Up-regulation of the expression of the gene for liver fatty acid-binding protein by long-chain fatty acids

Claire MEUNIER-DURMORT*‡, Hélène POIRIER†‡, Isabelle NIOT†, Claude FOREST* and Philippe BESNARD†§

*Centre de Recherche sur l'Endocrinologie Moléculaire et le Développement, CNRS, 9 rue Jules Hetzel, 92190 Meudon, France,
and †Laboratoire de Physiologie de la Nutrition, E.A. DRED 580, École Nationale Supérieure de Biologie Appliquée à la Nutrition et à l'Alimentation (ENSBANA), Université de Bourgogne, 1 esplanade Erasme, 21000 Dijon, France

The role of fatty acids in the expression of the gene for liver fatty acid-binding protein (L-FABP) was investigated in the well-differentiated FAO rat hepatoma cell line. Cells were maintained in serum-free medium containing 40 μM BSA/320 μM oleate. Western blot analysis showed that oleate triggered an approx. 4-fold increase in the cytosolic L-FABP level in 16 h. Oleate specifically stimulated L-FABP mRNA in time-dependent and dose-dependent manners with a maximum 7-fold increase at 16 h in FAO cells. Preincubation of FAO cells with cycloheximide prevented the oleate-mediated induction of L-FABP mRNA, showing that protein synthesis was required for the action of fatty acids. Run-on transcription assays demonstrated that the control of L-FABP gene expression by oleate was, at least in part, transcriptional. Palmitic acid, oleic acid, linoleic acid, linolenic acid and arachidonic acid were similarly potent whereas octanoic acid was inefficient. This regulation was also found in normal hepatocytes. Therefore long-chain fatty acids are strong inducers of L-FABP gene expression. FAO cells constitute a useful tool for studying the underlying mechanism of fatty acid action.

INTRODUCTION

Liver fatty acid-binding protein (L-FABP) is a small cytosolic protein that binds long-chain fatty acids (LCFAs) with high affinity. L-FABP is found in various tissues including liver, small intestine and kidney, in which it is thought to play a crucial role in intracellular fatty acid (FA) trafficking and in protection of the cell against the potential adverse effect of free FAs (reviewed in [1]). L-FABP content is essentially affected by diet composition and peroxisome-proliferator hypolipidaemic drugs of the fibrate family [1]. High-fat diets enhance L-FABP expression in liver [2] and in small intestine [3]. Recently we have reported a transcriptional induction of the L-FABP gene after direct infusion of a dietary oil in the terminal part of the ileum in mice [4]. Fibrates also stimulate the rate of transcription of the L-FABP gene in small intestine and liver [4,5]. This increase is probably due to the activation of specific nuclear receptors termed peroxisome-proliferator-activated receptors (PPARs) [6]. Because LCFAs are the main components of dietary oils and can also activate PPARs [7–9], we hypothesize that L-FABP gene expression is regulated by FAs, as reported for several genes involved in lipid metabolism [10–17]. The results reported here provide the first evidence that FAs might be physiological mediators of L-FABP gene expression and demonstrate that the FAO hepatoma cell line constitutes a useful tool for studying the regulation of L-FABP gene expression *in vitro*.

EXPERIMENTAL

Cell culture and treatments

The FAO cell line is a well-differentiated subclone derived from the rat hepatoma H4 II EC3 line. Cells were cultured at 37 °C, under a humidified atmosphere composed of 10 % CO_2 in air, in Ham F-12 medium (Gibco–BRL) containing 2.3 g/l bicarbonate, 10 % (v/v) fetal calf serum, 200 i.u./ml penicillin and 50 mg/ml streptomycin in accordance with published procedures [14]. The culture medium was changed every 2 days. The doubling time was approx. 24 h. Experiments were performed on subconfluent cells maintained in serum-free medium for 24 h. FAs were complexed to FA-free BSA in Ham F-12 medium before adding to the cells.

Hepatocytes from male Wistar rats (200–220 g) were isolated by collagenase perfusion by the method of Williams et al. [18]. Hepatocytes were plated (1.5×10^7 in each 80 cm^2 dish) and cultured at 37 °C in Leibovitz L-15 medium (Sigma) containing 10 % (v/v) fetal calf serum, 0.276 % BSA, 2 mM glutamine, 3 g/l glucose and 0.05 g/l gentamicin. After a 4 h preincubation period, cells were treated with 320 μM oleic acid for 16 h in serum-free medium. Control cultures were exposed to BSA alone.

Molecular probes

cDNA probes for L-FABP and intestinal fatty acid-binding protein (I-FABP) were generously provided by Dr. J. I. Gordon (Washington University, St. Louis, MO, U.S.A.) and the fatty acid synthase (FAS) plasmid by Dr. A. Goodridge (University of Iowa, Iowa City, IA, U.S.A.). Mouse β-actin cDNA was donated by Dr. A. Alonso (Institut Pasteur, Paris, France). These probes were labelled with [α-^{32}P]dCTP (3000 Ci/mmol; Amersham) by random priming (Megaprime kit, Amersham). A 24-residue oligonucleotide specific for rat 18 S rRNA [19] was used to ensure that equivalent amounts of RNA were loaded and transferred. This oligonucleotide was 5′ end-labelled by using T_4 polynucleotide kinase and [γ-^{32}P]ATP (3000 Ci/mmol, Amersham).

Abbreviations used: ETYA, 5,8,11,14-eicosatetraynoic acid; FA, fatty acid; FAS, fatty acid synthase; I-FABP, intestinal fatty acid-binding protein; LCFA, long-chain fatty acid; L-FABP, liver fatty acid-binding protein; PPAR, peroxisome-proliferator-activated receptor.

* C. M. D. and H. P. should be considered equal first authors.
§ To whom correspondence should be addressed.

Northern blot analysis

Total RNA was extracted by the method of Chomczynski and Sacchi [20]. Total RNA (30 μg) was denatured, subjected to electrophoresis on a 1% (w/v) agarose gel and transferred to Gene Screen membrane (NEN) with 20 × SSC (where 1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7) (solution A). Filters were prehybridized for 4 h then hybridized for 16 h at 42 °C in accordance with published procedures [21]. They were washed successively twice in 2 × solution A at room temperature, twice in 2 × solution A with 1% SDS at 50 °C for 30 min, and finally once in 0.1 × solution A at room temperature. Autoradiograms were quantified with an automatic densitometric scanner [CS-9000; Shimadzu, (Scientific Instruments section)].

Western blot analysis

Homogenates were prepared in ice-cold 154 mM KCl, 10 mM sodium phosphate, pH 7.4, containing 1 mM PMSF, in a glass–Teflon Potter–Elvehjem homogenizer, then centrifuged for 10 min at 18000 ***g*** at 4 °C. The resulting supernatants were centrifuged for 60 min at 105000 ***g*** before storage at −20 °C until required for analysis. The protein concentration was measured by the bicinchoninic acid ('BCA') method (Pierce) and 10 μg of denatured proteins were separated by SDS/PAGE as described elsewhere [5]. The rabbit anti-(rat L-FABP) antiserum was generously provided by Dr. J. I. Gordon. After transfer to the filter, the antigen–antibody complexes were detected by enhanced chemiluminescence (ECL system; Amersham).

Nuclear run-on transcription assay

The nuclei were isolated by lysis of plasma membrane with the non-ionic detergent NP40 [22]. Nuclear transcription assays were performed in accordance with previously described procedures [5]. Elongation of nascent mRNA *in vitro* was performed with 2×10^7 nuclei. They were incubated at 30 °C for 20 min in the presence of [α-^{32}P]UTP (800 Ci/mM; Amersham) (100 μCi per reaction). After their amplification by PCR, 5 μg of L-FABP and I-FABP, and 1 μg of β-actin cDNA, were denatured and immobilized on Gene Screen membrane using a dot–blot apparatus (Gibco–BRL). The filters were hybridized in the presence of 10^7 c.p.m. of labelled mRNA at 47.5 °C for 3 days in a rotary hybridization incubator. After extensive highly stringent washes and RNase treatment [5], the filters were subjected to autoradiography. L-FABP, I-FABP and β-actin cDNA fragments were the same as those used as labelled probes in the Northern blot analysis.

Statistical methods

Wherever possible the results are expressed as means ± S.E.M. The significance of differences between groups was determined by Student's t test.

RESULTS

Oleate stimulates L-FABP gene expression

To investigate whether FAs modulate L-FABP gene expression, we first treated FAO hepatoma cells with 40 μM BSA or with 320 μM oleate complexed with 40 μM BSA for 16 h and analysed L-FABP content by Western blot. As shown in Figure 1, oleate treatment induced a large increase in the cytosolic L-FABP concentration.

This might reflect a change in the stability of the protein and/or in the steady-state level of L-FABP mRNA. To distinguish between the two regulatory mechanisms, Northern blot analysis was performed. When the oleate concentration was varied between 80 and 400 μM, the level of L-FABP mRNA increased in a concentration-dependent manner (Figure 2). A significant induction was noted with the lowest oleate concentration used (50 μM; results not shown). A maximal increase occurred with concentrations of oleate ranging from 240 to 400 μM. The induction was gene-specific because the level of β-actin mRNA remained constant whatever the treatment (Figure 2A). A concentration of 320 μM oleate was chosen to study the time course of this induction. L-FABP mRNA increased after 2 h, reaching a maximum at 16 h, and decreased at 24 h. In contrast, the content of L-FABP mRNA did not differ significantly throughout the time course in control cultures (Figure 3).

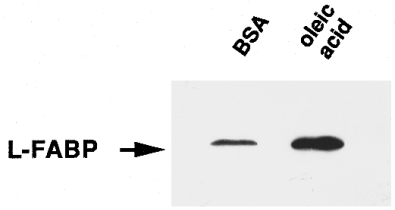

Figure 1 Effect of oleic acid on the cytosolic L-FABP level in FAO cells

Cells were cultured for 16 h in the presence of 320 μM oleic acid complexed with 40 μM BSA. Control cultures were performed in the presence of 40 μM BSA alone. Cytosolic proteins (10 μg) were analysed by Western blotting. The figure shows a representative blot analysis of two separate experiments.

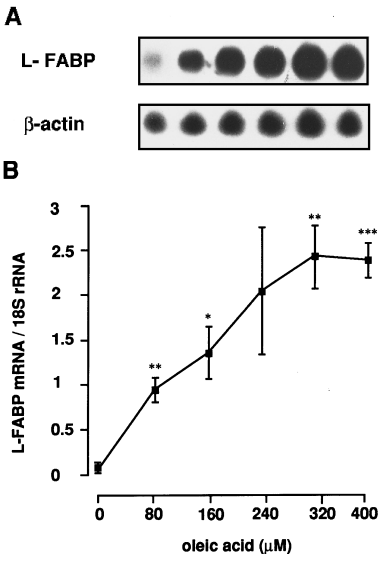

Figure 2 Effect of oleic acid concentration on mRNA species for L-FABP and β-actin in FAO cells

Cells were cultured for 6 h with various concentrations of oleic acid (80–400 μM) complexed with 40 μM BSA. Control cultures were performed in the presence of 40 μM BSA alone. RNA was extracted from two 60 mm dishes. (**A**) Typical Northern blot analysis performed with 30 μg of total RNA. (**B**) Data normalized to 18 S rRNA to compensate for differences in RNA loading. Each value represents the mean ± S.E.M. of results obtained from three independent experiments. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

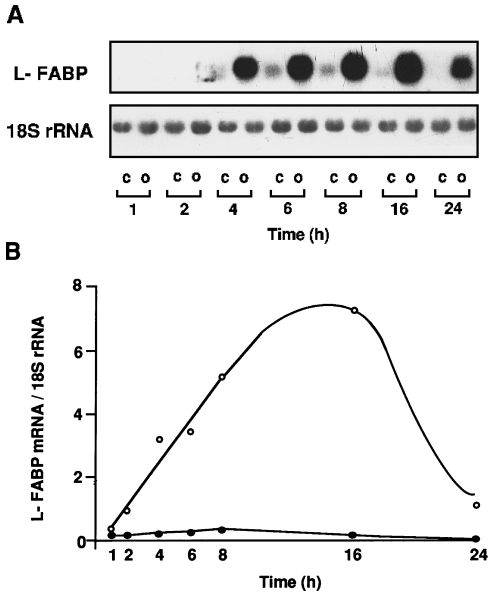

Figure 3 Time course of oleic acid action on L-FABP mRNA in FAO cells

Subconfluent cells were maintained in serum-free medium throughout the 24 h experiment. Oleic acid (320 μM) complexed with 40 μM BSA, or 40 μM BSA alone (control cultures), was added to the medium for 24, 16, 8, 6, 4, 2 or 1 h before they were harvested. RNA was extracted from two 60 mm dishes and analysed as described in the Experimental section. (**A**) Typical Northern blot analysis performed with 30 μg of total RNA. Lanes C, Control cultures; lanes O, cultures including oleate. (**B**) Each point represents the mean value obtained from two independent experiments with nearly identical results (3.4% difference). ●, Control cultures; ○, cultures including oleate. Data were normalized to 18 S rRNA to compensate for differences in RNA loading.

The FA-induced increase in L-FABP mRNA is transcriptional, requires protein synthesis and is dependent on FA chain length

The increase in L-FABP expression might be the consequence of a modification of gene transcription and/or of mRNA stability.

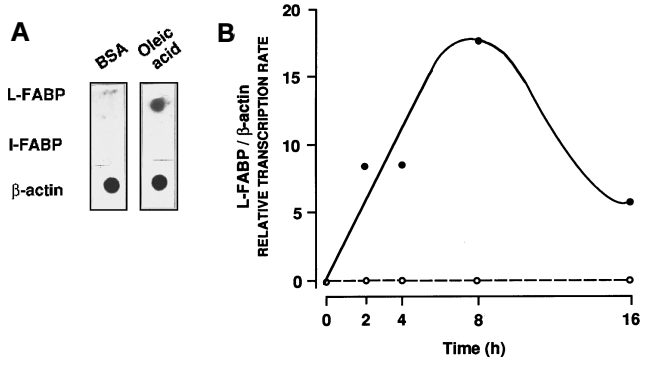

Figure 4 Regulation of L-FABP gene transcription rate by oleic acid in FAO cells

(**A**) Nuclear run-on assays were performed with nuclei extracted from FAO cells cultured for 8 h in the presence of 320 μM oleic acid complexed with 40 μM BSA. Control cultures were performed in the presence of 40 μM BSA alone. [α-^{32}P]UTP-labelled transcripts were hybridized with denatured cDNA probes bound to filters, then analysed by autoradiography. Mouse β-actin cDNA was used as an internal control probe and rat I-FABP as a negative probe (i.e. background). (**B**) Time course of the action of oleic acid on the transcription rate of L-FABP. Results in (**A**) and (**B**) are representative of two independent experiments. ○, Control cultures; ●, culture including oleate.

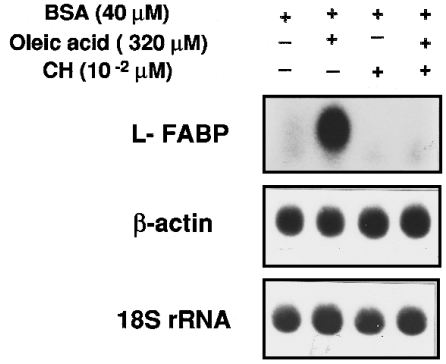

Figure 5 Effect of cycloheximide (CH) on the induction of L-FABP mRNA by oleic acid in FAO cells

Cells were preincubated for 30 min with 10 μM CH, then for an additional 6 h with 320 μM oleic acid or 40 μM BSA alone, with or without CH. Cells from two 60 mm dishes were pooled and RNA was extracted and analysed as described in the Experimental section. The figure is a representative blot analysis of two separate experiments.

To address this question, transcription run-on assays were performed on nuclei isolated from FAO cells treated with 320 μM oleate for 6 h. Because the level of β-actin mRNA remained constant after the addition of FA (Figure 2), β-actin cDNA probe was used as a control. To assess for the specificity of the hybridization, the I-FABP cDNA was also spotted on to the filters because this protein is not expressed in the liver [1]. As shown in Figure 4(A), oleate treatment specifically enhanced the transcription rate of the L-FABP gene. This increase was time-dependent with a maximum of 17-fold at 8 h. Stimulation decreased thereafter and became only 6-fold 8 h later (Figure 4B).

To explore whether the FA effect on L-FABP gene transcription required protein synthesis *de novo*, FAO cells were preincubated with 10 μM cycloheximide for 30 min and subsequently cultured for 6 h in the presence of 320 μM oleate and 10 μM cycloheximide. The induction of L-FABP mRNA by oleate was suppressed by cycloheximide (Figure 5). This effect was found to be specific because cycloheximide did not alter the level of β-actin mRNA (Figure 5). The prevention of oleate action by cycloheximide indicated that the effect of FA was dependent on continuing protein synthesis. Similar results had previously been found with bezafibrate [4].

The potencies of various FAs and of the PPAR activator, 5,8,11,14-eicosatetraynoic acid (ETYA), to stimulate L-FABP mRNA were next examined. The induction of L-FABP mRNA accumulation was clearly dependent on the length of the FA chain, whereas the degree of unsaturation seemed to be less important. Indeed, a short-chain FA such as octanoic acid was inefficient whereas all the LCFAs checked shared similar potencies and produced a 4–6-fold induction (Table 1). ETYA was more potent and produced a 14-fold increase in the level of L-FABP mRNA. These inductions were specific to the L-FABP mRNA because the level of β-actin mRNA remained similar whatever the treatment. Moreover, the level of FAS mRNA was not affected by oleic acid treatment but was significantly decreased when linolenic acid was used (Table 1).

Finally, to assess whether FAs also regulated L-FABP gene expression in non-transformed hepatic cells, primary cultures of rat hepatocytes were performed. Exposure to 320 μM oleic acid for 6 h resulted in a significant increase in L-FABP mRNA

Table 1 Effect of various FAs and of ETYA on levels of L-FABP, β-actin and FAS mRNA in FAO cells

Subconfluent cells were exposed for 6 h to various FAs or ETYA at a concentration of 320 μM complexed with 40 μM BSA. Control cultures were performed in presence of 40 μM BSA alone. RNA was extracted from two 60 mm dishes and analysed as described in the Experimental section. Data obtained by scanning densitometry were normalized for differences in RNA loading by using the 18 S rRNA signal and expressed as percentages of the L-FABP signal from BSA-treated cells. Each value is the mean ± S.E.M. for three independent experiments. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; no asterisks, not significant..

Condition	L-FABP mRNA	β-Actin mRNA	FAS mRNA
Control	1.00 ± 0.32	1.00 ± 0.14	1.00 ± 0.17
Octanoic acid	1.19 ± 0.49	0.97 ± 0.06	
Palmitic acid	4.40 ± 0.77***	0.88 ± 0.12	
Oleic acid	6.67 ± 1.30***	0.95 ± 0.10	1.16 ± 0.28
Linoleic acid	3.82 ± 0.78**	0.84 ± 0.06	
Linolenic acid	4.17 ± 0.82**	0.84 ± 0.03	0.65 ± 0.02*
Arachidonic acid	5.02 ± 1.49**	0.86 ± 0.03	
ETYA	13.96 ± 3.54***	0.84 ± 0.03	

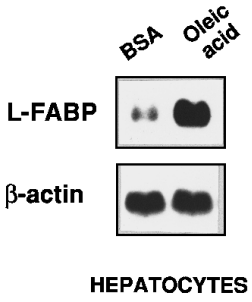

Figure 6 Effect of oleic acid on L-FABP and β-actin levels in cultured hepatocytes

Cultured hepatocytes were exposed to 320 μM oleic acid for 6 h. Control cutures were exposed to 40 μM BSA. The figure is a representative Northern blot analysis of two separate experiments.

expression compared with control cultures, whereas the level of β-actin mRNA remained unchanged (Figure 6).

DISCUSSION

Several proteins involved in lipid metabolism have recently been reported as being directly modulated by LCFAs. In cultured cells, negative regulation was described for FAS [11], S14 [11], L-pyruvate kinase [11], stearoyl-CoA desaturase I [12] and Apo A-I [16], whereas positive regulation was found for the adipocyte lipid-binding protein [10], cytochrome *P*-450-IVA1 [13], phosphoenolpyruvate carboxykinase [14,15], acyl-CoA oxidase [16] and carnitine palmitoyltransferase I [17]. Our results constitute the first demonstration that LCFAs are also involved in the regulation of L-FABP gene expression. This induction is triggered, at least in part, by an enhancement of the transcription rate of the L-FABP gene and requires protein synthesis *de novo*, as already shown for the adipocyte lipid-binding protein [10,23].

Two lines of evidence demonstrate that the LCFA-mediated induction of L-FABP is specific. First, β-actin mRNA remains stable whatever the treatment. Secondly, FAS mRNA is diminished by the polyunsaturated FA linolenate (Table 1). This latter observation is reminiscent of results from experiments *in vivo* in which a fish-oil diet rich in polyunsaturated FAs induces a large decrease in FAS mRNA in liver [11]. The specificity of FA action and the response of the L-FABP gene to unesterified FAs over the range of concentrations that can be found in the blood enhance the physiological significance of our results. Indeed, the half-maximal effect is reached at a concentration lower than 160 μM (Figure 2), which represents an FA/BSA ratio of 4, a value easily attained *in vivo*. We show in addition that the oleate-induced increase in L-FABP mRNA is also found in primary rat hepatocytes (Figure 6), arguing against a cell-type-specific effect of FAs. Such a result is at variance with the observation reported by Kaikaus et al. [24] that oleic acid has no effect on L-FABP mRNA in primary hepatocytes from rats. In the same report, no modification of acyl-CoA oxidase and cytochrome *P*-450IVA1 mRNA levels was found in FA-treated cells [24], whereas these genes have been reported [13,16] as inducible by various LCFAs in primary rat hepatocytes. These discrepancies are probably the result of the difference in the length of treatment time with FAs used in the experiments of Kaikaus et al. (3 days) [24]. In the present study, the induction of L-FABP mRNA grew weaker after 16 h and became less than 2-fold at 24 h (Figure 3). Interestingly, the induction of L-FABP by LCFAs is not restricted to hepatic cells because it has also been found in the colonic carcinoid cell line Caco-2 (H. Poirier, I. Niot and P. Besnard, unpublished work).

In contrast with the repressive effect of FAs on hepatic lipogenic genes, which seems to be restricted to polyunsaturated FAs [11,12,16], the transcriptional induction of the L-FABP gene seems independent of the degree of unsaturation of the LCFAs. Similar results have been found for other FA-induced genes in hepatocytes or in pre-adipocytes [10,17] but not in adipocytes, in which the phosphoenolpyruvate carboxykinase gene is stimulated solely by unsaturated FAs, through a process independent of protein synthesis *de novo* [15]. It is therefore clear that different mechanisms of FA action are likely to affect different genes in different cell types. It is also clear from our results that FAs can regulate the expression of two genes in an opposite fashion in the same cell type (Table 1). One of the postulated mechanisms of action of FAs on gene transcription is via PPAR activation [25]. Such a mechanism can occur for the FA effect on the L-FABP gene which contains a peroxisome-proliferator response element in its non-coding 5′ flanking sequence [26]. Indeed, bezafibrate, a peroxisome-proliferator hypolipidaemic drug, also stimulates L-FABP gene expression in primary hepatocytes [27], and ETYA, a strong PPAR activator, is an inducer of L-FABP mRNA in FAO cells (Table 1). PPARα and the FA-activated receptor [28], the murine homologue of NUC-1 [29], are expressed in FAO hepatoma cells (H. Poirier, I. Niot and P. Besnard, unpublished work). The co-localization of these two PPAR isoforms in FAO cells might be the basis for the differential response of different genes to peroxisome proliferators in the same cell type. These PPARs are also good candidates for mediating the action of FAs on the transcription of the L-FABP gene.

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