Long-chain fatty acids regulate liver carnitine palmitoyltransferase I gene (*L-CPT I*) expression through a peroxisome-proliferator-activated receptor α (PPARα)-independent pathway

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Liver carnitine palmitoyltransferase I (L-CPT I) catalyses the transfer of long-chain fatty acid (LCFA) for translocation across the mitochondrial membrane. Expression of the *L-CPT I* gene is induced by LCFAs as well as by lipid-lowering compounds such as clofibrate. Previous studies have suggested that the peroxisome-proliferator-activated receptor α (PPARα) is a common mediator of the transcriptional effects of LCFA and clofibrate. We found that free LCFAs rather than acyl-CoA esters are the signal metabolites responsible for the stimulation of *L-CPT I* gene expression. Using primary culture of hepatocytes we found that LCFAs failed to stimulate *L-CPT I* gene expression both in wild-type and PPARα-null mice. These results suggest that the PPARα-knockout mouse does not represent a suitable model for the regulation of *L-CPT I* gene expression by LCFAs in the liver. Finally, we determined that clofibrate stimulates L-CPT I through a classical direct repeat 1 (DR1) motif in the promoter of the *L-CPT I* gene while LCFAs induce L-CPT I via elements in the first intron of the gene. Our results demonstrate that LCFAs can regulate gene expression through PPARα-independent pathways and suggest that the regulation of gene expression by dietary lipids is more complex than previously proposed.

Key words: cultured hepatocyte, long-chain fatty acyl-CoA, PPARα-null mice, transient transfection.

INTRODUCTION

Recent evidence suggests that long-chain fatty acids (LCFAs) rapidly modulate the transcription of several genes involved in their own metabolism (reviewed in [1,2]). However, the nature of the signal and the molecular mechanisms by which LCFAs regulate gene transcription is still a matter of controversy. For instance, it has been reported that long-chain fatty acyl-CoAs were the intracellular metabolites responsible for the transcriptional effects of LCFAs in prokaryotes (*Escherichia coli*) or in lower eukaryotes (yeast) [3,4]. However, in mammalian cells, indirect evidence suggests differently: (i) in COS-7 cells transfected with the promoter of peroxisomal acyl-CoA oxidase (ACO) linked to chloramphenicol acetyltransferase (CAT), the stimulation of ACO transcription was substantially greater with free arachidonic acid than with its CoA ester [5]; (ii) in the Ob 1771 adipose cell line, the stimulation of *aP2* gene expression by LCFA preceeds the expression of the gene encoding long-chain acyl-CoA synthetase (ACS) [6]. On the basis of *in vitro* assays and/or comparison between the effects of LCFA and peroxisome proliferators, it was suggested that LCFAs modulated gene transcription through the activation of the peroxisome-proliferator-activated receptor α (PPARα, reviewed in [7,8]). The role of PPARα in mediating the effects of peroxisome proliferators has been clearly established in PPARα-null ($-/-$) mice, but the contribution of PPAR in mediating the effects of LCFAs remains controversial. In fetal rat hepatocytes, LCFAs induced the transcription of liver carnitine palmitoyltransferase I (L-CPT I; the rate-limiting enzyme in mitochondrial LCFA oxidation) but not carnitine palmitoyltransferase II (CPT II; the second enzyme involved in the transfer of LCFAs into mitochondria) [9]. In contrast, peroxisome proliferators (such as clofibrate) induced both *L-CPT I* and *CPT II* gene expression [9]. In addition, LCFA-induced *L-CPT I* gene transcription was antagonized by insulin whereas clofibrate-induced *L-CPT I* and *CPT II* gene expression was not [9]. These data suggested that peroxisome proliferators and LCFAs control gene expression through different mechanisms.

The aim of the present work was to determine the respective roles of free LCFAs and their CoA esters in the induction of gene expression in hepatoma cells and to investigate the contribution of PPARα in LCFA-induced gene expression.

EXPERIMENTAL PROCEDURES

Animals

Female Wistar rats bred in our laboratory were used. Experiments were performed in 12-day-old suckling rats obtained as described previously [10]. Wild-type or PPARα-null ($-/-$) mice (3–5 months old) on a C57 BL/6 genetic background were also used. They were housed at 23 °C in individual plastic cages with

Abbreviations used: L-CPT I, liver carnitine palmitoyltransferase I; CPT II, carnitine palmitoyltransferase II; mtHMG-CoA synthase, mitochondrial hydroxymethylglutaryl-CoA synthase; PPARα, peroxisome-proliferator-activated receptor α; PPRE, peroxisome-proliferator-responsive element; RXR, retinoid X receptor; LCFA, long-chain fatty acid; PEPCK, phosphoenolpyruvate carboxykinase; ACS, acyl-CoA synthetase; ACO, acyl-CoA oxidase; BFE, bifunctional enzyme; PEI, polyethylenimine; CAT, chloramphenicol acetyltransferase; PUFA, polyunsaturated fatty acids; L-PK, liver pyruvate kinase.

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light from 07:00 to 19:00. They had free access to water and food (energy, 63% carbohydrate/5% fat/22% protein; A03, UAR, Epinay, France).

Isolation and primary culture of suckling rat and adult mouse hepatocytes

Isolation and culture of 12-day-old suckling rat hepatocytes was performed as described previously [11]. As the liver phenotype was different in male and female PPARα-null mice [12], hepatocytes were isolated from either males or females. The livers from two wild-type and two PPARα-null (−/−) mice were perfused simultaneously with Hanks' balanced salt sodium medium (5.4 mM KCl/0.45 mM KH_2PO_4/138 mM NaCl/4.2 mM $NaHCO_3$/0.34 mM Na_2HPO_4/5.5 mM glucose/1 M Hepes/50 mM EGTA/50 mM $CaCl_2$, pH 7.4). Livers were washed out at a rate of 5 ml/min using the portal vein. Then collagenase (0.025%) was added and the perfusion rate was reduced to 4 ml/min. Hepatocytes (2.5×10^6–3×10^6 cells/dish) were plated in 60 mm Petri dishes in Williams' E/Ham's F12 medium (v/v, Gibco-BRL, Cergy-Pontoise, France) supplemented with 0.28 mM ascorbic acid, 2.4 mM L-glutamine, 0.07 mM ethanolamine, 1 mg/ml bovine transferin, 0.4 mM sodium pyruvate, 1.2 g/l glucose, 0.7 mM arginine, 0.4 mM ornithine and antibiotics (100 units/ml penicillin/10 g/ml streptomycin). During cell attachment, a fetal calf serum substitute (Ultroser G, 2%; IBF, Villeneuve la Garenne, France) and dexamethasone (10^{-7} M) were present. After 4 h, the non-adhering cells were removed and the medium was replaced by an Ultroser G and dexamethasone-free medium. Then hepatocytes from both genotypes were cultured for 6, 24 or 48 h in either the absence or presence of linoleate (0.5 mM) or clofibrate (0.5 mM). To avoid the detergent effect of non-esterified fatty acids, they were bound to defatted BSA (final concentration 0.2%).

Hepatoma cell culture

Fao is a well-differentiated subclone derived from the rat hepatoma H4IIE C3 line. These cells were cultured in Ham's F12 medium supplemented with 100 units/ml penicillin, 50 μg/ml streptomycin, 2 mM glutamine and 10% fetal calf serum and equilibrated with 5% CO_2/95% O_2 in a humidified incubator at 37 °C. Cells were detached with 1 ml of 0.5% trypsin/5.4 mM EDTA and replated in 60 mm Petri dishes for total RNA extraction and transfection assays and in 100 mm dishes for isolation of mitochondria and microsomes. All experiments were performed on subconfluent cells at the same stage of differentiation. Cells were deprived of fetal calf serum during the last 24 h of the treatment. Effectors were added 1–24 h before the end of the experiment.

Plasmid description and preparation

Three plasmids driving the expression of the gene encoding luciferase were used. pGL3-CPT11 and pGL3-CPT20 contain, respectively, 964 bp and 6870 bp of the regulatory sequence from the start site of transcription and pGL3-CPT12 includes 964 bp plus the first intron and the beginning of the second exon, as described previously [13]. The peroxisome-proliferator-responsive element (PPRE) consensus sequence of the bifunctional enzyme (*BFE*) gene linked to the luciferase reporter gene (BFE-Luc −2952/−2918 bp) was used as a positive PPARα target construct [14]. This plasmid was provided by Dr Norbert Latruffe (Laboratoire de Biologie Moléculaire et Cellulaire, Dijon, France). Plasmids were prepared by two successive equilibrium centrifugations in a caesium/ethidium bromide gradient.

The heterologous promoter–reporter construct L-CPT I (−2967/−2773)-TKCAT was constructed by ligation of a PCR product, an amplification of a L-CPT I sequence using synthetic oligonucleotides (upper strand, 5′-GGGGCATGCTATCCTT-GTGACAACGCCGC-3′; lower strand, 5′-AACTCTAGAC-CATAATAATGTTG-3′) into *Sph*I (5′) and *Xba*I (3′) sites in the PBLCAT5 polylinker [15]. Automated DNA sequencing confirmed that the nucleotide sequence of the PCR product was 100% identical with the template sequence.

Transient transfection protocol

Subconfluent Fao cells were transfected using the technique described by Boussif et al. [16]. Briefly, cells were transfected with 5 μg of plasmid DNA/plate and polyethylenimine (PEI; 0.56 μl of PEI/μg of DNA) in serum-free medium. In some experiments, cells were co-transfected with 0.5 μg of an expression vector containing full-length cDNA encoding mouse PPARα. This plasmid was provided generously by Dr Johan Auwerx (CNRS, Laboratoire de Génétique Moléculaire des Eucaryotes, Strasbourg, France). Transfection was accomplished by dilution of PEI and plasmid DNA in 50 μl of NaCl (150 mM). They were mixed together and the polycation/DNA complex was diluted into 2 ml of serum-free medium in 60 mm culture dishes.

Primary cultures of rat hepatocytes were transfected with 5 μg of L-CPT I-TKCAT constructs and PEI as described for Fao cells. In order to increase transfection efficiency, 200 plaque-forming units/plate of Ad-RSV-nlsLacZ (Rous sarcoma virus promoter driving the *nlsLacZ* gene) was added simultaneously [17]. This adenovirus was provided by M. Perricaudet (CNRS, Villejuif, France) [18].

After transfection (7 h), the medium was changed and experimental conditions (LCFA or clofibrate) were tested over a 14 h period (for luciferase assay) or 48 h period (for CAT assay). The cells were lysed using the reporter lysis buffer (Promega, Charbonnière, France), scraped off and centrifuged at 8000 ***g*** and 4 °C for 5 min. Proteins, luciferase and CAT activity were determined for the supernatant. Luciferase activity was assayed with 25 μl using the Promega kit (Promega) and CAT activity was assayed as described by Seed and Sheen [19].

Electrophoretic mobility-shift assays

Proteins for electrophoretic mobility-shift assays were obtained by *in vitro* transcription and translation using the TNT T7-coupled reticulocyte lysate system (Promega). *In vitro*-translated proteins PPARα and retinoid X receptor (RXR) α were incubated at 4 °C for 20 min with 50000 c.p.m. of labelled double-stranded oligonucleotides in a buffer containing 20 mM Tris/HCl, pH 7.5, 100 mM KCl, 14% glycerol, 2 mM dithiothreitol and 0.5 μg of poly(dI-dC) in a final volume of 20 μl. The complexes were then separated on a 6% native polyacrylamide gel with 0.25 × Tris/borate/EDTA running buffer at 200 V for 3 h. For DNA-binding competition experiments, a 1–50-fold molar excess of the unlabelled double-stranded competitor oligonucleotide was added to the incubation reaction. After the electrophoretic separation, gels were dried and exposed to X-ray films. The sense oligonucleotides used were: 5′-AGCTGATCCTTCC-CGAACGTGACCTTTGTCCTGGTCCCCTTTTGCTC-3′ for ACO-PPRE and 5′-AGTCAAAAGTGTACAGGAGCTCAA-AGTTCAAGTTCAG-3′ for L-CPT I-PPRE.

Effects of fatty acids and peroxisome proliferator on L-CPT I mRNA levels

The effects of medium-chain fatty acid (octanoate, $C_{8:0}$), and LCFAs (*cis*-9-oleate, $C_{18:1}$, and *cis*-9,12-linoleate, $C_{18:2}$) were tested on *L-CPT I* gene expression. Dose–response (40–320 μM) and time-course (1–8 h) curves of the oleate effect were performed to determine the optimal concentration and length of exposure. The effects of non-metabolizable fatty acids (2-bromopalmitate, $C_{16:0}$, bromostearate, $C_{18:0}$, and bromooctanoate, $C_{8:0}$) were tested at the concentrations indicated in the Results section. Non-esterified fatty acids were bound to BSA (final concentration 40 μM).

Clofibrate (500 μM) was dissolved in DMSO and its effects on gene transcription was tested after 6 h of culture. Prior to this it was checked that DMSO had no effect on *L-CPT I* gene expression (results not shown).

Effects of ACS inhibitors on *L-CPT I* gene expression

Two different inhibitors of long-chain ACS were used: bromooctanoate (320 μM [20]) and the sulpho-conjugate metabolite (M1) of troglitazone, an oral anti-diabetic agent and a potent non-competitive inhibitor of ACS [21]. As metabolite M1 does not cross the plasma membrane, Fao cells were permeabilized according to the following design.

Fao cell permeabilization

Cells plated in 100 or 60 mm Petri dishes were washed once with 150 mM NaCl, and then permeabilized with, respectively, 5 or 2 ml of freshly prepared saponin (50 μg/ml in PBS) for 5 min at 37 °C with occasional swirling. The culture monolayer was then washed carefully to remove saponin and the experimental conditions were tested. Metabolite M1 of troglitazone was added to permeabilized Fao cells either 1 h prior to oleate addition or at the same time as oleate addition. In both cases, cells were scraped off 6 h after oleate addition.

Extraction and Northern-blot analysis of total RNA

Total RNA was extracted from cultured hepatoma cells according to Chomczynski and Sacchi [22]. Briefly, the cells from two Petri dishes were lysed with 4 M guanidine thiocyanate, 25 mM sodium citrate, 0.5 % sodium sarcosyl and 0.1 M 2-mercaptoethanol solution. After homogenization of the resulting suspension, total RNA was extracted with phenol/chloroform and precipitated with isopropanol. Northern-blot analysis of total RNA (20 μg) was performed after 1 % agarose gel electrophoresis in 2.2 M formaldehyde as described previously [23]. After transfer to nylon membranes (Hybond N; Amersham International, Amersham, Bucks., U.K.), the filters were prehybridized (6 h at 42 °C) and then hybridized overnight at 42 °C in the presence of formamide. cDNA probes of rat L-CPT I, mitochondrial hydroxymethylglutaryl-CoA synthase (mtHMG-CoA synthase) and cytosolic phosphoenolpyruvate carboxykinase (PEPCK) described previously [24–26] were labelled with [α-^{32}P]dATP by using the Multiprime labelling system kit (Amersham International). Hybridization of the blots with a [γ-^{32}P]ATP-labelled synthetic oligonucleotide specific for the 18 S rRNA subunit [27] allowed us to correct for possible variations in the amount of RNA transferred on to the membranes. Quantifications were performed by scanning densitometry of the autoradiographs.

Isolation of mitochondria and microsomes

Mitochondria and microsomes were isolated from cultured hepatoma cells plated on to 100 mm Petri dishes, in a sucrose buffer (300 mM sucrose/5 mM Tris/1 mM EGTA, pH 7.4) using the differential centrifugation technique as described previously [28,29]. The final mitochondrial and microsomal pellets were resuspended at a protein concentration of 1–2 mg/ml.

Measurement of enzyme activities

Mitochondrial L-CPT I activity was assayed as described previously [29]. In all experiments, the formation of palmitoylcarnitine from palmitoyl-CoA (80 μM) and L-[methyl-^{3}H]-carnitine (1 mM, 1.6 Ci/mol) was almost completely suppressed by a high concentration of malonyl-CoA (100 μM), suggesting that only L-CPT I activity was measured without any significant contribution of CPT II.

Mitochondrial and microsomal long-chain ACS were assayed according to Krisans et al. [30] as described previously [21]. Briefly, ACS activity was measured in the presence of [1-^{14}C]oleate (100 μM) on 5 and 10 μg of mitochondrial and microsomal proteins for 6 min at 37 °C.

Chemicals

The sulpho-conjugate derivative (metabolite M1) of the troglitazone was provided kindly by Dr H. Hashimoto (Sankyo, Tokyo, Japan). [1-^{14}C]Oleate and [^{3}H]carnitine were purchased from Amersham International. Saponin, fatty acids and 25 kDa PEI were obtained from Sigma (St Quentin-Fallavier, France) and Fluka.

Analytical methods and statistical analysis

Protein concentrations were determined by the method of Lowry et al. [31] with BSA as standard.

Results are expressed as means $\pm$ S.E.M. Statistical analysis was performed using the Wilcoxon rank-order test [32].

RESULTS

Effects of various fatty acids on L-CPT I mRNA levels in Fao hepatoma cells

When Fao hepatoma cells were cultured in fetal-calf-serum-free medium for 24 h, the L-CPT I mRNA concentration was very low. The L-CPT I mRNA level was increased in a time- and dose-dependent manner by oleate (Figure 1). The maximal effect of oleate was achieved after 6 h of culture with a half-maximal effect at a concentration of 100 μM (Figure 1). Similar results were obtained with linoleate (results not shown).

To test whether the accumulation of L-CPT I mRNA in response to LCFAs required their intramitochondrial metabolism, Fao hepatoma cells were cultured for 6 h in the presence of two non-metabolizable fatty acids, either 2-bromopalmitate or 2-bromostearate. Under these conditions, the increase of L-CPT I mRNA was 50–60 % greater than in the presence of the metabolizable fatty acid used at the same concentration (fold of stimulation above control i.e. in the absence of fatty acid: bromopalmitate, 3.2 ± 0.5, versus oleate, 2.5 ± 0.4; bromostearate, 3.7 ± 0.1, versus stearate, 2.4 ± 0.3, $n = 5$). This suggested that either non-esterified fatty acids or their CoA esters were responsible for the stimulation of *L-CPT I* gene expression. To answer this question, Fao hepatoma cells were cultured in the presence of LCFAs and inhibitors of long-chain ACS.

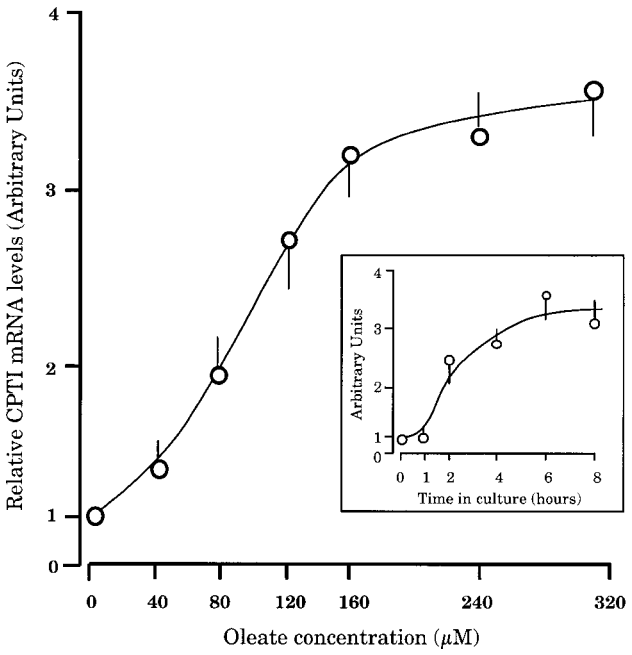

Figure 1 Time-course and dose–response curves of the effect of oleate on *L-CPT I* gene expression in Fao hepatoma cells

Fao cells were cultured either for various times in the presence of 320 μM oleate (insert) or for 6 h in the presence of the indicated concentrations of oleate. Results are expressed as arbitrary units, the reference value (1) being the concentration of L-CPT I mRNA in cells cultured with 40 μM BSA alone. Results are means $\pm$ S.E.M. from 4–12 different cultures.

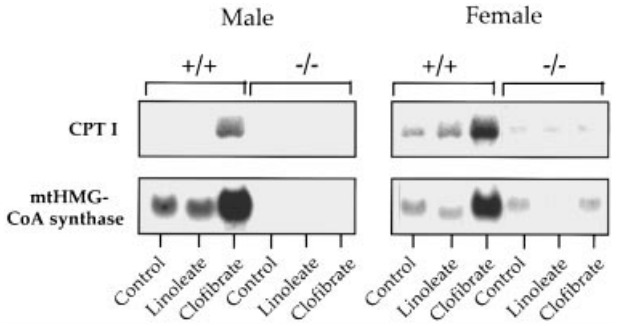

Figure 2 Effects of linoleate and clofibrate on *L-CPT I* and mtHMG-CoA synthase gene expression in cultured hepatocytes from wild-type and PPARα-null mice

Hepatocytes from male and female wild-type (+/+) or PPARα-null (−/−) mice were cultured for 24 h in the presence of linoleate or clofibrate (both 0.5 mM). This blot is representative of four different cultures.

Effects of inhibitors of ACS on LCFA-induced L-CPT I mRNA levels

When Fao hepatoma cells were cultured in the presence of bromo-octanoate, the activity of ACS was inhibited by 85 % in both isolated mitochondria and microsomes (Table 1). Under these conditions, bromo-octanoate increased L-CPT I mRNA levels whereas octanoate had no effects (Table 1). To confirm these results, we used another ACS inhibitor, the sulpho-conjugate metabolite (M1) of troglitazone [21]. As the plasma membrane was impermeable to this compound we first permeabilized the plasma membrane by using saponin. In preliminary experiments we checked that saponin had no deleterious effects on intracellular membrane. The activity of mitochondrial L-CPT I, which is particularly sensitive to detergent (reviewed in [33]), was not affected by saponin treatment (2.1 ± 0.3 nmol/min per mg of protein, $n = 5$; see Table 1 for comparison). In addition, saponin-permeabilization of Fao cells did not impair oleate-induced L-CPT I mRNA accumulation (2.9 ± 0.3, $n = 5$; see Table 1 for comparison) or cAMP-induced PEPCK mRNA accumulation (percentage of control: cAMP, 190 ± 20 %; saponin + cAMP, 160 ± 10 %, $n = 3$). When permeabilized cells were cultured in the presence of the metabolite M1, the activities of mitochondrial and microsomal ACS were inhibited by more than 90 % (Table 1). The same degree of inhibition of ACS was achieved whether metabolite M1 was added 1 h before or at the same time as oleate. Despite profound inhibition of ACS activity, the L-CPT I mRNA accumulation in response to oleate was not altered when compared with oleate alone (Table 1).

Effects of fatty acid and clofibrate in cultured mouse hepatocytes

In cultured hepatocytes from wild-type mice, the stimulatory effect of clofibrate on *L-CPT I* gene expression was maximal after 24 h of exposure (results not shown). As shown on Figure 2 the basal level of L-CPT I mRNA was higher in hepatocytes isolated from wild-type females than in age-matched males. Conversely, mtHMG-CoA synthase gene expression was higher in males than in age-matched female hepatocytes cultured under basal conditions (Figure 2). Despite these sex differences in the basal level of L-CPT I and mtHMG-CoA synthase gene ex-

Table 1 Effects of ACS inhibitors on fatty acid-induced L-CPT I mRNA levels in Fao hepatoma cells

Fao hepatoma cells were cultured for 6 h in the absence or presence of octanoate, bromo-octanoate or oleate, each used at 320 μM. In some experiments, Fao hepatoma cells were permeabilized for 5 min in the presence of saponin (50 μg/ml) to allow the entry of sulpho-conjugate metabolite (M1) of troglitazone. Metabolite M1 was added 1 h before the addition of oleate. Results are means $\pm$ S.E.M. from the number of experiments shown in parentheses. *$P < 0.01$ when compared with respective control conditions. ND, not determined.

	ACS activity (nmol/min per mg of protein)			
Culture conditions	Mitochondria	Microsomes	Mitochondrial L-CPT I (nmol/min per mg of protein)	L-CPT I mRNA levels (arbitrary units)
Control	19.5 ± 5.5 (12)	36 ± 4 (11)	1.9 ± 0.4 (5)	1
Octanoate	ND	ND	ND	1.2 ± 0.3 (8)
Bromo-octanoate	2.8 ± 0.6 (5)*	6 ± 1 (5)*	2.4 ± 0.4 (5)	3.4 ± 0.4 (5)*
Oleate	19.8 ± 6.3 (10)	30 ± 4 (9)	1.9 ± 0.4 (5)	3.1 ± 0.3 (10)*
M1	2.7 ± 0.8 (5)*	7 ± 2 (5)*	1.8 ± 0.2 (5)	1.1 ± 0.1 (5)
Oleate + M1	2.3 ± 1.0 (5)	6 ± 3 (5)*	1.8 ± 0.2 (5)	2.8 ± 0.2 (5)*

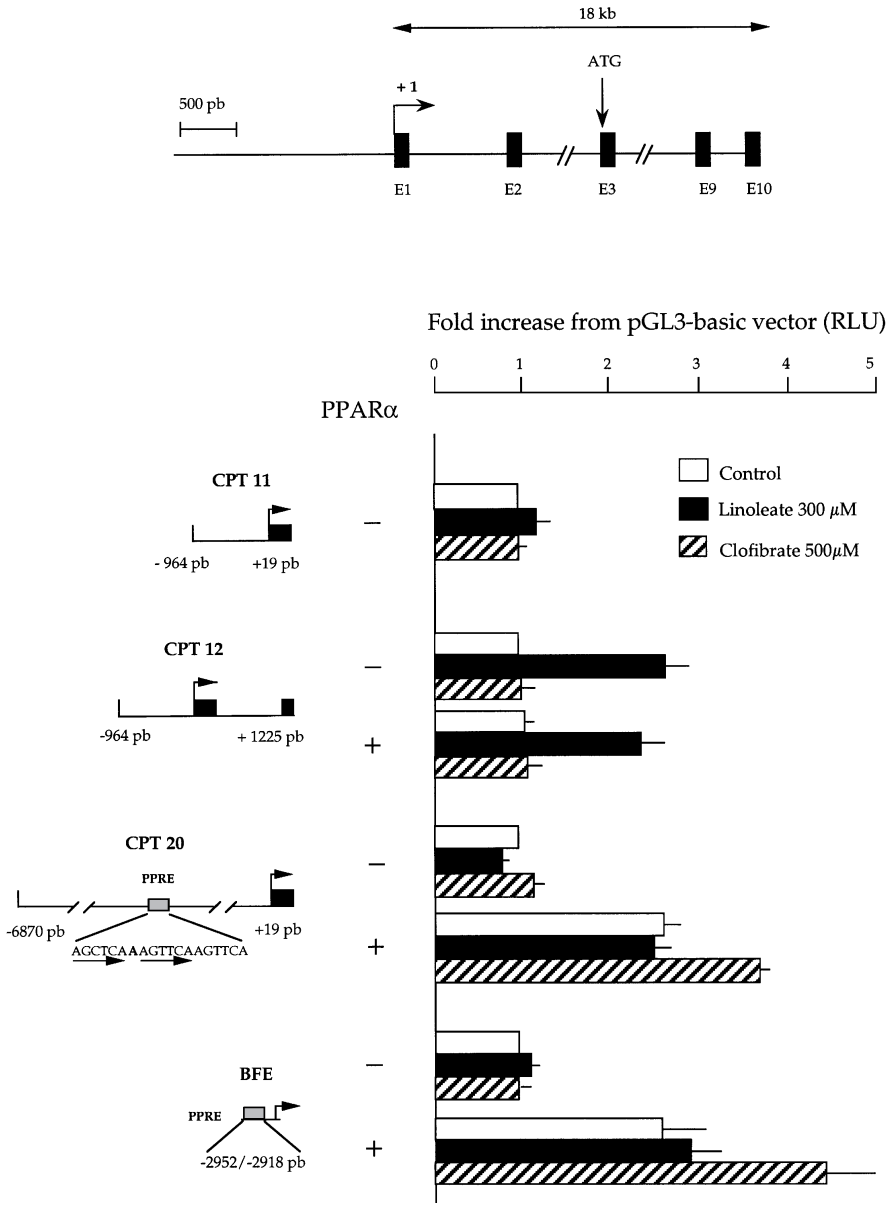

Figure 3 Effects of linoleate and clofibrate on transcriptional activity of *L-CPT I* gene promoter constructs

Fao cells were transfected with 5 μg of different reporter plasmids pGL3-CPT11 ($-964/+19$ bp), pGL3-CPT12 ($-964/+1225$ bp), pGL3-CPT20 ($-6870/+19$ bp) or the PPRE sequence of BFE ($-2952/-2918$ bp), which was used as a positive PPAR target construct. In some experiments cells were co-transfected with 0.5 μg of a plasmid expressing full-length cDNA encoding mouse PPARα. Cells were cultured in either the absence (control) or presence of linoleate (300 μM) or clofibrate (500 μM). After 14 h of culture, cells were harvested and assayed for protein concentrations and luciferase activity. Luciferase assays (relative light units, RLU) were normalized using the pGL3-enhancer vector activity standard. Results are means $\pm$ S.E.M. from 3–6 different cultures performed in duplicate.

pression, it is noteworthy that the degree of stimulation induced by clofibrate on L-CPT I or mtHMG-CoA synthase mRNA is similar whatever the sex of the animal and is totally lost in cultured hepatocytes from PPARα-null mice (Figure 2). Neither *L-CPT I* nor mtHMG-CoA synthase genes were induced by linoleate, whatever the origin of the hepatocytes (wild-type or PPARα-null) or the sex of animal [Figure 2; fold increase of L-CPT I mRNA levels above controls, wild-type male, clofibrate (clo) 2.8 ± 0.3, linoleate (lino) 0.9 ± 0.1; PPARα-null male, clo 1.3 ± 0.2, lino 1.0 ± 0.3; wild-type female, clo 3.1 ± 0.3, lino 0.9 ± 0.1; PPARα-null female, clo 1.2 ± 0.2, lino 0.7 ± 0.2, $n = 4$]. This did not result from the genetic background in which the knockout was done (C57 BL/6) since we found similar results in cultured hepatocytes from SV129 wild-type mice (fold increase of L-CPT I mRNA levels above controls: clofibrate 3.5 ± 0.3, linoleate 0.9 ± 0.2, $n = 3$). It is noteworthy that the basal expression of mtHMG-CoA synthase was lower in the liver of PPARα-null mice than in wild-type controls (Figure 2). Similar observations were reported in the liver of wild-type and PPARα-null mice [34].

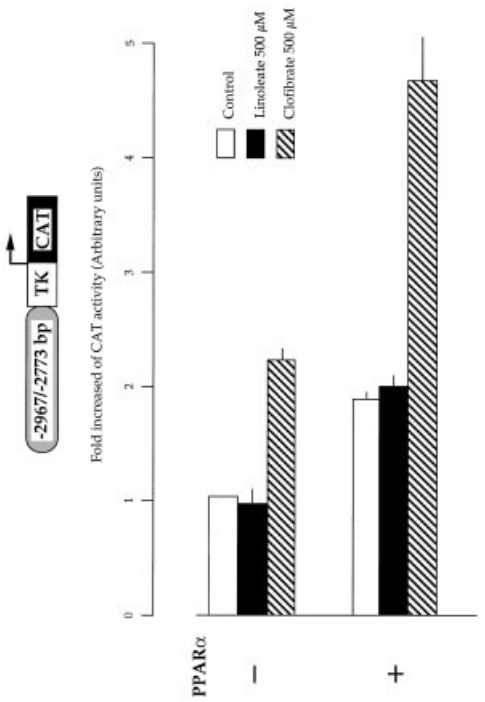

Figure 4 Effects of linoleate and clofibrate on the putative PPRE region of the *L-CPT I* gene

Hepatocytes from 12-day-old suckling rats were transfected with 5 μg of the heterologous promoter–reporter construct L-CPT I ($-2967/-2773$)-TKCAT. In some experiments hepatocytes were co-transfected with 0.5 μg of an empty plasmid (PPAR$\alpha-$) or with plasmid expressing full-length cDNA encoding mouse PPARα ($+$). Hepatocytes were cultured in either the absence (control) or presence of linoleate or clofibrate (both 500 μM). After 48 h of culture, cells were harvested and assayed for protein concentrations and CAT activity. CAT assays were normalized using the pSV2-CAT vector activity standard. Results are means $\pm$ S.E.M. from three different cultures.

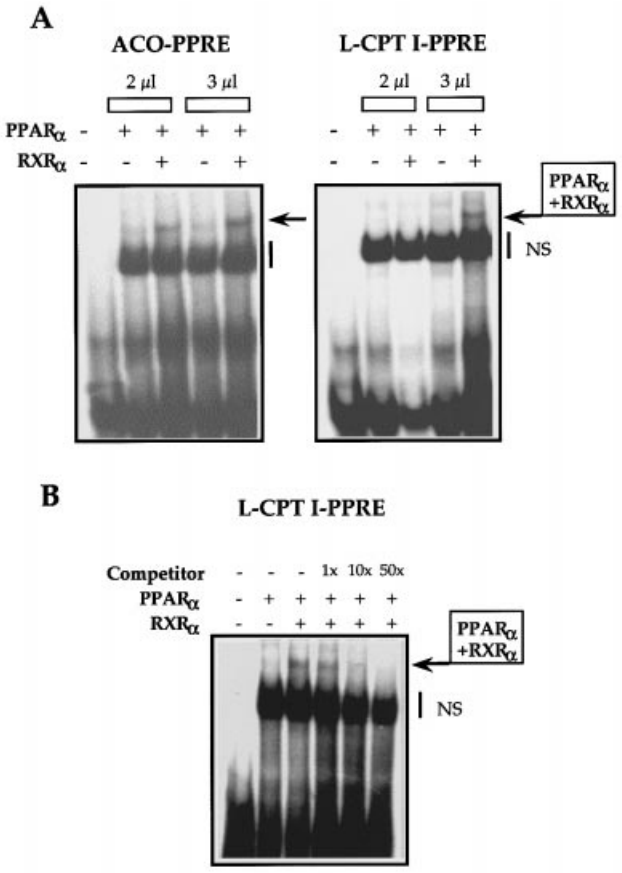

Figure 5 Electrophoretic mobility-shift analysis of PPARα binding to the L-CPT I PPRE sequence

(**A**) Direct electrophoretic mobility-shift assays: direct binding of *in vitro*-synthetized PPARα or PPARα–RXRα heterodimer was achieved using ^{32}P-labelled DNA sequences of ACO-PPRE or L-CPT I-PPRE with 2 or 3 μl of lysate. The oligonucleotide sequences are shown in the Experimental procedures section. (**B**) Competitive electrophoretic mobility-shift assays: competition assays were performed in the presence of a 1–50-fold molar excess of unlabelled L-CPT I-PPRE oligonucleotide. The results are representative of two separate experiments. NS, non-specific complexes.

Effects of fatty acids and clofibrate on *L-CPT I* gene promoter

To further investigate the molecular mechanisms involved in the regulation of *L-CPT I* gene expression by LCFAs and peroxisome proliferators, transient transfections of plasmids containing the L-CPT I promoter linked to luciferase were performed. As shown on Figure 3, the shorter construct CPT11 ($-964/+19$ bp) of the *L-CPT I* gene promoter showed a low luciferase activity which remained unchanged in cells cultured in the presence of either linoleate or clofibrate. When the first intron of the *L-CPT I* gene was added to the previous construct (CPT12, $-964/+1225$ bp), LCFA stimulated (2.5 ± 0.1-fold, $P < 0.01$, $n = 6$) the transcription of this construct (Figure 3). By contrast, this construct was not stimulated by clofibrate. The absence of a clofibrate effect did not result from the culture conditions since clofibrate stimulated the endogenous *L-CPT I* gene expression in transfected Fao hepatoma cells (2.5 ± 0.3, $P < 0.05$, $n = 4$). We then tested the possibility that endogenous PPARα could be rate-limiting for the effect of clofibrate. Indeed, this was suspected from the results obtained with the CPT20 construct, which contains a putative PPRE sequence of the *L-CPT I* gene ($-2859/-2846$ bp), and with the construct containing the PPRE sequence of BFE used as a positive control of the effect of clofibrate. None of these constructs were stimulated by clofibrate when transfected in Fao hepatoma cells (Figure 3). The co-transfection of the PPARα expression vector into Fao cells stimulated the basal rate of transcription of CPT20 and BFE constructs, both containing a PPRE sequence, whereas PPARα co-transfection had no effect on the CPT12 construct, which did not have a PPRE consensus sequence (Figure 3). Accordingly, the PPARα co-transfection allowed the stimulation by clofibrate of the CPT20 and BFE constructs but not that of the CPT12 construct (Figure 3). By contrast, the co-transfection of PPARα did not enhance the effect of linoleate on the CPT12 construct (Figure 3) nor on CPT20 or BFE (compare linoleate + PPARα with PPARα alone, Figure 3). Similar results have been obtained in rat hepatocytes transfected with these chimaeric DNA constructs (J.-F. Louet, J.-F. Decaux and J. Girard, unpublished work).

To further characterize the functionality of the PPRE of L-CPT I, the sequence between -2967 and -2773 bp of the promoter region was introduced into the heterologous thymidine kinase vector fused to *CAT* reporter gene (TKCAT), and transfected into cultured hepatocytes. In the absence of PPARα co-transfection, clofibrate induced a 2-fold increase in CAT activity (2.2 ± 0.2, $n = 3$; Figure 4), whereas linoleate had no effect on this construct. When PPARα was co-transfected,

there was a 2-fold increase in basal CAT activity (Figure 4) and an increase of clofibrate-induced CAT activity by 4.8 ± 0.4-fold ($n = 3$; Figure 4). By contrast, PPARα co-transfection did not enhance the effect of linoleate on the CAT construct above the basal level (Figure 4).

Morever, we have examined the ability of the heterodimer complex PPARα–RXRα to bind the ACO (as a control) and the L-CPT I-PPRE ($-2883/-2773$ bp) sequences in gel-shift experiments. We found that the PPARα–RXRα complex bound to the specific PPRE oligonucleotides of ACO and L-CPTI (Figure 5A). Competition assays were then performed using excess amounts of unlabelled specific L-CPT I oligonucleotide. A decrease of the intensity of one complex indicated that the binding between the heterodimer PPARα–RXRα and the L-CPT I-PPRE sequence was specific (Figure 5B).

DISCUSSION

The aim of the present work was to gain a better understanding of the effect of LCFAs and peroxisome proliferators on the regulation of *L-CPT I* gene expression. The following two questions were addressed. (i) What is the intracellular signal responsible for the LCFA effect on gene expression? (ii) Does LCFA-induced *L-CPT I* gene expression depend upon PPARα?

To answer the first question we developed a cellular model which allowed short-term exposure to a metabolic inhibitor, in order to understand the mechanisms involved in the stimulation of *L-CPT I* gene expression by LCFAs [9]. Fao hepatoma cells were chosen for their capacity to respond rapidly to physiological concentrations of fatty acids. This was demonstrated for liver fatty acid-binding protein and fatty acid synthase [35] and for L-CPT I (present study). Moreover, the specificity (long-chain versus short-chain fatty acids) and the intensity (3–4-fold increase) of the stimulation was similar to that found in cultured fetal rat hepatocytes [9]. The main difference was the time course of response to LCFAs and peroxisome proliferators (6 h for Fao cells versus 48 h for fetal hepatocytes).

Although the nature of the metabolite(s) triggering the transcriptional effect of LCFA remains unknown, the fact that *L-CPT I* gene expression was more stimulated by non-metabolizable than by metabolizable LCFAs suggests that either non-esterified fatty acids or acyl-CoA esters were the signal metabolite. Furthermore, when the activity of long-chain ACS is inhibited by more than 90 % the LCFA-induced *L-CPT I* gene expression is still observed. This suggested that non-esterified fatty acids rather than acyl-CoA esters are the active component regulating *L-CPT I* gene expression. It must be emphasized that the oleate-induced L-CPT I mRNA accumulation observed in the presence of troglitazone metabolite (M1) does not result from a PPARγ-mediated mechanism. Indeed, it has been shown that thiazolidinediones are potent modulators of gene expression in white adipose tissue secondarily to their high-affinity binding to PPARγ (reviewed in [36]). Despite the fact that PPARα and β are the predominant isoforms expressed in Fao hepatoma cells [35] we have verified that metabolite M1 did not induce *PPARγ* gene expression in these cells (C. Schoonjans, J. Auwerx, J. Girard and J. P. Pégorier, unpublished work). Moreover the same degree of stimulation of *L-CPT I* gene expression is achieved when ACS is inhibited by bromo-octanoate. This favours a role of non-esterified fatty acids as the metabolic signal for the control of *L-CPT I* gene expression. Similar observations have been made for the *aP2* gene whose expression was stimulated by LCFAs in Ob 1771 adipocyte cells before these cells expressed the *ACS* gene (reviewed in [6]). In addition, in COS-7 cells transfected with the peroxisomal ACO promoter linked to the *CAT* reporter gene, the transcription was stimulated more efficiently by free arachidonic acid than by arachidonyl-CoA [5]. Finally, non-esterified fatty acids are better ligands for PPARα and β than their CoA thioesters [37–39]. This led to the second goal of our work. Do LCFAs and peroxisome proliferators stimulate *L-CPT I* gene expression through a common pathway, i.e. via a PPARα-dependent mechanism? The role of PPARα in mediating the transcriptional effect of peroxisome proliferators in the liver has been clearly identified by studying PPARα-null mice [40–43]. By contrast, the contribution of PPARα in mediating the effects of LCFA has never been established. For instance, it was shown recently that the expression of *L-CPT I*, a putative PPARα target gene ([9] and the present work, see below), was normally induced during starvation in the livers of PPARα-null mice [34,44,45]. Similarly, dietary polyunsaturated fatty acids (PUFA) suppress lipogenic gene expression [40] and liver pyruvate kinase (*L-PK*) gene transcription [46] to the same extent in the livers of wild-type and PPARα-null mice. However, as starvation and high-fat diets are associated not only with increased delivery of LCFAs to the liver but also with marked changes in pancreatic hormone concentrations (rise in plasma glucagon, fall in plasma insulin), it is difficult to conclude whether *L-CPT I*, lipogenic enzyme or *L-PK* genes are controlled through a hormone-dependent mechanism and/or a LCFA-independent PPARα pathway in the liver of PPARα-null mice. Using primary cultures of hepatocytes isolated from wild-type and PPARα-null mice, we provide evidence supporting the former hypothesis. i.e. hormone-dependent control of *L-CPT I* gene expression. Indeed, cAMP stimulates L-CPT I mRNA accumulation to the same magnitude in wild-type and PPARα-null mouse hepatocytes (C. Le May, J. Girard and J.-P. Pégorier, unpublished work), whereas LCFAs have no stimulatory effect whatever the mouse genotype. These results suggest that the rise in L-CPT I mRNA levels in the livers of fasting mice of both genotypes [34,44,45] was due to pancreatic hormonal changes rather than to a LCFA-mediated mechanism. The absence of an LCFA effect on gene expression could be a general feature in mouse liver since mtHMG-CoA synthase, whose expression is also regulated by LCFAs in rat liver (reviewed in [47]), failed to be affected by fatty acids in cultured hepatocytes from wild-type mice. Thus our results underline the species-specific differences in the regulation of gene expression by LCFA and unfortunately suggest that the mouse would not be an adequate model in which to study such regulation in the liver.

By contrast, the transfection of chimaeric promoter DNA constructs into liver cells provides evidence for a separate mechanism of LCFA- and clofibrate-induced *L-CPT I* gene transcription. First, we show that LCFA specifically stimulates a DNA construct (pGL3-CPT12) that does not contain a PPRE consensus sequence. The absence of a PPRE-consensus sequence in the pGL3-CPT12 construct could explain the absence of a clofibrate effect on this construct, even in the presence of a co-transfected PPARα expression vector. Secondly, constructs containing the PPRE consensus sequence, either inside the *L-CPT I* gene (CPT20) or inserted into a heterologous reporter gene, are not activated by LCFAs even when PPARα is over-expressed in liver cells. Morever, using gel-shift experiments we have shown that the nuclear receptor complex PPARα–RXRα binds to the L-CPT I-PPRE sequence ($-2883/-2773$ bp). According to previous results, PPARα alone was unable to bind this sequence, indicating that its heterodimerization with RXRα is necessary for DNA binding. Similar data have been obtained for several PPARα-targeting genes, such as ACO, BFE and CYP4A1 [48,49]. Interestingly, these results differ markedly from those reported recently for the regulation of muscle-type CPT I (M-CPT I). Indeed, it was shown that M-CPT I was up-regulated by LCFA

through a PPARα-dependent mechanism [50–52]. This suggests that (i) *M-CPT I* and *L-CPT I* genes could be controlled by LCFAs through distinct regulatory sequences and (ii) the presence of a PPRE consensus sequence in a promoter region of a given gene does necessarily mean that it is regulated by fatty acids. Indeed, such discrepancies between the effect of LCFAs and peroxisome proliferators have been reported previously for the hepatic promoter of the *Spot*14 gene [40,53], which contains two distinct and functional sequences responsible for the effects of peroxisome proliferators and PUFA. Similarly it has been recently shown that PUFA-induced suppression of hepatic *L-PK* gene transcription does not require PPARα, as the heterodimer PPARα–RXRα did not bind the L-PK promoter sequences [46].

The identity of the sequence responsible for the stimulatory effect of LCFAs in the *L-CPT I* gene is currently under investigation. The homology with the PUFA-responsive element described for stearoyl-CoA desaturase [54] will be checked with particular attention.

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