Troglitazone	91 $\pm$ 11	85 $\pm$ 18	117 $\pm$ 22
Lactating	Vehicle	16 $\pm$ 5	81 $\pm$ 18	33 $\pm$ 6
	Bezafibrate	89 $\pm$ 11*	179 $\pm$ 22*	95 $\pm$ 24*
	WY-14,643	78 $\pm$ 15*	166 $\pm$ 18*	310 $\pm$ 29*
	Troglitazone	55 $\pm$ 6*	201 $\pm$ 17*	71 $\pm$ 22*

induced 10-fold by WY-14,643 and only 2–3-fold by bezafibrate or troglitazone.

DISCUSSION

We report that UCP1 mRNA is down-regulated during lactation but recovers after spontaneous or sudden weaning. This resulted in a decrease in UCP1 protein in BAT mitochondria from lactating mice and some recovery after spontaneous weaning but not 24 h after sudden weaning of mice in mid-lactation. These findings are similar to those described previously for rats or mice, except that late-pregnant rats already show a decrease in UCP1 mRNA levels [27,34]. The decreased expression of the gene encoding UCP1 has been proposed to be involved in the decrease of BAT thermogenesis during lactation. Our results show that, whereas UCP1 and UCP3 mRNA species were down-regulated during lactation, UCP2 was not. Thus either there is a post-transcriptional regulation of gene expression or UCP2 is not involved in the regulatory metabolic modifications taking place in BAT during the breeding cycle. To answer these points, further research on the content of the UCP2 protein in mitochondria will require the development of highly specific antibodies that are not yet available. In contrast, the potentially 'compensatory' up-regulation of UCP2 mRNA in mice when UCP1 mRNA expression is suppressed by targeted disruption of the gene encoding UCP1 [11] does not occur in association with the physiological suppression of UCP1 mRNA expression owing to lactation. In skeletal muscle of lactating mice, UCP3 mRNA is down-regulated [30], whereas UCP2 mRNA expression is unaltered (N. Pedraza and F. Villarroya, unpublished work). However, UCP2 mRNA is up-regulated in the uterus of pregnant rats [35]. These findings indicate a tissue-specific regulation of the gene encoding UCP2 during the breeding cycle.

The down-regulation of UCP3 mRNA and UCP3 protein levels during lactation and their recovery after weaning, which is similar to those for UCP1, might have various interpretations. It might be involved in the decrease in mitochondrial thermogenesis in lactation. Similarly, if UCP3 is specifically related to the fatty acid oxidation rates in mitochondria, a decrease in UCP3 content would be consistent with the low oxidation rate of fatty acids expected in BAT during lactation, when the oxidation of metabolic fuels for thermogenesis is impaired. The same reasoning can be applied to the experiments with litters of different sizes: when litters are small, both thermogenesis and lipid oxidation in the tissue are less depressed [36] and UCP1 mRNA and UCP3 mRNA are less down-regulated, although the impact of this modification on the levels of UCP proteins is observed only for UCP1. An identical conclusion can be reached from experiments in which the energy stress due to lactation was partly prevented by feeding dams with a high-fat diet: again, the down-regulation of UCP1 and UCP3 mRNA species and mitochondrial proteins was prevented, in whole or in part. Thus a parallel was observed for the regulation of expression of the genes encoding UCP1 and UCP3 in different periods of the breeding cycle and in response to nutritional manipulations, with the sole exception of the response to fasting.

UCP1 mRNA expression was down-regulated by fasting of virgin control mice for 24 h, as reported previously for fasted rats [37], although it did not lead to a significant modification in the content of the UCP1 protein in BAT mitochondria. However, UCP3 mRNA and protein levels were unaltered. Whereas the fasting of lactating dams did not further decrease the low levels of UCP1 mRNA expression, it caused a significant increase in UCP3 mRNA expression. This last observation was quite surprising and will require further consideration because neither thermogenesis nor the oxidation of fatty acids are expected to be enhanced in BAT when lactating dams are fasted for 24 h. Moreover, the different responses to fasting of UCP1 and UCP3 mRNA expression in lactating mice indicate that, although these two genes are likely to share common regulatory mechanisms, they must also have differential regulatory pathways. The findings that the decrease in UCP1 mRNA caused by fasting for 24 h did not lead to changes in UCP1 protein, and the up-regulation of UCP3 mRNA in fasted lactating dams did not modify mitochondrial UCP3 protein content, indicate that changes in the

mRNA levels need a longer duration for translation before they reach detectable protein levels. This has already been observed for changes in UCP3 gene expression in skeletal muscle [30].

Decreased sympathetic activity has been considered to be a major regulatory event leading to the down-regulation of the gene encoding UCP1 during lactation [38,39]. However, because sympathetic activity determines lipolysis rates and lipoprotein lipase activity [40,41] in BAT, it is difficult to assess to what extent the adrenergic regulation of gene expression in BAT might be due to a direct cAMP-dependent influence on gene promoters or to the action of lipid-derived molecules formed owing to lipid catabolism, via PPAR-responsive gene elements. We addressed the issue of whether the UCP genes were differently responsive to PPAR agonists *in vivo* and used acute injections of PPAR agonists with different specificities for PPAR subtypes. We observed previously that UCP1 mRNA expression is sensitive to PPARα or PPARγ agonists *in vivo* in lactating dams [42]. This is consistent with the presence of a PPARγ and PPARα response element in the UCP1 gene enhancer [42] and the highest sensitivity of lactating dams might be due to depressed basal levels of this signalling pathway in a physiological situation of depressed lipid catabolism and to the generation of endogenous PPAR-dependent signals. A similar observation was made for UCP2 mRNA regulation, which responds to PPARγ agonists in brown adipocytes in culture [17,18]. In the present study we observed that UCP3 mRNA expression was also responsive to PPARα and PPARγ agonists but it showed a much higher sensitivity to PPARα activators than the genes encoding UCP1 and UCP2: PPARα activators induced UCP3 mRNA expression even in virgin mice and they caused a maximal activation (10-fold induction) in lactating dams. The activation of expression of the gene encoding UCP3 by PPARα agonists has been reported in muscle in newborns [43] and PPARα-responsive elements are present in the human UCP3 gene promoter (G. Solanes, N. Pedraza and F. Villarroya, unpublished work). Target genes for PPARα usually encode components of the lipid oxidation machinery [44]; the preferential activation of the gene encoding UCP3 by PPARα activators supports the involvement of UCP3 in these metabolic pathways. Thus UCP3 mRNA down-regulation during lactation might be elicited by the decreased fatty acid catabolism in BAT during this period and might be part of the metabolic adaptations associated with the decrease in fuel oxidation when energy sparing is needed.

Recently, transgenic mice with targeted disruption of the UCP3 gene have been generated, and no major disturbances have been detected in thermogenesis and fuel energy metabolism [13,14] despite some impairment in the shift from fatty acid storage to oxidation induced in muscle in response to fasting [45]. A protective role for UCP3 in relation to the mitochondrial production of reactive oxygen species when fatty acids are oxidized has been described on the basis of the phenotype of these mice [13]. Moreover, transgenic mice overexpressing UCP3 in muscle have a decreased body weight despite hyperphagia [15]. Further research will be needed to establish the physiological meaning of the adaptive impairment in expression of the gene encoding UCP3 during lactation in the light of these new insights into the role of UCP3 in mitochondrial function.

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