Tissue-specific activity of lipoprotein lipase in skeletal muscle regulates the expression of uncoupling protein 3 in transgenic mouse models

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Uncoupling protein (UCP)-2 and UCP-3 are two recently discovered proteins similar to UCP-1, which regulates thermogenesis in brown adipose tissue (BAT). Whereas UCP-1 expression is restricted to BAT, UCP-2 is widely expressed. UCP-3 is found mainly in skeletal muscle and BAT. A large body of evidence exists that the expression of UCP-2 and UCP-3 in skeletal muscle of mice is regulated by feeding/fasting, and some studies have suggested that this effect might be caused by the changing concentration of plasma non-esterified fatty acids (NEFAs). In an attempt to determine whether the increased import of triacylglycerol-derived NEFAs can also affect UCP expression, we determined the mRNA levels of UCP-1, UCP-2 and UCP-3 in BAT and muscle of induced mutant mouse lines that overexpressed or lacked lipoprotein lipase (LPL) in these tissues. The expression levels of UCP-1 and UCP-2 in BAT and in skeletal and cardiac muscle respectively were not affected by variations in tissue LPL activities. In contrast, UCP-3 mRNA levels were induced 3.4-fold in mice with high levels of LPL in skeletal muscle, and down-regulated in mice that lacked LPL in skeletal muscle. The presence or absence of LPL in BAT had no effect on UCP-3 expression levels. The response of UCP-3 mRNA expression to variations in LPL activity in skeletal muscle was independent of the feeding status or of plasma NEFA concentrations. These findings indicated that NEFAs as lipolytic products of LPL-mediated triacylglycerol hydrolysis markedly affect UCP-3 expression and that increased LPL activities occurring during fasting in skeletal muscle contribute to the induction of UCP-3 expression by promoting the increased uptake of NEFAs. In addition, our results demonstrate that UCP-2 and UCP-3 are differentially regulated in response to LPL-mediated NEFA uptake in skeletal muscle of mice.

Key words: brown adipose tissue, energy metabolism, induced mutant mice, non-esterified fatty acids, thermogenesis.

INTRODUCTION

Uncoupling proteins (UCPs) are members of the mitochondrial anion carrier family with high sequence similarity to UCP-1. UCP-1 dissipates the proton electrochemical gradient while uncoupling respiration from oxidative phosphorylation and converting fuel into heat. It mediates this activity by transporting non-esterified fatty acid (NEFA) anions, thereby allowing NEFAs to function as cycling protonophores [1–3]. UCP-1 is expressed only in brown adipose tissue (BAT), where it regulates rodent thermogenesis. UCP-1 is responsible for energy expenditure during cold exposure [4,5]. Increased UCP-1 activity leads to the dissipation of more energy as heat for the regulation of body temperature. UCP-1 can be induced by cold exposure, food intake, β-adrenergic stimulation and thyroid hormone [6]. The lack of UCP-1 in mice results in cold sensitivity but not obesity [7], whereas the lack of BAT causes severe obesity [8,9].

UCP-2 and UCP-3 are recently identified members of the mitochondrial carrier family that show a high sequence similarity to the gene for UCP-1. They are of particular interest because they are expressed in tissues that might be important for energy expenditure. In contrast with the restricted tissue distribution of UCP-1, UCP-2 is expressed in many tissues including muscle, BAT and white adipose tissue [10], whereas in rodents UCP-3 mRNA is expressed preferentially in skeletal muscle and BAT, which are both thermogenic tissues that regulate energy expenditure in mammals [11,12]. Whether UCP-2 and UCP-3 also exhibit uncoupling activity is under current debate. Overexpression of UCP-2 and UCP-3 decreases the mitochondrial potential in yeast, which suggests strongly that the enzymes also possess respiratory-chain uncoupling activity [10,13]. However, recent studies on mice null for the UCP-2 and UCP-3 gene do not support a true uncoupling activity and thermogenic function for them [14,15]. Whether UCP-2 and UCP-3 are physiological mediators of thermogenesis in skeletal muscle [16] is still controversial, because exposure to cold up-regulates UCP-3 mRNA expression in BAT of rats but fails to do so in skeletal muscle [17,18]. In addition, fasting, a condition that decreases resting energy expenditure, has been reported to increase the expression of both UCP-2 and UCP-3 mRNA in rodents [19]. UCP-2 and UCP-3 expression in skeletal muscle of rodents decreases with the switch from enhanced lipid utilization during fasting to decreased lipid utilization during refeeding [20], which suggests that both of them act as regulators for lipids as fuel substrates for oxidation. UCP-3-deficient mice lack an observable phenotype and respond normally to fasting, β3-adrenergic stimulation and thyroid hormone treatment [15]. This indicates that UCP-3 is not a major determinant of metabolic rate but instead affects other metabolic pathways such as lipogenesis, ketogenesis and amino acid metabolism as well as the regulation of reactive oxygen species [21,22]. However, mice overexpressing UCP-3 exhibit decreased body weight despite hyperphagia, owing to increased energy expenditure [23].

The observation that increased plasma NEFA concentrations in humans and rats as a result of fasting or of Intralipid™ infusion caused an increase in UCP-3 mRNA levels in skeletal muscle suggested that NEFAs or a metabolite thereof can affect UCP-3 gene transcription *in vivo* [24–27]. In fact, a strong

Abbreviations used: BAT, brown adipose tissue; NEFA, non-esterified fatty acid; LPL, lipoprotein lipase; MCK, mouse creatine kinase; RT–PCR, reverse-transcriptase-mediated PCR; UCP, uncoupling protein.

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correlation between plasma NEFA concentrations and UCP-3 expression has been reported in several studies [16,27,28] and it was speculated that UCP-3 induction might be mediated by peroxisome-proliferator-activated receptor-γ [29,30]. The major enzyme for the hydrolysis of plasma triacylglycerols in adipose tissue and muscle is lipoprotein lipase (LPL). The enzyme generates NEFAs, which are taken up by the underlying tissue. In muscle, LPL is the major determinant for the quantity of NEFAs absorbed by the tissue [31]. In the present study, previously generated transgenic and knock-out mouse lines that overexpressed or lacked LPL in skeletal muscle [32,33] were used to determine the effects of triacylglycerol-derived NEFAs on the mRNA expression levels of UCP-1, UCP-2 and UCP-3 in BAT, skeletal and cardiac muscle.

EXPERIMENTAL

Animals

Table 1 summarizes the mouse lines used in this study. The generation of these lines and their detailed characterization have been published previously [32,33]. L2-MCK and L0-MCK mice contain a human LPL minigene under the control of the MCK (for mouse creatine kinase) promoter in the presence (L2-MCK) or absence (L0-MCK) of functional mouse LPL alleles [32]. L2-hLPL and L0-hLPL mice contain the identical minigene under the control of the mouse LPL promoter (8 kb 5′-flanking region of the mouse gene encoding LPL) in the presence (L2-hLPL) or absence (L0-hLPL) of functional mouse LPL alleles [33]. Interbreeding of heterozygous LPL knock-out mice (L1) resulted in progeny of the following genotypes: 25 % L2 (wild-type), 50 % L1 and 25 % homozygous LPL knock-out mice (L0). All offspring were injected immediately after birth with defective adenovirus containing the human LPL cDNA (J. G. Strauss, S. Frank, D. Kratky, G. Hämmerle, G. Knipping, A. von Eckardstein, G. M. Kostner and R. Zechner, unpublished work). This treatment resulted in animals that survived the suckling period and developed into normal adulthood when kept on a low-fat diet. Non-transgenic littermates (L2) were used as controls. Genetic screening of the genotypes was performed by a double-PCR screening protocol to analyse for the presence or absence of the human transgene and the knock-out mutation by using a protocol described previously [32]. All animals were maintained on a regular light–dark cycle (14 h light, 10 h dark) and kept on a standard laboratory chow diet.

Table 1 Summary of genotypes [31–33]

The relative changes in LPL activity levels in skeletal muscle (SM), cardiac muscle (CM), white adipose tissue (WAT) and BAT of the various transgenic mouse models are compared with those in control mice (arbitrarily set at 1). Non-transgenic littermates expressing two alleles of endogenous mouse LPL were used as controls (L2). L0-MCK and L2-MCK mice contain a human LPL transgene under the control of the MCK promoter in the absence (L0-MCK) or presence (L2-MCK) of functional mouse LPL alleles. L0-hLPL and L2-hLPL mice contain the same human LPL transgene under the control of the mouse LPL promotor in the absence (L0-hLPL) or presence (L2-hLPL) of functional mouse LPL alleles. L0 mice are adenovirus-treated LPL knock-out mice. Abbreviation: n.d., not detected.

			LPL level (fold of control)			
Genotype	Mouse LPL alleles	Transgene allele	SM	CM	WAT	BAT
L2	2	0	1	1	1	1
L0-MCK	0	1	3.6	0.2	0	< 0.2
L2-MCK	2	1	3.6	1.4	1	1
L0-hLPL	0	1	0	0.6	0	< 0.2
L2-hLPL	2	1	1	1.2	1	1
L0	0	0	n.d.	n.d.	n.d.	n.d.

RNA isolation and Northern blot analysis

Tissues were removed surgically, weighed and subsequently frozen in liquid nitrogen. For each 50–100 mg of wet tissue, 1 ml of TRI Reagent (MRC, Karlsruhe, Germany) was used for homogenization. Total tissue RNA was precipitated with propan-2-ol. After centrifugation the RNA pellet was washed with 75 % (v/v) ethanol, recentrifuged and dissolved in diethyl pyrocarbonate-treated water. RNA was prepared from various tissues of adult transgenic and control mice. For Northern blot analysis, 10 μg of total RNA were separated by formaldehyde/1 % (w/v) agarose-gel electrophoresis and subsequently blotted overnight to nylon membranes (Hybond N^+; Apbiotech, Freiburg, Germany). RNA was cross-linked to membranes by UV irradiation. Blots were prehybridized for 4 h at 65 °C in a buffer containing 0.15 M sodium phosphate (pH 7.2), 1 mM EDTA, 7 % (w/v) SDS and 1 % (w/v) BSA, then hybridized overnight in the same buffer at 65 °C with appropriate UCP probes. After hybridization, the blots were washed for 20 min at room temperature in 2 × SSC (SSC is 0.15 M NaCl/0.015 M sodium citrate) containing 0.1 % SDS, followed by two additional washing steps in SSC/0.1 % SDS for 10 min at 65 °C each. Specific hybridization was detected by autoradiography on X-ray films (Cronex; Siemens, Vienna, Austria) or by exposure to a PhosphorImager Screen (Apbiotech).

For the detection of mouse UCP-1, rat UCP-1 cDNA that hybridized also to mouse UCP-1 mRNA was kindly provided by Dr Daniel Ricquier (Centre National de la Recherche Scientifique, Meudon, France). The probe was labelled radioactively with [^{32}P]dCTP (NEN, Boston, MA, U.S.A.) by using a random priming kit (Prime-a-Gene Kit; Promega, Mannheim, Germany).

A partial UCP-2 cDNA (GenBank accession number U69135) was generated from total murine white adipose tissue RNA by reverse-transcriptase-mediated PCR (RT–PCR) with two specific primers: 5′-GATCCAAGGGGAGAGTCA-3′ (forward) and 5′-GTGACCTGCGCTGTGGTACT-3′ (reverse). The RT–PCR reaction resulted in a cDNA probe 640 bp long; this was subcloned into pCR2.1 (Invitrogen, Groningen, The Netherlands). The identity of the clone was confirmed by DNA sequencing. A cDNA probe for UCP-3 (GenBank accession number AF019883) was obtained from total murine skeletal muscle RNA by RT–PCR with the specific primers 5′-CAACGGTTGTGAAGTTCCTG-3′ (forward) and 5′-AATCGGACCTTCACCAC-ATC-3′ (reverse). The RT–PCR reaction was performed with a kit by Invitrogen in accordance with the manufacturer's protocol. The 388 bp cDNA was subcloned into pCR2.1 and sequenced.

LPL activity

Tissues were excised, weighed, minced with scissors and transferred to ice-cold tubes containing 1 ml of Dulbecco's modified Eagle's medium/2 % (w/v) BSA containing 2 units/ml heparin. The tissue samples were incubated at 37 °C for 1 h. LPL activity was determined in extracts from skeletal and cardiac muscle, white adipose tissue and BAT from mice. In brief, 0.6 μCi of radioactive triolein (NEN) and 4.6 μl of non-radioactive glycerol trioleate (triolein; Sigma, Vienna, Austria) [20 % (w/v) triolein in chloroform] per sample were evaporated under a stream of nitrogen for approx. 1 h. Tris/HCl, pH 8.6 (20 μl, 1 M), 20 μl of 1 % (v/v) Triton X-100 and 4 ml of doubly distilled water were added and the mixture was sonicated six times (1 min on, 1 min

off) on an ice-cold water bath at 80 mW. Then 40 μl of heat-inactivated human serum containing apoCII as activator (obtained from a pool of donors, then heated at 50 °C for 1 h and stored at −20 °C) and 40 μl of 10 % (w/v) BSA were added to the substrate. For analysis, 0.2 ml of the substrate was incubated at 37 °C with 100 μl of sample for 1 h. The reaction was stopped by the addition of 3.25 ml of a mixture of methanol/chloroform/n-heptane (1.41:1.25:1, by vol.) and 1 ml of 0.1 M K_2CO_3/H_3BO_3, pH 10.5. NEFAs were extracted by vortex-mixing for 20 s and phases were separated by centrifugation. A 1 ml sample of the upper phase was added to 10 ml of ReadySafe Cocktail and counted in the β-counter. For inhibition of LPL activities, samples were preincubated with 1 M NaCl or 0.1 ng of 5D2 monoclonal antibody (kindly provided by Dr J. D. Brunzell, University of Washington, Seattle, WA, U.S.A.) for 1 h at 4 °C.

Statistics

Results are expressed as means ± S.D. Student's t test was used to calculate statistical significance between groups. Significance levels were set at $P < 0.05$ (*) and $P < 0.01$ (**).

RESULTS AND DISCUSSION

Several studies have provided convincing evidence that fasting/feeding and/or plasma NEFA levels regulate the expression of UCP-3 and, potentially, UCP-2 [13,24–26]. However, the major source of NEFAs taken up by muscle and adipose tissue originate from the LPL-mediated hydrolysis of plasma triacylglycerols transported in chylomicrons and very-low-density lipoprotein. In the present study we investigated the effects of variations of LPL activity in muscle and adipose tissue of genetically modified mice on the expression of UCP-1, UCP-2 and UCP-3.

Table 1 summarizes the genotypes and the LPL activities in muscle and adipose tissue of the mouse lines used. As described previously, L2-MCK animals harbour a human LPL-minigene under the control of the MCK promoter [31,32]. Accordingly, these mice exhibited a 3.6-fold increase in LPL activity in skeletal muscle and a 1.4-fold increase in cardiac muscle compared with control mice (L2). LPL activities in white adipose tissue and BAT were identical with those in L2 animals. L0-MCK mice are the result of breeding L2-MCK animals with LPL knock-out mice (L0) [32]. L0-MCK mice express only the human transgene but lack both alleles of the endogenous mouse gene encoding LPL. Therefore L0-MCK mice exhibited increased LPL activity in skeletal muscle (3.6-fold), whereas in cardiac muscle LPL was decreased by 80 % and in BAT by 83 % compared with L2 mice. The very low level of LPL activity in BAT might have been due to contamination by muscle fibres that had not been totally removed during the isolation of BAT. Importantly, these mice completely lack LPL activity in white adipose tissue.

L2-hLPL animals harbour the same human LPL-minigene but under the control of the mouse LPL promoter region (8 kb of 5′-flanking region of the mouse gene encoding LPL) [33]. This minigene was found to be expressed in cardiac muscle, but not in skeletal muscle and adipose tissue. L2-hLPL mice exhibited a 1.2-fold increase in LPL activity in cardiac muscle and normal LPL activities in skeletal muscle and adipose tissue in comparison with L2 controls. L0-hLPL mice, as a result of breeding L2-hLPL mice with LPL knock-out mice, lack the endogenous mouse alleles and exhibit half-normal LPL activities in cardiac muscle [33]. In BAT, LPL was decreased by 83 % compared with controls. Importantly, these animals completely lack LPL activity in skeletal muscle and white adipose tissue.

Finally, the L0 mice used in this study were LPL knock-out animals that were rescued from post-natal death [34,35] by adenovirus gene transfer (J. G. Strauss, S. Frank, D. Kratky, G. Hämmerle, G. Knipping, A. von Eckardstein, G. M. Kostner and R. Zechner, unpublished work). These animals expressed

Table 2 Fasted plasma NEFA concentrations

Blood was taken in the morning from animals that had fasted overnight. The concentrations of NEFA were measured enzymically. Results are means ± S.D. The male animals were 11 weeks old. $^{**}P < 0.01$ compared with the controls.

Genotype	n	NEFA concentration (mM)
L2	7	0.92 ± 0.17
L0-MCK	7	0.82 ± 0.15
L2-MCK	7	0.82 ± 0.15
L0-hLPL	7	1.0 ± 0.16
L2-hLPL	7	0.82 ± 0.15
L0	7	2.69 ± 0.40**

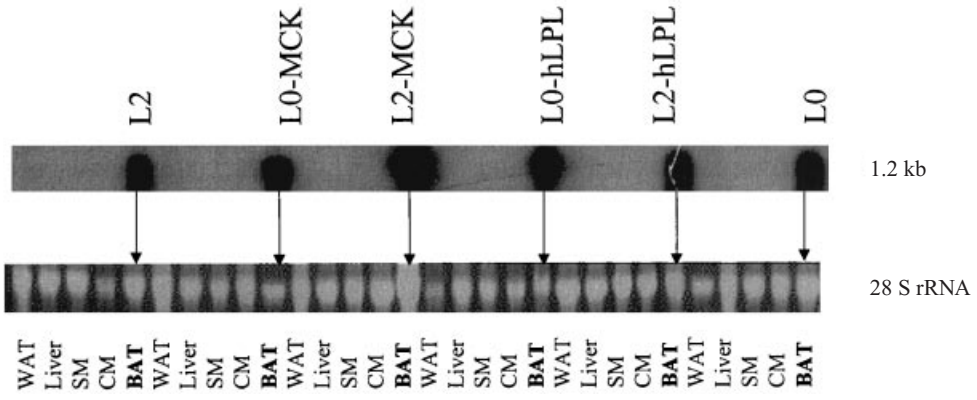

Figure 1 Expression of UCP-1 in murine tissues

Northern blotting analysis was performed with RNA samples from various murine tissues. Total RNA (10 μg) was subjected to formaldehyde/agarose-gel electrophoresis and blotted to nylon membrane. After hybridization of the blot with a ^{32}P-labelled rat UCP-1 cDNA it was subjected to autoradiography. Abbreviations: WAT, white adipose tissue; SM, skeletal muscle; CM, cardiac muscle. For definitions of the various genetically modified mouse lines, see the legend to Table 1.

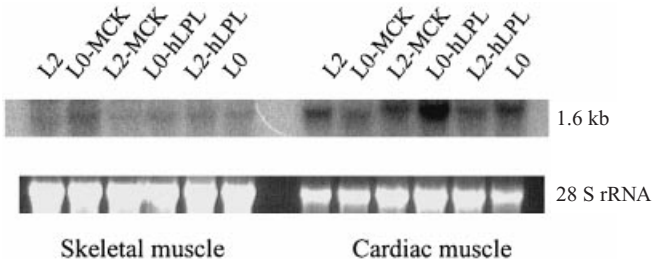

Figure 2 Expression of UCP-2 in skeletal and cardiac muscle

Northern blotting analysis was performed with RNA samples from various murine tissues. Total RNA (10 μg) was subjected to formaldehyde/agarose-gel electrophoresis and blotted to nylon membrane. After hybridization of the blot with a ^{32}P-labelled murine UCP-2 cDNA it was subjected to autoradiography. For definitions of the various genetically modified mouse lines, see the legend to Table 1.

LPL only during the suckling period owing to adenoviral infection. In adult animals LPL activity is undetectable in all body tissues including skeletal muscle, cardiac muscle, white adipose tissue and BAT.

Despite the large differences in the tissue-specific LPL activity levels between the various mouse lines, the NEFA concentrations in plasma were identical in L2, L2-MCK, L0-MCK, L2-hLPL and L0-hLPL mice (Table 2). Only L0 animals exhibited increased NEFA levels in plasma (2.9-fold). To investigate whether variations in tissue-specific LPL activities could affect the expression of UCP-1, mRNA levels of UCP-1 were determined in various tissues by Northern blot analysis (Figure 1). In all mouse lines, UCP-1 was expressed exclusively in BAT and the expression level was not affected by changes in the LPL-mediated hydrolysis of triacylglycerol-rich lipoproteins. Additionally, the increased NEFA levels in L0 mice also had no effect on UCP-1 expression in BAT. This finding indicated that the modulation of NEFA uptake from albumin–NEFA complexes or as a result of the hydrolysis of triacylglycerols cannot induce UCP-1 expression in white adipose tissue or muscle and does not affect UCP-1 mRNA levels in BAT.

UCP-2 is expressed in many tissues, including muscle and adipose tissue. However, for thermogenesis and energy homoeostasis its expression in muscle is considered most important [21]. Therefore the effect of LPL-mediated NEFA production on UCP-2 mRNA concentrations was analysed in skeletal and cardiac muscle (Figure 2). Identical UCP-2 mRNA concen-

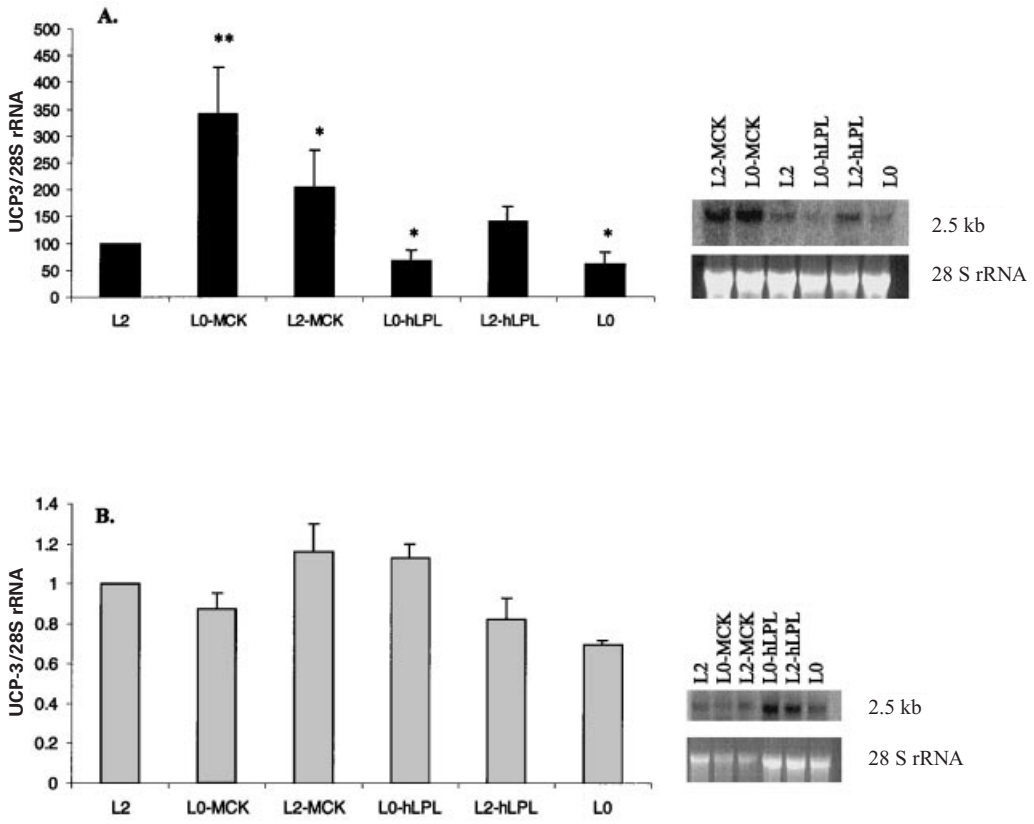

Figure 3 Expression of UCP-3 mRNA in murine skeletal muscle and BAT

Left panels: relative abundances of UCP-3 mRNA in skeletal muscle (**A**) or in BAT (**B**) of mice expressing different amounts of LPL. Results are means $\pm$ S.D. for the relative intensities of hybridization signals from four (**A**) or two (**B**) Northern blots. Results are expressed as percentages of the expression of UCP-3 in control mice (L2). $^{*}P < 0.05$; $^{**}P < 0.01$ compared with controls. Right panels: Northern blotting analysis was performed with RNA samples from murine skeletal muscle (**A**) or BAT (**B**). Total RNA (10 μg) was subjected to formaldehyde/agarose-gel electrophoreses and blotted to nylon membrane. After hybridization of the blot with a ^{32}P-labelled murine UCP-3 cDNA it was subjected to autoradiography. For definitions of the various genetically modified mouse lines, see the legend to Table 1.

trations were observed in all mouse lines, suggesting that variations in NEFA delivery to muscle in response to changing LPL activities have no effect on the regulation of UCP-2 mRNA levels. Identically, UCP-2 expression in white adipose tissue was also not affected (results not shown). Although L0 animals had markedly increased NEFA levels in plasma, their UCP-2 mRNA concentrations in skeletal and cardiac muscle were unchanged from control mice. The latter result is in agreement with studies in humans that reported the absence of a correlation between plasma NEFA concentrations and UCP-2 mRNA levels in muscle [36]. Accordingly, our results provide additional evidence that the effect of fasting on UCP-2 mRNA levels is not mediated by changes in NEFA levels.

UCP-3 is expressed predominantly in skeletal muscle and BAT and has been shown to be highly responsive to fasting. When UCP-3 mRNA levels were determined in the various mouse lines used in the present study (Figure 3A), UCP-3 mRNA levels were increased 3.4-fold in skeletal muscle of L0-MCK and 2.1-fold in L2-MCK mice. Evidently, overexpression of LPL in skeletal muscle of these mice resulted in a marked up-regulation of UCP-3 expression. In contrast, increased levels of circulating NEFAs in plasma did not induce UCP-3 mRNA levels (Figure 3A). In fact, L0 mice with 2.9-fold elevated plasma NEFA concentrations exhibited decreased UCP-3 mRNA levels in muscle. These animals, just like L0-hLPL mice, lack LPL in muscle with the consequence of low levels of UCP-3 mRNA. Fasting induces LPL activity in both skeletal and cardiac muscle [37], a process that is presumably mediated by catecholamines. In addition, it causes increased levels of circulating NEFAs owing to increased fat lipolysis in adipose tissue [38]. Our results suggest that it is the induction of LPL that affects UCP-3 gene transcription rather than the level of circulating NEFAs in plasma. These observations might also explain why essentially all studies so far have found that fasting induces UCP-3 expression in skeletal muscle [24–26], whereas controversial findings were obtained when UCP-3 mRNA levels were correlated with plasma NEFA concentrations [16,27,36,39,40].

The effect of fasting on UCP-3 expression in BAT was found to be the opposite of that in skeletal muscle. Fasting decreased UCP-3 mRNA levels in BAT of rats but increased UCP-3 expression 6-fold in skeletal muscle [13,17]. In our experiments, variations in LPL activity in BAT did not affect UCP-3 mRNA levels (Figure 3B), suggesting a fundamental difference between UCP-3 gene regulation in skeletal muscle and that in BAT in response to absorbed NEFAs.

In conclusion, our results demonstrate that UCP-2 and UCP-3 are differentially regulated in muscle in response to changes in LPL activity. In contrast with UCP-2, UCP-3 is markedly up-regulated when LPL activities are high and is repressed at low LPL levels. These results suggest that, in addition to other mechanisms, increased LPL activity in muscle during fasting might contribute to the substantial induction of UCP-3 expression during this condition.

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