Impaired Expression of the Uncoupling Protein-3 Gene in Skeletal Muscle During Lactation

Fibrates and Troglitazone Reverse Lactation-Induced Downregulation of the Uncoupling Protein-3 Gene

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The expression of uncoupling protein (UCP)-3 mRNA in skeletal muscle is dramatically reduced during lactation in mice. The reduction in UCP-3 mRNA levels lowers the amount of the UCP-3 protein in skeletal muscle mitochondria during lactation. Spontaneous or abrupt weaning reverses the downregulation of the UCP-3 mRNA but not the reduction in UCP-3 protein levels. In lactating and virgin mice, however, fasting increases UCP-3 mRNA levels. Changes in UCP-3 mRNA occur in parallel with modifications in the levels of free fatty acids, which are reduced in lactation and are upregulated due to weaning or fasting. Modifications in the energy nutritional stress of lactating dams achieved by manipulating litter sizes do not influence UCP-3 mRNA levels in skeletal muscle. Conversely, when mice are fed a high-fat diet after parturition, the downregulation of UCP-3 mRNA and UCP-3 protein levels due to lactation is partially reversed, as is the reduction in serum free fatty acid levels. Treatment of lactating mice with a single injection of bezafibrate, an activator of the peroxisome proliferator-activated receptor (PPAR), raises UCP-3 mRNA in skeletal muscle to levels similar to those in virgin mice. 4-chloro-6-[(2,3-xylidine)-pirimidinylthio] acetic acid (WY-14,643), a specific ligand of the PPAR-α subtype, causes the most dramatic increase in UCP-3 mRNA, whereas troglitazone, a specific activator of PPAR-γ, also significantly increases UCP-3 mRNA abundance in skeletal muscle of lactating mice. However, in virgin mice, bezafibrate and WY-14,643 do not significantly affect UCP-3 mRNA expression, whereas troglitazone is at least as effective as it is in lactating dams. It is proposed that the UCP-3 gene is regulated in skeletal muscle during lactation in response to changes in circulating free fatty acids by mechanisms involving activation of PPARs. The impaired expression of the UCP-3 gene is consistent with the involvement of UCP-3 gene regulation in the reduction of the use of fatty acids as fuel by the skeletal muscle and in impaired adaptative thermogenesis, both of which are major metabolic adaptations that occur during lactation. *Diabetes* 49:1224–1230, 2000

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ANT, adenine nucleotide translocase; COII, cytochrome oxidase subunit II; ECL, enhanced chemiluminescence; PPAR, peroxisome proliferator-activated receptor; UCP, uncoupling protein; UCP-3L, the long isoform of UCP-3.

Uncoupling protein (UCP)-2 and UCP-3 are 2 recently cloned genes that have a high sequence homology with the brown adipose tissue UCP-1. They uncouple oxidative phosphorylation when transfected into yeast, and, by analogy with UCP-1, they are considered to be potentially involved in regulatory thermogenesis (1–4). The UCP-2 and UCP-3 genes are located adjacent in a region of human chromosome 11, which coincides with quantitative trait loci for obesity and type 2 diabetes (5). The UCP-3 gene has attracted attention due to its preferential expression in thermogenic tissues, brown fat, and skeletal muscle in rodents and skeletal muscle in humans. However, recent studies indicate that the physiological role of UCP-3 as a mediator of thermogenesis in muscle is quite complex. For example, UCP-3 mRNA abundance is unaltered in situations of enhanced regulatory thermogenesis in muscle, such as long-term cold exposure (6), whereas UCP-3 mRNA is upregulated in situations of depressed muscle thermogenesis, such as starvation (7,8). In fact, most of the physiological or pathological situations reported to date in which UCP-3 gene expression in skeletal muscle is altered (i.e., during instances of high-fat diets, postnatal development, or streptozotozin-induced diabetes) are associated with parallel changes in circulating free fatty acids (9–11). Fatty acids themselves have been reported to upregulate UCP-3 gene expression in muscle (7,12), most likely by activating peroxisome proliferator-activated receptor (PPAR)-α (12), and it has been proposed that UCP-3 could be specifically involved in the regulation of the use of lipids as fuel substrates in skeletal muscle. Decreased UCP-3 mRNA expression has been described in the muscle of type 2 diabetic patients (13), a situation associated with decreased rates of lipid oxidation by skeletal muscle (14), although other studies did not confirm this finding (15). Moreover, several studies in obese and type 2 diabetic humans show an association between polymorphisms in the UCP-3 gene and a reduction in basal lipid oxidation (16,17).

The breeding cycle is a physiological situation associated with dramatic metabolic adaptations and changes in energy balance. A major increase in energy requirements occurs during lactation due to the needs of milk production. Well-nourished women meet most of the additional energy costs of lactation by increasing food intake, and there is little evidence of energy-sparing adaptations involving basal metabolic rate or adaptative thermogenesis (18). However, a reduction in diet-induced thermogenesis has been demonstrated in lactating women under nutritionally unfavorable conditions (19), and it has been interpreted as a way to save energy in periods when food restriction overlaps with a high-energy output because of lactation. The molecular mechanisms for this adaptation are not known, but skeletal muscle has been reported to be involved (20). In rodents, an overall increase in the efficiency of energy utilization and a reduction in adaptative thermogenesis develop in lactation, even in well-nourished animals (21). Functional atrophy of brown fat, a major thermogenic tissue in rodents, and a reduced expression of the UCP-1 gene take place during lactation (22,23), and these have been proposed to contribute to energy sparing during this period. Lactation is a unique physiological situation in which an adaptative decrease in energy expenditure appears in association with hyperphagia. This phenomenon also occurs in many cases of obesity, and lactation constitutes an excellent model to assess the molecular mechanisms that determine adaptative changes in energy expenditure and inter-organ metabolic partitioning. During lactation, metabolic adaptations develop to promote the utilization of metabolic fuels by the mammary gland. In lactating mothers, glucose and fatty acids are channeled to the mammary gland for the synthesis of milk, whereas other tissues, including skeletal muscle, reduce their use of metabolic fuels for oxidation (24).

Here, we report that the expression of the UCP-3 gene is suppressed in the skeletal muscle of lactating mice, even though UCP-3 mRNA expression remains highly sensitive to induction by physiological (fasting, weaning) or pharmacological (PPAR activators) stimuli.

RESEARCH DESIGN AND METHODS

Materials. Bezafibrate was acquired from Sigma (St. Louis, MO) and 4-chloro-6-[(2,3-xylidine)-pirimidinylthio]acetic acid (WY-14,643) was acquired from Cayman Chemicals (Ann Arbor, MI). Troglitazone was provided by Glaxo Wellcome (Greenford, U.K.).

Animals. Adult female Swiss mice were used. They were maintained under standard conditions of illumination (12-h light/dark cycle) and temperature (21 ± 1°C) and were fed a standard diet composed of 72% (in gross energy) carbohydrate, 6% fat, and 22% protein (B.K. Universal, Barcelona, Spain), unless otherwise indicated. Female mice were mated with adult males, and the day of pregnancy was determined by the presence of spermatozoa in vaginal smears. When lactating mice were studied, 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. Pregnant (day 19), lactating (days 1, 7, 15, and 30), abruptly weaned dams (24 h after removal of 15-day lactating pups), and virgin control mice were studied in basal conditions. The effects of fasting were determined after 24-h suppression of food to 15-day lactating dams and virgin controls. The effects of a high-fat diet during lactation were assessed by replacing the standard diet by a diet composed of 36% (in gross energy) carbohydrate, 42% fat, and 22% protein (Harlan Teklad, Madison, WI) after parturition, and dams were studied on day 15 of lactation. When indicated, mice were treated with a single intraperitoneal injection of bezafibrate (100 µg/g body wt), WY-14,643 (50 µg/g body wt), or troglitazone (100 µg/g body wt) in 50% dimethyl sulfoxide/saline. Controls were given equivalent volumes of the 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 6-h treatments. Mice were killed by decapitation at the beginning of the light cycle, except for those mice in experiments with PPAR activators, in which injections were performed at the beginning of the light cycle and animals were killed 6 h later. The gastrocnemius, extensor digitorum longus, and tibialis anterior skeletal muscles were dissected and blood was collected. Skeletal muscle from mouse fetuses and spleen from virgin controls were also obtained for comparative purposes in immunoblot assays of mitochondrial proteins.

RNA isolation and Northern blot hybridization. RNA from skeletal muscles was prepared using a guanidine thiocyanate method (25). RNA (20 µg) was denatured, electrophoresed on 1.5% formaldehyde-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 equal loading of gels and transfer efficiency. Prehybridization and hybridization were performed at 55°C using 0.25 mol/l Na_2HPO_4 (pH 7.2), 1 mmol/l EDTA, 20% SDS, and 0.5% blocking reagent (Boehringer Mannheim) solution. Blots were hybridized using as probes the human cDNA for UCP-3 (4) and the mouse cDNA for the mitochondrial genome–encoded cytochrome oxidase subunit II (COII) (26) that was used as a control. The DNA probes were labeled with an [α-^{32}P]dCTP using a random oligonucleotide-primer method. Hybridization signals were quantified using Molecular Image System GS-525 (Bio-Rad, Richmond, CA).

Immunoblot analysis. Mitochondria were isolated from gastrocnemius and whole skeletal muscle from the legs of fetuses and from adult mouse spleens (27). Samples containing 20 µg mitochondrial protein were mixed with equal volumes of 2 × SDS loading buffer, incubated at 90°C for 5 min, and electrophoresed on SDS/12%-polyacrylamide gels. Proteins were transferred to polyvinylidene difluoride membranes, and immunological detection was performed using a rabbit affinity-pure UCP-3 antiserum (Alpha Diagnostic, San Antonio, TX). This antibody was generated against a 17–amino acid peptide sequence located at the second and third transmembrane domain of human UCP-3, showing 71% homology with mouse UCP-3 and no significant homology with UCP-1 or UCP-2. It was used at a 4-µg/ml dilution, and detection was achieved using the enhanced chemiluminescence (ECL) detection system (Amersham, Amersham, U.K.). As a positive control, 293 cells stably transfected with a tetracycline-inducible (Tet-On; Clontech, Palo Alto, CA) construct driving the long isoform of human UCP-3 (UCP-3L) cDNA expression (293-U3 cells) were used (B. Sibille, G.S., F.V., unpublished data). Blots were stripped thereafter and probed with a rabbit antiserum against bovine heart adenine nucleotide translocase (ANT), a gift of Dr. G. Brandolin (DBMS/Biochimie, Grenoble, France), which, according to previously established procedures (27), was used as a control of equal abundance of mitochondrial membrane protein in the samples. The sizes of the proteins detected were estimated using protein molecular-mass standards (Bio-Rad). Quantitation of autoradiographs and ECL signals was performed by scanning densitometry.

Serum free fatty acid levels were quantified using a colorimetric acyl-CoA synthase and acyl-CoA oxidase-based method (Wako Chemicals, Neuss, Germany).

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

RESULTS

UCP-3 mRNA expression in skeletal muscle is suppressed in lactating mice. Weaning reverses the down-regulation of UCP-3 mRNA expression. Figure 1 shows the UCP-3 mRNA levels in the gastrocnemius muscle of female virgin controls, late-pregnant mice, and lactating mice at different days of lactation. UCP-3 mRNA levels were similar in female virgin and late pregnant mice. Lactating mice showed a dramatically lowered expression of UCP-3 mRNA, and, in mid-lactating dams, UCP-3 mRNA was hardly detectable in the conditions of Northern blot assays that allow an optimal detection in virgin controls (Fig. 1). The impairment in UCP-3 mRNA expression in mid-lactating mice was equally observed in other skeletal muscles: UCP-3 mRNA levels in tibialis anterior and extensor digitorum longus muscles from 15-day lactating dams were, respectively, 8 ± 4 and 14 ± 5% of those in the corresponding virgin controls. The drop in UCP-3 mRNA expression in skeletal muscle occurred suddenly after birth, and, after only 1 day of lactation, UCP-3 mRNA abundance decreases to less than one-tenth of that in pregnant dams just before parturition. Thirty days after parturition, when pups had spontaneously substituted milk for standard diet, UCP-3 mRNA levels in dams

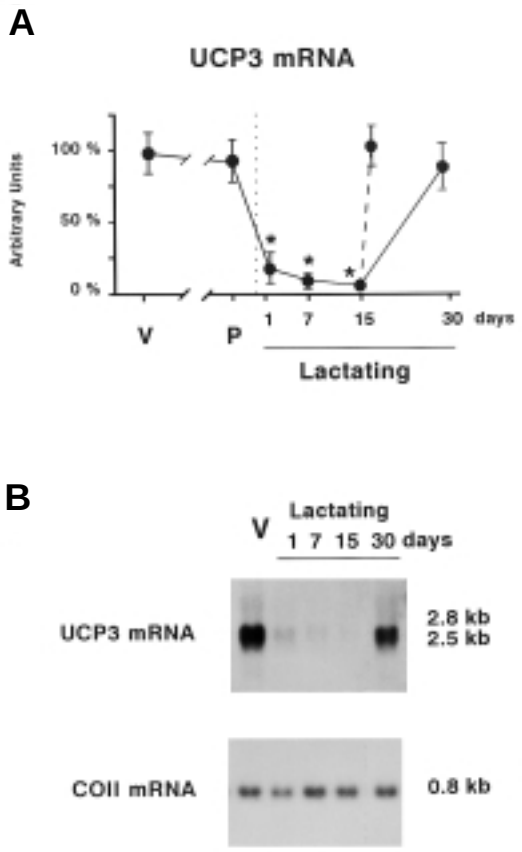

FIG. 1. Changes in UCP-3 mRNA levels in the gastrocnemius skeletal muscle of late pregnant and lactating mice. *A*: Representation of the abundance of UCP-3 mRNA in virgin control mice (V), 19-day pregnant mice (P), and mice at the indicated days of lactation. Discontinuous line indicates abrupt weaning of 15-day lactating dams. Points are means ± SE (indicated by bars) of the hybridization intensity signals of 3–4 samples. The 2.8- and 2.5-kb transcripts for UCP-3 were additively quantified as a single point. Data are expressed as the percentage relative to the virgin control value. Statistical comparisons between virgin controls and experimental groups are shown by *$P \leq 0.05$. *B*: Representative Northern blot analysis of 20 µg/lane of RNA from gastrocnemius. The sizes of the UCP-3 and COII transcripts are depicted to the right.

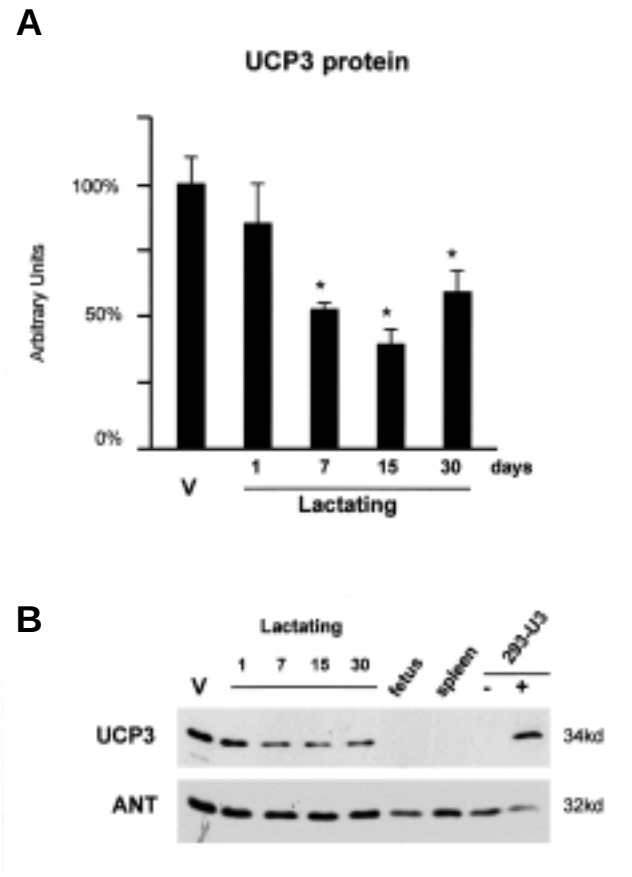

FIG. 2. Changes in mitochondrial UCP-3 abundance in gastrocnemius skeletal muscle during lactation. *A*: Abundance of UCP-3 in virgin control mice (V) and mice at the indicated days of lactation. Bars are means ± SE of the Western blot intensity signals of 3–4 samples. Data are expressed as the percentage relative to the virgin control value. Statistical comparisons between virgin controls and experimental groups are shown by *$P \leq 0.05$. *B*: Representative immunoblot analysis of the UCP-3 content in mitochondrial preparations of gastrocnemius from virgin mice (V), mice at the indicated days of lactation, and mitochondria from 18-day fetal mouse skeletal muscle (fetus) or adult spleen. 293-U3 lanes are mitochondria preparations from 293 cells stably transfected with a tetracycline-inducible human UCP-3L cDNA construct nonexposed (–) or exposed (+) to tetracycline. Of mitochondrial protein, 20 µg was loaded in each lane, except for 293 cells (10 µg), and was immunoblotted using a rabbit anti–UCP-3 antibody (upper lane) and, subsequently, a rabbit anti-ANT antibody as a control (lower lane). The sizes of the signals obtained are shown to the right of the lanes.

returned to control values. However, the effects of weaning on UCP-3 mRNA did not require a long duration. When 15-day lactating dams were abruptly separated from their offspring for 24 h, UCP-3 mRNA abundance was upregulated and reached levels similar to those of controls. The reduction in the expression of UCP-3 mRNA during lactation and the upregulation due to weaning occurred in parallel for the 2.5- and 2.8-kb mRNA species of UCP-3 (Fig. 1*B*). All of these changes occurred without modifications in the mRNA levels for COII, which were used as a control of overall mitochondrial biogenesis.

UCP-3 abundance is reduced in skeletal muscle mitochondria from lactating mice. To assess whether the reduction in UCP-3 mRNA expression results in a decrease in the relative amount of UCP-3 protein in skeletal muscle mitochondria from lactating mice, immunoblot assays of UCP-3 were performed using a specific antibody (see RESEARCH DESIGN AND METHODS). Analysis of mitochondrial protein from adult mouse gastrocnemius resulted in the detection of a single 34-kD band. 293 cells stably transfected with a tetracycline-inducible construct driving the expression of human UCP-3L. cDNA showed no signal (noninduced) or a strong (tetracycline-induced) 34-kD signal, the same size as that observed in muscle samples (Fig. 2*B*). No 34-kD signal was detected in mitochondria from fetal muscle or spleen in which UCP-2 mRNA but not UCP-3 mRNA is expressed (6,12). This confirms the specificity of UCP-3 detection. Figure 2*B* shows an immunoblot analysis of UCP-3 protein levels in mitochondria isolated from the gastrocnemius muscle of virgin or lactating mice at different days of lactation. Homogeneity of mitochondrial preparations of muscles from virgin and lactating mice was assessed as equal signals in immunoblot analysis of the ANT, a mitochondrial protein unaltered in skeletal muscle during lactation (N.P., F.V.,

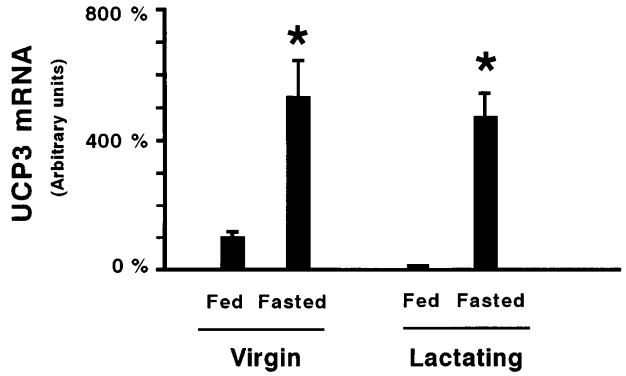

FIG. 3. Effects of 24-h fasting of virgin and 15-day lactating mice on UCP-3 mRNA expression in gastrocnemius muscle. Bars are means ± SE of the hybridization intensity signals of at least 3 samples. Data are expressed as the percentage relative to the fed virgin value. Statistical significance of comparisons between fed and fasted groups for each experimental situation are shown by *$P \leq 0.05$.

unpublished data). A significant decrease in UCP-3 protein levels was achieved on days 7 and 15 of lactation, and spontaneously weaned 30-day lactating dams already showed lower UCP-3 protein levels than those of virgin controls (Fig. 2*A*). 24-h weaning of 15-day lactating dams did not reverse UCP-3 protein downregulation, which remained at 44 ± 11% of virgin control values.

Effects of fasting on UCP-3 gene expression in skeletal muscle of lactating mice. The effects of 24-h starvation on UCP-3 mRNA in gastrocnemius from virgin and lactating mice were determined to assess whether the impairment in UCP-3 mRNA expression of lactating mice affects their response to fasting. Fasting of female virgin mice caused a significant rise in UCP-3 mRNA expression (Fig. 3), which is in agreement with previous reports (6,28). Although the basal levels of UCP-3 mRNA were very low in lactating dams, after 24 h of fasting, they rose dramatically to similar levels to those in fasted virgin mice. Thus, there was a >50-fold induction of UCP-3 mRNA in gastrocnemius from fasted lactating dams compared with fed lactating controls. The same behavior was observed for extensor digitorum longus and tibialis anterior muscles (data not shown). COII mRNA levels were essentially unaltered due to fasting in any of the experimental groups. However, the relative content of UCP protein in mitochondrial preparations of either fasted virgin or fasted lactating mice, as compared with fed animals, was unaltered: 115 ± 25% in fasted vs. fed virgins and 52 ± 12% in fasting vs. fed lactating dams.

Changes in litter size modify energy nutritional stress in lactating mice but do not affect UCP-3 mRNA expression in skeletal muscle. As an experimental approach to assess the role of the nutritional stress during lactation in the UCP-3 gene downregulation in muscle, litter sizes were manipulated. Litters were adjusted after birth to a low size (4 pups) or a high size (18 pups) as a way to decrease or increase, respectively, the output of milk by lactating dams. This experimental model is known to raise or lower, respectively, nonshivering thermogenesis and brown adipose tissue thermogenic activity in lactating mothers (29). The impact of litter size on nutritional stress was revealed by the fact that, on day 15 of lactation, the total weight of the litters consisting of 4 pups was 45.6 ± 1.2 g, 91% of the dam's body weight, whereas it was 108.0 ± 3.6 g in litters consisting of 18 pups, 216% of the dam's weight. However, UCP-3 mRNA expression in skeletal muscle of dams was not significantly altered by these changes in litter size. UCP-3 mRNA levels in dams nursing small or large litters were 128 ± 23 and 106 ± 19%, respectively, vs. lactating controls. It is concluded that modulation of overall energy nutritional stress is not a major determinant of UCP-3 mRNA expression during lactation.

TABLE 1
Free fatty acid levels in serum of virgin control, late pregnant, and mid-lactating mice under different experimental conditions

Animals	Serum free fatty acid levels (μmol/l)
Virgin control mice	
Baseline	1,278 ± 96
+ 24-h fasting	1,779 ± 113*
Late pregnant mice	
Day 19	972 ± 174
Mid-lactating mice	
Day 1	763 ± 102*
Day 7	494 ± 79*
Day 15	411 ± 14*
+ 24-h Fasting	729 ± 110*†
+ 24-h Weaning	1,017 ± 127†
+ High-fat diet	820 ± 134*†
Low litter size (4 pups)	534 ± 133*
Large litter size (18 pups)	525 ± 101*
Day 30	892 ± 98†

Data are means ± SE of 4 or 5 samples. *$P < 0.05$ vs. virgin controls; †$P < 0.05$ vs. 15-day lactating mice.

A high-fat diet during lactation partially reverses UCP-3 mRNA and UCP-3 protein downregulation in skeletal muscle. As a second approach to assess the role of nutrition on UCP-3 gene downregulation in skeletal muscle during lactation, dams were exposed after parturition to a high-fat diet and studied at mid-lactation. This treatment resulted in an average increase in caloric intake of 22.5% with respect to lactating mice fed a regular high-carbohydrate diet. The weight of pups from 15-day lactating mice fed a high-fat diet was higher than that of control pups (high-fat diet 8.43 ± 0.4 g, controls 6.9 ± 0.2 g, $P \leq 0.05$), thus indicating the impact of this dietary treatment on the amount of energy delivered as milk by lactating dams. UCP-3 mRNA levels in skeletal muscle of mid-lactating dams fed the high-fat diet were significantly higher (451 ± 59%, $P \leq 0.05$) than mid-lactating mice fed a regular diet, although they did not reach the levels found in virgin controls. Similar behavior was observed for UCP-3 protein: the relative concentration in lactating dams fed the high-fat diet was higher (164 ± 21%, $P \leq 0.05$) than that in lactating dams fed a regular diet.

Serum nonesterified fatty acid levels in pregnant and lactating mice: the effects of weaning, fasting, high-fat diet, and changes in litter size. Table 1 shows the changes in serum free fatty acids due to the different physiological and nutritional situations studied. Late pregnant mice did not show significant changes in serum free fatty acid levels, whereas a progressive decrease in serum free fatty acid concentrations occurred after parturition. The lowest levels of

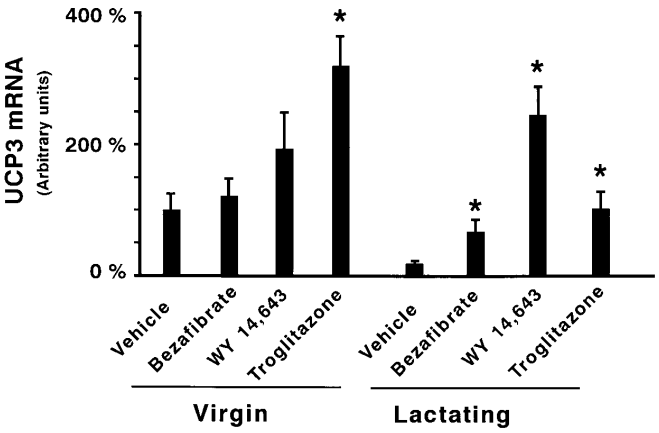

FIG. 4. Effects of PPAR activators on UCP-3 mRNA expression in skeletal muscle of virgin and lactating mice. Representation of the relative abundance of UCP-3 mRNA in gastrocnemius from virgin and 15-day lactating mice 6 h after being injected intraperitoneally with bezafibrate (100 µg/g body wt), WY-14,643 (50 µg/g body wt), troglitazone (100 µg/g body wt), or vehicle solution. Data are expressed as the percentage relative to the fed virgin value. Statistical significance of comparisons between groups of PPAR activator–treated mice and their respective vehicle-treated controls are shown by *$P \leq 0.05$.

serum free fatty acids were attained in mid-lactation; this finding is in agreement with previous reports (30). Both 24 h after abrupt weaning of mid-lactating dams or after spontaneous weaning (day 30 of lactation), serum free fatty acid levels rose to levels that were not significantly different from those in virgin controls. Fasting induced a rise in serum free fatty acid concentrations in virgin and mid-lactating mice. Litter size had no effect on serum free fatty acids. Feeding dams a high-fat diet during lactation resulted in levels of serum free fatty acids higher than those in lactating dams fed a regular diet. In conclusion, there was a close positive association between changes in serum free fatty acid levels elicited by lactation and nutritional manipulations and changes in UCP-3 mRNA levels in skeletal muscle.

Differential effects of fibrates and troglitazone on UCP-3 mRNA expression in skeletal muscle of lactating mice. The present results suggest that free fatty acids may be responsible for adaptative changes in UCP-3 gene expression in skeletal muscle during lactation, and we have reported recently that fibrates, which are activators of PPAR, may mediate free fatty acid effects on the UCP-3 gene (12). The effects of single injections of the hypolipidemic drug bezafibrate, a preferential PPAR-α activator (31), WY-14,643, a highly specific activator of PPAR-α (32), and troglitazone, a thiazolidinedione specific for PPAR-γ (33), were studied in mid-lactating mice (Fig. 4). Bezafibrate or WY-14,643 administration did not significantly alter UCP-3 mRNA expression in skeletal muscle of virgin mice, whereas troglitazone induced it. When injected into lactating mice, bezafibrate induced UCP-3 mRNA levels and almost reversed the downregulation of UCP-3 gene expression in lactation. WY-14,643 caused the most dramatic increase in UCP-3 mRNA abundance in lactating dams, which achieved levels even higher than those in virgin controls. Troglitazone increased UCP-3 mRNA expression in skeletal muscle of lactating mice to a similar extent as that in virgin mice (3- to 4-fold induction in comparison with vehicle-injected lactating mice). The acute injections with the PPAR activators did not significantly modify serum nonesterified fatty acid levels in either virgin or lactating mice (data not shown).

Unaltered PPAR-α mRNA expression in skeletal muscle of lactating mice. Considering the different sensitivity to fibrates and particularly to PPAR-α–specific activation in virgin and lactating mice, PPAR-α gene expression in skeletal muscle of lactating mice was determined in comparison with virgin controls. Northern blot analysis showed that PPAR-α gene was expressed in mouse skeletal muscle as a single transcript of 8.5 kb, as already reported (12). No difference was observed in the levels of PPAR-α mRNA expression in gastrocnemius skeletal muscle between virgin and 15-day lactating mice. Densitometric scanning of 4 independent Northern blot assays indicated that PPAR-α mRNA signals in mid-lactating mice was 92 ± 28% of those found in virgin controls.

DISCUSSION

In the present study, a dramatic reduction in UCP-3 mRNA expression resulting in a decrease in the relative abundance of UCP-3 in the skeletal muscle mitochondria is described as part of the metabolic regulatory events that take place in skeletal muscle during lactation. The comparison of changes in UCP-3 mRNA and UCP-3 protein levels indicates that, as a general rule, long-term modifications in UCP-3 mRNA, such as those observed in lactation or high-fat diet, lead to significant changes in UCP-3 protein abundance in mitochondria. However, short-term modifications in UCP-3 mRNA levels, such as those elicited by 24-h fasting or 24-h weaning, had no significant effect on UCP-3 protein abundance. Indeed, previous reports on the effects of fasting showed a modest increase in UCP-3 protein only after 2-day starvation (28). In the absence of UCP-3 protein turnover studies, these results indicate a slow-acting translational and/or posttranslational regulation of the UCP-3 gene.

In the context of the current debate on the physiological role of UCP-3, two physiological events occur in lactation that are compatible with the downregulation of UCP-3 gene expression: a reduction in nonshivering thermogenesis (21) and a decrease in the utilization of fatty acids by muscle, which favors the use of these substrates by the mammary gland for milk production (24). The time-course of UCP-3 protein downregulation throughout lactation fits well with the adaptative reduction in nonshivering thermogenesis, which develops progressively as lactation proceeds (21–23). Thus, UCP-3 protein abundance is slightly decreased in dams just after parturition, when milk production is very low, whereas it attains minimal levels in mid-lactation when milk production is maximal. However, the drop in UCP-3 mRNA after parturition is abrupt and UCP-3 mRNA expression is extremely low in lactating mice at any moment of lactation, indicating that regulatory events leading to a low expression of the UCP-3 gene start long before substantial energy-sparing mechanisms are required. In this sense, circulating fatty acids are already reduced just 1 day after parturition.

Our results indicate that different stages of lactation or nutritional manipulations in the breeding period (fasting, weaning, and high-fat diet) were associated with changes in UCP-3 mRNA expression in skeletal muscle only when the levels of free fatty acids were modified in the same direction and regardless of the lipolytic activity of adipose tissue. For instance, both fasting and weaning activate UCP-3 mRNA

expression in skeletal muscle of lactating dams because they cause an increase in free fatty acid levels. However, lipolysis is activated during fasting, but it is reduced in weaning, when the rise in free fatty acids is caused by the sudden impairment of their use by the mammary gland (34). This supports the notion that fatty acids themselves regulate the expression of the UCP-3 gene in skeletal muscle during lactation. Other hormonal or metabolic signals previously reported to induce the UCP-3 gene in muscle, such as leptin or thyroid hormones, are unlikely to play a major role in UCP-3 downregulation during lactation. The levels of circulating leptin are unaltered during lactation (35), and, although lactation is associated with a mild hypothyroid state, changes in serum thyroid hormones due to fasting (36) or litter size manipulation (37) do not correlate with changes in UCP-3 mRNA expression in muscle.

Activators of PPAR reverse the UCP-3 mRNA downregulation during lactation, and they are likely to mimic the positive action of fatty acids on the UCP-3 gene. Whereas the effects of long-term treatments with fibrates or thiazolidinediones may rely on indirect metabolic mechanisms (38,39), the effectiveness of PPAR activators in the very short-term exposure shown here suggests direct effects on skeletal muscle. The ability of fibrates to induce the UCP-3 gene in muscle has been reported in newborn mice (12) and adult rats (39), and the highest potency of WY-14,643, a specific ligand of PPAR-α in lactating mice, supports a major involvement of this PPAR subtype in UCP-3 gene regulation in skeletal muscle. According to the present results, the higher sensitivity to PPAR-α activation of the UCP-3 gene expression in skeletal muscle of lactating mice was not attributable to changes in PPAR-α receptor expression. A potential explanation for these findings would be that if PPAR-α activation mimics the effect of free fatty acids on UCP-3 mRNA in skeletal muscle (12), then this pathway of stimulation would already be quite active in virgin mice because virgin mice have higher levels of serum free fatty acid levels and UCP-3 mRNA expression. As a result, a lack of sensitivity to PPAR-α activators is observed here. In most cases, target genes of PPAR-α regulation code for enzymes and proteins that are part of the lipid oxidation machinery of the cell (40), and the identification of the UCP-3 gene as a target of PPAR-α activation further supports its putative involvement in the regulation of fatty acid oxidation. Troglitazone, an antidiabetic thiazolidinedione and a specific activator of PPAR-γ (33), could also induce UCP-3 gene expression in skeletal muscle of lactating mice and normalize UCP-3 mRNA levels, but, in contrast to fibrates, it was also effective in virgin animals. This finding indicates a different pathway of regulation of UCP-3 gene expression by PPAR-γ activation, with respect to the PPAR-α–dependent pathway. Moreover, it suggests that the PPAR-γ–dependent activation of the UCP-3 gene is not related to the physiological action of fatty acids promoting UCP-3 gene expression. Although PPAR-γ is extremely low in skeletal muscle (12,33), chronic treatments of humans or rodents with troglitazone are known to improve insulin sensitivity and reduce triacylglyceride content in skeletal muscle (41). Several recent reports claim there is a direct effect of thiazolidinediones on this tissue (42,43), which would be consistent with the effects on the UCP-3 gene observed in this study. However, newborn mice, which are highly sensitive to upregulation of UCP-3 gene expression in response to PPAR-α activators, are poorly sensitive to the action of thiazolidinediones (12). Further research will be necessary to establish the precise mechanisms of action of thiazolidinediones on the UCP-3 gene in muscle and how they are modified in different physiological situations.

Insulin resistance of skeletal muscle is fundamental to the development of type 2 diabetes, and excessive exposure of muscle to free fatty acids appears to play a prominent role in the appearance of the insulin-resistant state associated with obesity (44). Understanding the regulatory mechanisms of fatty acid oxidation by the skeletal muscle cells is essential for the development of strategies to avoid an insulin-resistant state. In the present work, we report that impaired UCP-3 gene expression in skeletal muscle is a physiological event associated with the metabolic adaptations of lactation and, particularly, with the reduced utilization of fatty acids by the tissue as an energy source. Both fibrates and troglitazone reverse the downregulation of the UCP-3 mRNA expression. Further research should be undertaken to determine the involvement of the UCP-3 gene upregulation in skeletal muscle in the hypolipidemic and antidiabetic effects of these drugs.

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