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De novo expression of uncoupling protein 3 is associated to enhanced mitochondrial thioesterase-1 expression and fatty acid metabolism in liver of fenofibrate-treated rats

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Abstract Uncoupling protein 3 (UCP3) is a member of the mitochondrial carrier superfamily, preferentially expressed in skeletal muscle. Its function is not fully understood and it is debated whether it uncouples oxidative phosphorylation as does UCP1 in brown adipose tissue. Recent evidences suggest a role for UCP3 in the flux of fatty acids in and out mitochondria and their utilization in concert with mitochondrial thioesterase-1 (MTE-1). In fact, mice overexpressing muscle UCP3 also show high levels of MTE-1. Fenofibrate is a hypolipidemic drug that prevents body weight gain in diet-induced obese rats and enhances lipid metabolism by activating peroxisome proliferator-activated receptors (PPARs). Because fatty acids and fenofibrate stimulate PPARs and in turn UCP3, we investigated whether UCP3 expression might be induced 'de novo' in situations of increased hepatic mitochondrial fatty acid utilization caused by a combined effect of a high-fat diet and fenofibrate treatment. We also investigated whether *Mte*-1 expression and β-oxidation were affected. We show here that *Ucp*3 is induced in liver of fenofibrate-treated rats at the mRNA and protein level. Expression was restricted to hepatocytes and was unevenly distributed in the liver. No increase in cell proliferation, inflammatory or fibrotic responses was found. *Mte*-1 expression and mitochondrial β-oxidation were upregulated. Thus, *Ucp*3 can be transactivated in tissues where it is normally silent and fenofibrate can attain this effect in liver. The data demonstrate that UCP3 is involved in fatty acid utilization and support the notion that UCP3 and MTE-1 are linked within the same metabolic pathway. **© 2002 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: Uncoupling protein; Fibrate; Mitochondrion; Fatty acid; Liver; Mitochondrial thioesterase

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Abbreviations: UCP, uncoupling protein; MTE-1, mitochondrial thioesterase-1; PPAR, peroxisome proliferator-activated receptor; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; RT-PCR, reverse transcriptase-polymerase chain reaction; PCNA, proliferating cell nuclear antigen; FFA, free fatty acid; CoA, coenzyme A

1. Introduction

Maintenance of a stable body weight depends on the balance between energy intake and expenditure and is governed by genetic, physiological and behavioral factors [1]. The discovery of uncoupling proteins (UCPs), and their isoforms (UCP1–5) in animal and human tissues prompted studies of the mechanisms that regulate energy expenditure [2–4].

UCP1 was the first member of these mitochondrial carriers to be identified and its function in thermogenesis is well established. In brown adipose tissue it dissipates the proton gradient across the inner mitochondrial membrane [2]. UCP2 is widely distributed in a number of tissues and cell types. Albeit its high homology with UCP1 and overexpression experiments in yeast demonstrating uncoupling activity, the biological role of UCP2 is still debated [5]. UCP3 is predominantly expressed in skeletal muscle and may be important in energy balance. Mice overexpressing UCP3 are hyperphagic and lean with increased mitochondrial proton conductance [6]. In contrast, body weight was unaffected in mice in which the UCP3 gene was ablated, despite increased mitochondrial respiration coupling in skeletal muscle [7].

UCP3 function appears then not completely elucidated. Based on its hypothesized uncoupling function, several studies have investigated this activity in situations where UCP3 expression was physiologically altered but contrasting results have been obtained [8–11]. Recent evidences indicate that UCP3 may play functional roles unrelated to the basal uncoupling [12]. In conditions of elevated lipid metabolism, UCP3 might export fatty acid anions, released from an excess of acyl-coenzyme A (CoA) by mitochondrial thioesterase-1 (MTE-1), from the mitochondrial matrix to the cytosol [13]. Thus, UCP3 gene may be important in situations where β-oxidation becomes the main metabolic pathway.

Fenofibrate, a hypolipidemic drug that activates peroxisome proliferator-activated receptors (PPARs), in particular the α-isoform, prevents and reduces body weight gain in diet-induced obese rats [14,15]. This effect is due to enhanced lipid catabolism in the liver mainly through PPARs transcriptional induction of the genes for mitochondrial and peroxisomal oxidative enzymes [16]. UCP genes contain functional PPAR-responsive elements [17,18] and may be targets of fenofibrate.

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Because fatty acids and fenofibrate stimulate PPARα and in turn UCPs, we investigated whether UCP3 expression was induced in rat liver when hepatic lipid metabolism is strongly enhanced by the combined effect of the high-fat diet and fenofibrate. Such a condition, that is metabolically and not genetically determined, would contribute to identify a role for UCP3.

2. Materials and methods

2.1. Animal treatment and tissue sampling

Eight-week-old male Wistar rats (Charles River, Lecco, Italy), weighing about 200 g, were used in this study. The animals, housed in a conventional temperature-controlled animal room (28°C), one per cage, on a 12 h light–darkness cycle, were treated according to the national guidelines regarding animal experiments and underwent 1 week of adaptation. They were divided into four groups of seven rats each and monitored for 2 months. During this time, group C was kept on the standard diet (15.9 kJ gross energy/g), and group D, DF1, and DF2 were fed a palatable high-fat diet (19.8 kJ gross energy/g) [19]. Groups DF1 and DF2 were also fed fenofibrate (kindly provided by Fournier Pharma, SpA., Segrate, Italy) at a dose of 320 mg/kg body weight daily suspended in 0.1% carboxymethylcellulose. DF1 rats received the drug only during the second month of dietary treatment, while DF2 rats received fenofibrate for 2 months. C and D rats were fed the suspension vehicle once a day. Body weight and food intake were monitored daily. Animals were weighed and decapitated. Excised liver and gastrocnemius muscles were weighed, snap-frozen in liquid nitrogen and stored at −80°C.

2.2. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted from frozen tissues [15]. Superscript™ II (Life Technologies Italia, San Giuliano Milanese, Italy) was used to reverse transcribe 2 μg of RNA and was omitted in the negative controls. cDNAs were amplified by standard PCR, using primers selective for UCP3 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), used for normalization as previously reported [15]. Specific primers for rat MTE-1 were as follows: CCTCGTCTTTC-GCTGTCCTG (sense), GTGTCCGTCCAGCACCTCCA (antisense). The *Mte*-1 DNA fragment was amplified by running 35 cycles of 40 s at 95°C, 50 s at 61°C, and 50 s at 72°C using 1.5 mM $MgCl_2$, and 5% DMSO. PCR products were 662, 452, and 876 bp long DNA fragments for UCP3, MTE-1, and GAPDH genes, respectively.

2.3. Mitochondrial protein extracts and Western blotting

About 500 mg of frozen tissue were homogenized and processed for mitochondrial protein extraction as previously described [10]. Western blot analysis was carried out on 30 μg of protein for each sample incubated with a rabbit anti-human UCP3 polyclonal antibody (1:500 dilution; Research Diagnostic, Inc., Flanders, NJ, USA). Detection was obtained by chemiluminescence (Amersham Pharmacia Biotech Italia, Cologno Monzese, Italy).

2.4. Histochemistry and immunohistochemistry

4-μm-thick sections from formalin-fixed, paraffin-embedded liver specimens were used for histochemical analysis using the hematoxylin–eosin staining. Immunohistochemistry was performed after heat-induced antigen retrieval for all antibodies. The antibodies used were: anti-UCP3 (the same antibody used for Western blotting, 1:200 dilution), proliferating cell nuclear antigen (mouse anti-human PCNA monoclonal antibody, PC10; 1:200 dilution; Dako, Carpinteria, CA, USA), PPARα (rabbit anti-human PPARα polyclonal antibody; dilution 1:200; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Immunodetection was performed with a biotinylated secondary antibody, followed by peroxidase-labelled streptavidin (LSAB-DAKO, Carpinteria, CA, USA) with diaminobenzidine chromogen as substrate. Steatosis was evaluated by hematoxylin–eosin staining which resulted in visualization of vacuoles in hepatocytes and was expressed as percentage of vacuolized hepatocytes (for each high power field (HPF), considering at least 10 HPF). Inflammation was evaluated by the number of Kupffer cells per HPF (at least 10 HPF were evaluated) in portal, periportal and intra-acinar regions. Fibrosis was evaluated as presence/absence of significant amounts of collagen fibers stained with Red Sirius in the portal and periportal regions as described [20]. The proliferation index was the mean percentage of PCNA-positive cells in 10 HPF (40×). The distribution of UCP3 and PPARα was measured in the portal space area (zone 1), central vein area (zone 3) and in the intermediate area (zone 2).

2.5. Mitochondrial preparation, measurement of fatty acid-dependent respiration and determination of the kinetic response of the proton leak to changes in membrane potential

Liver specimens were homogenized; after differential centrifugations, mitochondria were isolated at 3000×*g*. The isolated organelles were used for fatty acid-dependent respiration (β-oxidation) and respiratory control ratio (RCR) measurements, as previously reported [9]. Membrane potential ($\Delta\Psi$) was determined from the distribution of the lipophilic cation triphenylmethylphosphonium ($TPMP^+$), which was measured using a $TPMP^+$-sensitive electrode [21]. To measure the basal proton leak the incubation medium was supplemented with 1% w/v fatty acid-free bovine serum albumin (BSA). To evaluate the kinetic response of the mitochondrial proton leak to a change in $\Delta\Psi$, we supplemented the incubation medium with oligomycin and the respiration rate was inhibited by sequential addition of malonate up to 2.5 mM.

2.6. Statistics

Results are expressed as means ± S.E.M. The statistical significance of differences between groups was determined by ANOVA followed by a Student–Neuman–Keuls test. The level of significance was set at $P < 0.05$.

3. Results

3.1. Fenofibrate regulates UCP3 mRNA and protein levels

To determine whether fenofibrate modulates UCP3 gene expression, we analyzed by RT-PCR total RNA extracted from the gastrocnemius muscle and liver of rats kept as described. *Ucp*2 mRNA levels in liver and *Ucp*3 mRNA levels in skeletal muscle did not differ in fenofibrate-treated and control animals (data not shown) as previously shown [15]. Unexpectedly, *Ucp*3 transcription was induced in the livers of rats treated with fenofibrate, for 1 or 2 months (Fig. 1A, lanes DF1 and DF2). Interestingly, *Ucp*3 induction was detected also in rats treated with fenofibrate and fed a standard control chow diet (data not shown). *Ucp*3 mRNA was instead absent from livers of animals fed a standard diet (C) or a high-fat diet (D). The same result was obtained when the PCR was carried out in saturating conditions, i.e. for 45–50 cycles.

To verify that the *Ucp*3 mRNA induction was paralleled by an increase in the corresponding protein, we analyzed mitochondria-enriched liver extracts from rats of the four groups by Western blotting. A band of the apparent molecular weight of 33 kDa was detected only in extracts from the livers of fenofibrate-treated rats fed a high-fat diet (Fig. 1B, lanes DF1 and DF2) and a standard chow diet (data not shown). No *Ucp*3 signal was detected in rats not treated with fenofibrate, whereas a band of the same molecular weight was found in the mitochondria-enriched extracts prepared from the gastrocnemius muscle, which served as positive control. The intensity of this signal remained unchanged in mitochondria-enriched extracts from muscle of treated and control rats (data not shown) and in general was similar in extracts from rats of the same group, indicating a low variability among individuals.

3.2. Fenofibrate upregulates Mte-1 in liver

The same liver cDNAs obtained as described were used to amplify a specific *Mte*-1 fragment. The high-fat diet was as-

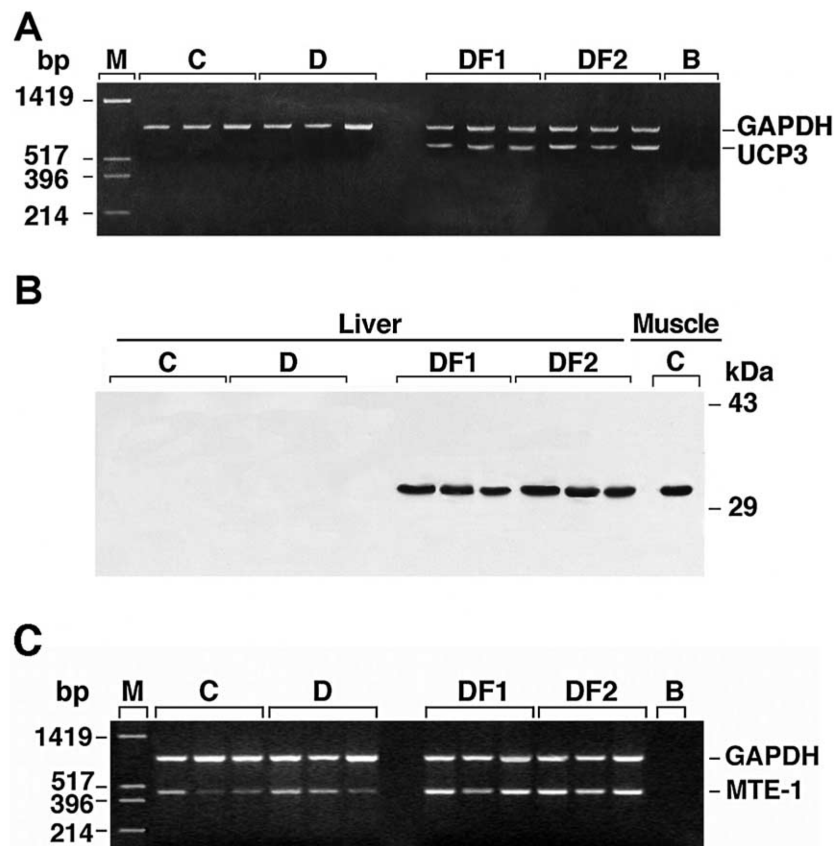

Fig. 1. UCP3 mRNA and protein expression in rat liver. A: RT-PCR analysis of UCP3 gene expression in liver from rats on a standard diet (C), high-fat diet (D), high-fat diet plus fenofibrate administered for 1 (DF1) or 2 months (DF2). GAPDH amplified in parallel served as internal control. The negative control was loaded in lane B. The size marker (M) is shown on the left. B: Western blotting analysis of UCP3 in liver mitochondrial protein extracts from C, D, DF1, and DF2 rats. Mitochondrial protein extracts from skeletal muscle of a rat on a standard diet were used as a positive control. The molecular weight marker is shown on the right. C: RT-PCR analysis of MTE-1 gene expression in rat liver. M, C, D, DF1, DF2, and B are as in panel A.

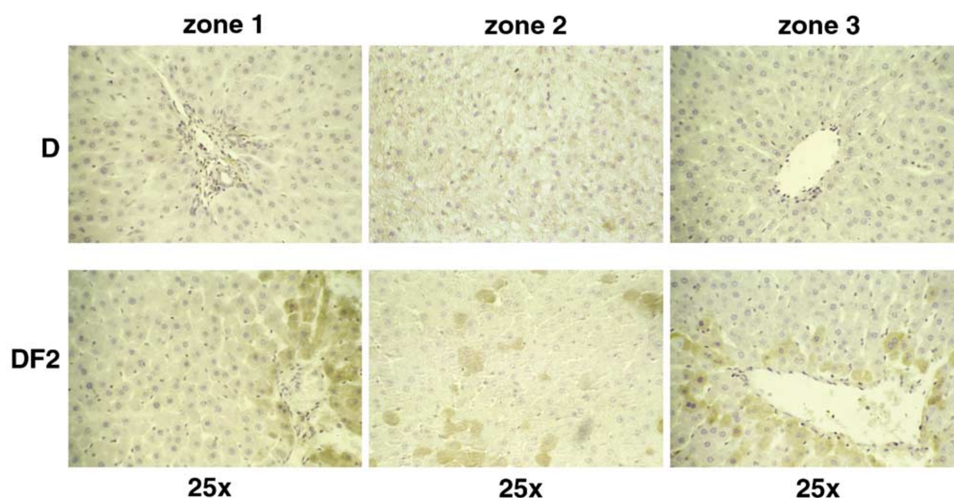

Fig. 2. Immunohistochemistry detection of UCP3 in liver. 4-µm-thick liver sections of rats fed a high-fat diet (D), and high-fat diet plus fenofibrate administered for 2 months (DF2) were treated with hematoxylin–eosin, immunostained with an antibody against UCP3 and visualized with the chromogen diaminobenzidine. The three panels reported for each group represent the portal space area (zone 1), the central vein area (zone 3) and the intermediate area between zones 1 and 3 (zone 2). Only sections from fenofibrate-treated rats (DF2) stained positive; the signal was particularly intense and widespread in the cytoplasm of the cells around the vessels.

Table 1
Histochemical and immunohistochemical analysis of livers from C, D, DF1, and DF2 rats

Group	PCNA[a]	Steatosis[a]	Collagen fibers			Kupffer cells[b]	UCP3		
			zone 1	zone 2	zone 3		zone 1	zone 2	zone 3
C	5%	< 5%	N	N	–	1	–	–	–
C	8%	10%	N	N	–	1	–	–	–
C	10%	7%	N	N	–	2	–	–	–
C	15%	< 5%	N	N	–	1	–	–	–
D	15%	25%	N	N	–	1	–	–	–
D	25%	30%	N	N	–	3	–	–	–
D	5%	40%	N	N	–	1	–	–	–
D	5%	30%	N	N	–	1	–	–	–
DF1	20%	30%	N	N	–	1	–	–	+
DF1	5%	20%	N	N	–	1	+	–	+
DF1	15%	35%	N	N	–	1	+	–	+
DF1	10%	40%	N	N	–	2	–	–	++
DF2	10%	20%	N	N	–	2	++	++	+++
DF2	15%	10%	N	N	–	1	++	+	+++
DF2	15%	15%	N	N	–	1	++	+	+++
DF2	10%	15%	N	N	–	1	++	+	+++

4-μm-thick formalin-fixed, paraffin-embedded liver sections were evaluated for steatosis, inflammation (Kupffer cells) and fibrosis. UCP3 and PCNA were detected by immunohistochemistry. PCNA was used as a proliferation index. + = < 10% of immunopositive hepatocytes (250×). ++ = > 10%, < 20% of immunopositive hepatocytes (250×). +++ = > 20% of immunopositive hepatocytes (250×). N = normal.
[a]% of hepatocytes (450×).
[b]Evaluated for 10 hepatocytes.

sociated to a small, not significant, increase of *Mte*-1 mRNA levels. However, the addition of fenofibrate to the dietary regimen increased the *Mte*-1 mRNA by 3.9- and 3.6-fold after 1 and 2 months of treatment, respectively.

3.3. Histochemistry and immunohistochemistry

Liver sections from treated and control animals were analyzed to confirm *Ucp*3 expression and to identify the cells where induction occurred (Fig. 2). UCP3 was detected only in the hepatocytes from groups DF1 and DF2 (only sections of DF2 rats are shown). Interestingly, zone 1 and zone 3 (the portal and center-lobular region, respectively) were intensely stained, whereas the interconnecting zone 2 showed only a slight staining. There was no staining in liver of rats fed a standard or a fat-enriched diet (groups C and D).

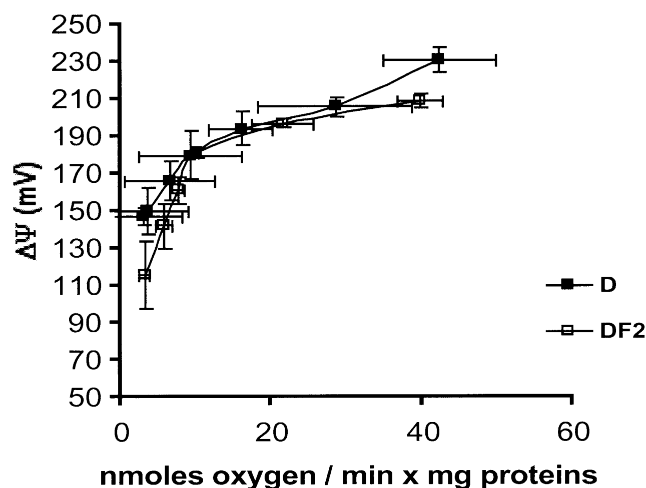

Fig. 3. Kinetic response of the proton leak to changes in membrane potentials ($\Delta\Psi$). The analysis was carried out in liver mitochondria incubated with 1% BSA: comparison between D and DF2 rats is shown. Each point represents the mean ± S.E.M. of duplicate determinations in four different mitochondrial preparations.

Hepatic steatosis was significantly higher in groups D and DF1 than group C (Table 1). Differently, the amount of fat in hepatocytes of DF2 animals was clearly reduced, even though each individual cell remained enlarged. The proliferating index, as measured by PCNA-positive cells, was similar in all groups, indicating no increase in cell proliferation. Likewise, there were no differences among the groups in the number neither of Kupffer cells (an index of the inflammatory response) nor in the size and amount of collagen fibers (a fibrotic index), ruling out chronic hepatitis or incipient cirrhosis.

3.4. Mitochondrial β-oxidation rate and proton leak kinetics

Fenofibrate treatment induced a significant increase in liver mitochondrial β-oxidation rate as compared to animals on the high-fat or the standard diet. The values of State 4 were 20.7 ± 0.9, 12.4 ± 0.9 and 13.0 ± 1.5 (natoms oxygen/min/mg protein) in groups DF2, D and C, respectively. These values correspond to an increase of 68% in DF2 as compared to D rats ($P < 0.05$). The RCR values were 5.4 ± 0.4, 4.3 ± 0.4 and 5.1 ± 0.1 for DF2, D, and C rats, respectively (P = not significant). There were no differences in the kinetic responses of the proton leak to changes in $\Delta\Psi$ in liver mitochondria from D and DF2 rats in the presence of fatty acid-free BSA (Fig. 3).

4. Discussion

The UCP3 gene, predominantly expressed in muscle, is involved in energy homeostasis [2]. It could be a molecular determinant of the T_3-regulated resting metabolic rate [10] and mice overexpressing UCP3 in muscle are lean and hyperphagic [6]. In this latter model, the white adipose tissue mass was reduced, as in our system based on the effects of fenofibrate on diet-induced obese rats [15]. However, under no circumstances UCP3 was detected in liver. Here we present the first experimental evidence that UCP3 protein expression can be induced in a cell where it is normally silenced. The effect is mediated by fenofibrate at mRNA and protein levels. Immu-

nohistochemical analysis confirmed that the gene is induced in hepatocytes. Interestingly, UCP3-positive staining was more abundant in liver zones 1 and 3 than in zone 2. Similar results were obtained with PPARα (data not shown), thereby linking UCP3 and PPARα expression. This pattern might reflect the accumulation of the ligand deriving from the intestine in zone 1 and after diffusion through the hepatic lobular system in zone 3. Additional histochemical and immunohistochemical data ruled out that cell proliferation, inflammation and fibrosis occurred in liver.

The mechanisms regulating *Ucp*3 expression in different tissues and its silencing in liver are unknown. In liver of fenofibrate-treated animals, de novo gene induction has been reported only for the lipoprotein lipase gene [22]. Tissue-specific factors may be responsible for this expression pattern. Because PPARs regulate UCP gene expression [17,18], it is possible that the PPREs in UCP genes have a low binding affinity for the liganded PPAR. Consequently, inordinately high concentrations of the correct ligand can transactivate target genes. Fenofibrate may fulfill this function. Alternatively, at the dose used, fenofibrate may promiscuously activate PPARγ present at low levels in hepatocytes that in turn may activate the UCP3 gene. Finally, pathways distinct from PPAR activation might regulate fenofibrate-mediated UCP3 gene expression.

Whatever the mechanism underlying UCP3 gene activation, it remains to be established whether 'basal uncoupling' is the primary function of the protein [9,23–25]. In our fenofibrate-treated animals, the mitochondrial proton leak kinetics, measured in the presence of albumin, did not show marked differences between the D and DF1 or DF2 groups. This finding indicates a lack of basal uncoupling activity due to UCP3, in agreement with previous data [9,26]. However, the extreme points of the curves, corresponding to the minimum and maximum State 4 oxygen consumption, showed a lower mitochondrial membrane potential in fenofibrate-treated animals ($p < 0.05$). This result, coupled with the RCR decrease (even if not significant) in mitochondria from the same rats, would suggest a possible increase in the basal proton leak due to fenofibrate and UCP3. Nevertheless, we do not support this possibility because a significant difference should be found for all oxygen consumption states. The lack of a basal uncoupling activity in vitro, on the other hand, does not exclude that UCP3 might catalyze an inducible proton conductance when activated by some cofactors such as coenzyme Q, free fatty acids (FFA) and reactive oxygen species in vivo [27,28]).

Alternatively, UCP3 may play functional roles not strictly related to the uncoupling. UCP3 can act as a FFA translocator and a role as a regulator of lipids as fuel substrate has been hypothesized in situations where lipid metabolism predominates [12]. In concert with MTE-1 that would produce FFA^- and CoASH from acyl-CoA molecules, UCP3 would export the FFA^- molecule from the mitochondrial matrix to the cytosol. Therefore, sufficient levels of CoASH molecules will become available for β-oxidation and tricarboxylic acid cycle, especially when high rates of fatty acid oxidation occur in the cell. The three-fold increase in MTE-1 mRNA in mice overexpressing UCP3 further supported this hypothesis. In our experimental conditions, fenofibrate induces de novo expression of UCP3, no variations in UCP2 mRNA, more than a three-fold increase in MTE-1 expression and a significant elevation (68%) in mitochondrial β-oxidation in liver. These results provide strong evidence that UCP3 and MTE-1 are linked within the same metabolic pathway as regulators of fatty acid metabolism.

These data further explain why fenofibrate causes reduction of body weight in our experimental setting [15]. The exported FFA^- anions can be activated to acyl-CoA and either transported back into the mitochondria or metabolized by peroxisomes whose number and activity are strongly induced by fenofibrate [15,29]. The cytoplasmic fatty acid activation would require ATP hydrolysis and a concomitant entry of H^+, generating a futile ATP hydrolysis and a H^+ re-entry, if any, with the net result of a reduced ATP production and additional energy wasting.

In conclusion, we demonstrate a de novo expression of UCP3 in liver, provide evidence that UCP3 and MTE-1 may concur in regulating fatty acid metabolism and enhancing energy dissipation in liver mitochondria and describe a suitable animal model to deepen comprehension of the physiological role of UCP3. The findings reported might prompt studies aimed at verifying whether fenofibrate or other compounds interfere with energy metabolism in man.

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