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9-*cis*-Retinoic acid enhances fatty acid-induced expression of the liver fatty acid-binding protein gene

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Abstract The role of retinoic acids (RA) on liver fatty acid-binding protein (L-FABP) expression was investigated in the well differentiated FAO rat hepatoma cell line. 9-*cis*-Retinoic acid (9-*cis*-RA) specifically enhanced L-FABP mRNA levels in a time- and dose-dependent manner. The higher induction was found 6 h after addition of 10^{-6} M 9-*cis*-RA in the medium. RA also enhanced further both L-FABP mRNA levels and cytosolic L-FABP protein content induced by oleic acid. The retinoid X receptor (RXR) and the peroxisome proliferator-activated receptor (PPAR), which are known to be activated, respectively, by 9-*cis*-RA and long chain fatty acid (LCFA), co-operated to bind specifically the peroxisome proliferator-responsive element (PPRE) found upstream of the L-FABP gene. Our result suggest that the PPAR-RXR complex is the molecular target by which 9-*cis*-RA and LCFA regulate the L-FABP gene.

Key words: Liver fatty acid-binding protein; 9-*cis*-Retinoic acid; Fatty acid; Nuclear receptor; Gene expression; FAO hepatoma cell

1. Introduction

Liver fatty acid-binding protein (L-FABP) is a small cytosolic protein which binds long chain fatty acids (LCFA) with a high affinity. Although the precise function of this abundant protein is not yet known, it is thought to play an important role in the intracellular fatty acid (FA) trafficking and metabolism (reviewed in [1]). Recently, we have reported that LCFA can directly trigger the transcriptional induction of the L-FABP gene in rat FAO hepatoma cells [2]. Similar results have also been reported for other proteins involved in lipid metabolism (reviewed in [3]). Several lines of evidence suggest that this up-regulation is likely mediated through the interaction of specific nuclear receptors termed peroxisome proliferator-activated receptors (PPARs) with a specific response element (PPRE) located in the promoter of target genes [4–6]. The investigation of key genes involved in the peroxisomal β-oxidation, i.e. acyl-CoA oxidase (ACO) and bifunctional enzyme (BFE), has shown that retinoid X receptors (RXRs) are required for PPARs to bind to the PPRE [7–9]. Since 9-*cis*-retinoic acid (9-*cis*-RA) specifically activates the RXRs [10,11] and a PPRE-like sequence exists in the L-FABP promoter [12], we have explored the effect of this retinoid on L-FABP expression in the FAO cells. The data reported here provide evidence that 9-*cis*-RA is involved in the regulation of the L-FABP gene and enhances the up-regulation triggered by LCFAs most likely through a PPAR-RXR complex.

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Abbreviations: L-FABP, liver fatty acid-binding protein; ACO, acyl-CoA oxidase; BFE, bifunctional enzyme; RA, retinoic acids; 9-*cis*-RA, 9-*cis*-retinoic acid; FA, fatty acid; LCFA, long chain fatty acid; PPAR, peroxisome proliferator-activated receptor; FAAR, fatty acid-activated receptor; RXR, retinoid X receptor; PPRE, peroxisome proliferator-responsive element

2. Materials and methods

2.1. Cell cultures and treatments

FAO cells are a well-differentiated subclone derived from the rat hepatoma H4 II EC3 line. They were cultured at 37°C in a humidified atmosphere of 5% CO_2, 95% air in Ham F-12 medium (Gibco-BRL) containing 10% fetal calf serum, penicillin (200 IU/ml) and streptomycin (50 mg/ml) according to previously published procedures [2]. The culture medium was changed every 48 h. Experiments were performed on subconfluent cells maintained in culture under serum-free conditions. All *trans*-RA and 9-*cis*-RA (gift from Hoffman-La Roche) were dissolved in dimethyl sulfoxide (DMSO) while oleic acid was complexed to FA-free bovine serum albumine (BSA).

2.2. Molecular probes

The L-FABP cDNA probe was generously provided by Dr J.I. Gordon (Washington University, St. Louis, MO, USA). Mouse PPARα, PPARβ/FAAR and PPARγ1 were generously provided by S. Green (Imperial Chemical Industries, Cheshire, UK), P. Grimaldi (Université de Nice, France) and R. Evans (Howard Hughes Medical Institute, La Jolla, CA 92037), respectively. Mouse RXRβ was a kind gift of K. Ozato (National Institute of Health, Bethesda, MA 20892) and mouse β-actin cDNA was a gift from A. Alonso (Institut Pasteur, Paris, France). These probes were labeled with [α^{32}P]dCTP (3000 Ci/mmol; Amersham) by random priming (Megaprime kit, Amersham). A 24-residue oligonucleotide specific for rat 18S rRNA was used to test the loading of equivalent amounts of RNAs and their efficient transfer. This oligonucleotide was 5′ end-labeled using T_4 polynucleotide kinase and [γ^{32}P]ATP (3000 Ci/mmol, Amersham).

2.3. Northern blot analysis

Total RNA was extracted from according to Chomczynski and Sacchi [13]. Thirty μg of Total RNA were denatured, electrophoresed on a 1% agarose gel and transferred to Gene Screen membrane (NEN) using 20-fold concentrated 150 mM NaCl and 15 mM trisodium citrate, pH 7 (NaCl/Cit). Filters were prehybridized for 4 h then hybridized for 16 h at 42°C according to previously published procedures [14]. For L-FABP, PPARβ/FAAR and β-Actin probes, filters were washed successively two times in 2×NaCl/Cit at room temperature for 5 min, in 2×NaCl/Cit with 1% SDS at 55°C for 30 min and in 0.1×NaCl/Cit at room temperature for 30 min. For PPARα and PPARγ$_1$ probes, they were washed three times with 2×NaCl/Cit at 37°C for 15 min. Autoradiograms were quantified with an automatic densitometric scanner (CS-9000, Shimadzu, Scientific Instruments).

2.4. Western blot analysis

Cellular homogenates were prepared in ice-cold 50 mM Tris-HCl, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF) in a

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glass teflon potter. The homogenates were centrifuged for 10 min at 18 000 g. The resulting supernatants were centrifuged for 60 min at 105 000 g before to be stored at −20°C until analysis. The rabbit anti-rat L-FABP antiserum and the purified rat L-FABP were generously provided by Dr. J.I. Gordon (St Louis, MO, USA). The cytosolic protein concentration was measured by BCA (Pierce) and 10 μg denatured proteins were separated by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) as described elsewhere [14]. After transfer on filter, the antigen-antibody complexes were detected by chemiluminescence (ECL-system; Amersham).

2.5. Production of nuclear proteins and gel shift analysis

Nuclear receptors (i.e. mPPARα, mPPARβ/FAAR, mPPARγ1 and mRXRβ) were produced from the pSG5 expression plasmid using the TNT rabbit reticulocytes translation system as directed by the supplier (Promega). A sense strand oligonucleotide containing the PPRE sequence (underlined) of the L-FABP gene (5′-AAGCTTCAATCACTGACCTATGGCCTATATTTGTCTAGA-3′) was annealed with the corresponding antisense strand oligonucleotide, and 5′ end-labeled using T4 polynucleotide kinase and [γ^{32}P]ATP (3000 Ci/mmol, Amersham). Aliquots (2 μl) of in vitro translated proteins were preincubated on ice for 20 min in 18 μl of the binding buffer (10 mM HEPES pH 7.4, 50 mM KCl, 1 mM DTT, 5% glycerol) containing 10 μg of poly dI-dC and 10 μg of heat treated salmon sperm DNA. Samples were incubated for an additional 20 min at room temperature after addition of labeled probe(s) (40 000 cpm/reaction). The DNA-protein complexes were resolved by 5% non-denaturing polyacrylamide gel electrophoresis in 0.5-fold concentrated TBE (45 mM Tris, 45 mM boric acid, 1 mM EDTA). Gels were dried and autoradiographied at −70°C.

2.6. Statistical methods

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

3. Results

3.1. 9-cis-RA enhances L-FABP mRNA levels

To investigate whether 9-*cis*-RA was involved in the regulation of L-FABP expression, FAO cells were cultured for 6 h with varying concentrations of this RA. Control cultures were performed with the vehicle alone (0.1% v/v DMSO). As shown in Fig. 1A, 9-*cis*-RA induced L-FABP mRNA in a concentration-dependent manner, the maximal increase occurring at 10^{-6} M. Next, the time-course of induction was analysed. Cells were treated with 10^{-6} M 9-*cis*-RA for 1 to 24 h. Under these conditions, increased L-FABP mRNA levels were first detected at 1 h, reached a maximum at 6 h, and then decreased (Fig. 1B). A weaker induction also occurred when all *trans*-RA was used as an inducer (data not shown).

3.2. Additive effects of 9-cis-RA and LCFA on L-FABP expression

L-FABP gene expression is enhanced by LCFA in FAO cells [2]. In order to determine whether RA may potentiate this FA action, the effects of 9-*cis*-RA (10^{-6} M) and/or oleic acid (8×10^{-5} M) on L-FABP expression were studied next. Taken separately, compounds triggered an increase in L-FABP mRNA level after a 6 h treatment. An additive effect was found when the two inducers were applied together (Fig. 2A). Furthermore, increase in cytosolic L-FABP protein contents paralleled that of L-FABP mRNA levels (Fig. 2B).

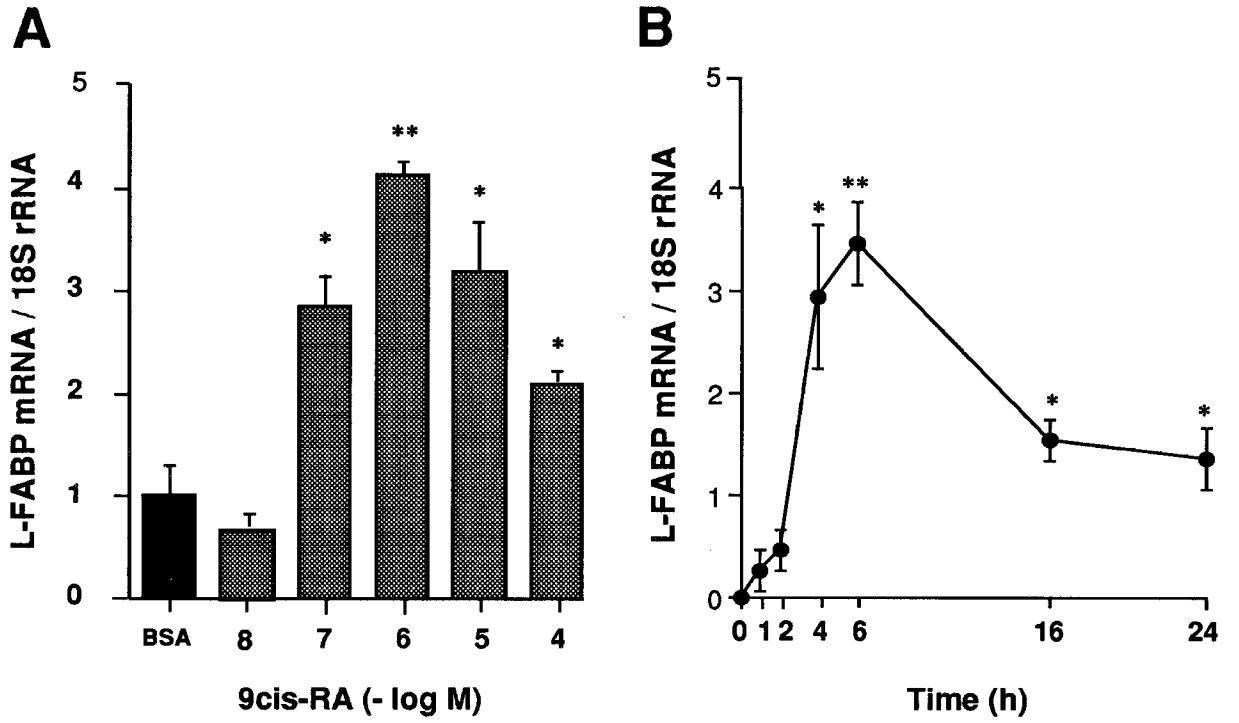

Fig. 1. Effect of 9-*cis*-RA on L-FABP mRNA in FAO cells. RNAs were extracted from three 60 mm dishes and analyzed as described in Section 2. Data were normalized to the amount of 18S rRNA in the samples. (A) Cells were cultured for 6 h with various concentrations of 9-*cis*-RA. Control cultures received the vehicle alone DMSO (0.1% v/v). (B) Time course of 9-*cis*-RA action. Subconfluent cells were maintained in serum-free medium throughout the 24 h experiment. 10^{-6} M 9-*cis*-RA was added to the medium for 24, 16, 8, 6, 4, 2 or 1 h before the cells were harvested. $^{*}P<0.05$; $^{**}P<0.01$, $n=3$.

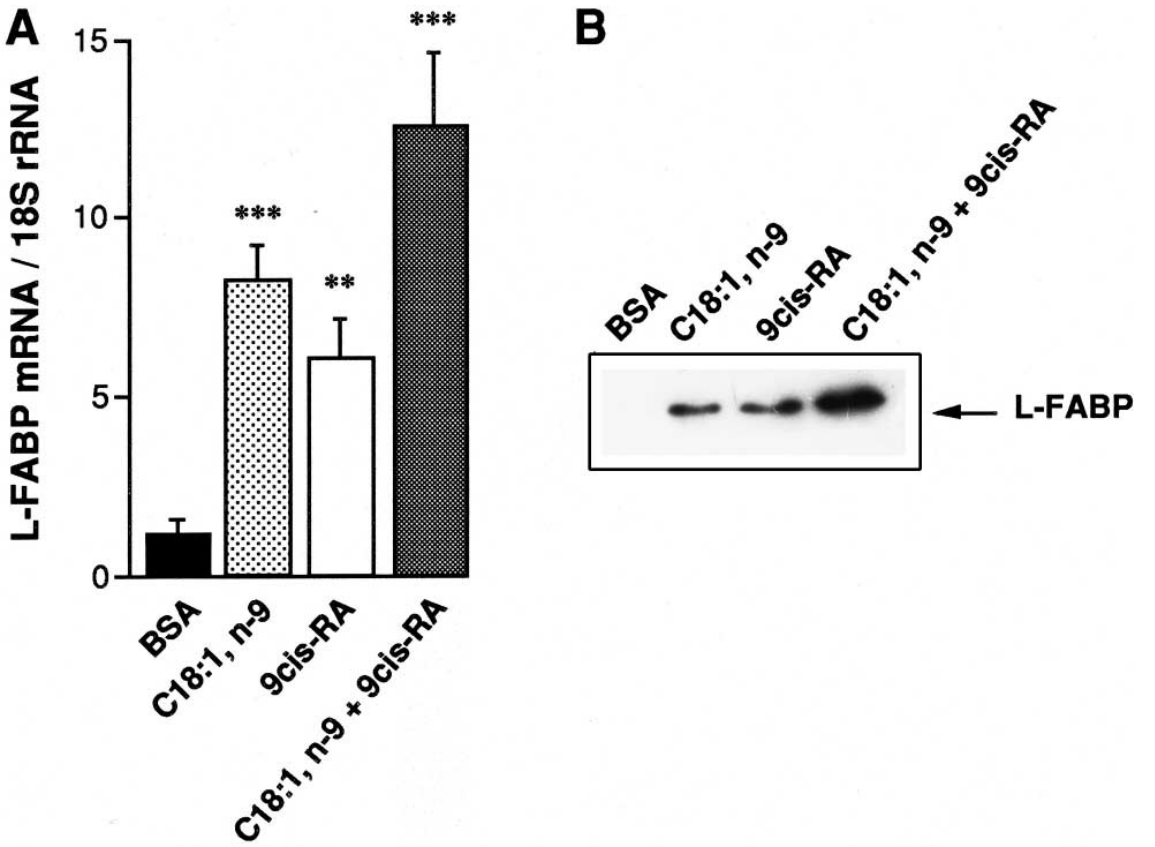

Fig. 2. Effect of 9-*cis*-RA and/or oleic acid on L-FABP expression in the FAO cells. Cells were cultured for 6 h in presence of 10^{-6} M 9-*cis*-RA in DMSO (0.1% v/v) and/or 8×10^{-5} M oleic acid complexed with 2×10^{-5} M BSA. Control cultures were performed in presence of DMSO and BSA. (A) Bar graph representation derived from 9 independent experiments. Control cultures (black column); cultures plus oleic acid (light shaded); cultures plus 9-*cis*-RA (open); cultures plus oleic acid and 9-*cis*-RA (dark shaded). $^{**}P<0.01$; $^{***}P<0.001$. (B) Effect of 9-*cis*-RA and/or oleic acid on the cytosolic L-FABP levels in the FAO cells. Ten μg of cytosolic proteins were analyzed by Western blot as described in Section 2.

3.3. *Various PPAR-RXR heterodimers bind the L-FABP PPRE*

The additive effect of 9-*cis*-RA and LCFA on L-FABP expression, suggested that a PPAR-RXR heterodimer interacts with the promoter of L-FABP gene, as already found for the acyl-CoA oxidase and bifunctional enzyme promoters [7–9]. Therefore, using the L-FABP PPRE-like sequence as a probe, gel shift experiments were performed in order to test this working hypothesis and to study the binding properties of PPAR-RXR complex to this nucleotide sequence. RXRβ was used since it is found in all the tissues where L-FABP is expressed, e.i. liver, small intestine and kidney [15]. No specific retardation was detected when either PPAR (α, β or γ1) or RXRβ were used alone (Fig. 3, lanes 2–5). In contrast, specific shifts were systematically found when RXRβ was mixed with one of the three different PPAR sub-types (Fig. 3, lanes 6–8) indicating that RXR and PPAR co-operatively bind to the L-FABP PPRE. Differences in the migration of the complexes reflect the specific molecular mass of each PPAR checked. Similar data were found with the ACO-PPRE taken as control (data not shown).

3.4. *FAO cells express both PPARα and PPARβ/FAAR*

The expression of different PPAR sub-types in FAO cells, was studied next with respect to stimulation with 9-*cis*-RA end LCFA. As shown in Fig. 4 and according to previous data performed in rat liver [16], both the mRNA for PPARα and PPARβ/FAAR were expressed in hepatoma FAO cell line while PPARγ1 was undetectable. However treatment of the cells with oleic acid and/or 9-*cis*-RA did not modified the PPARα and PPARβ/FAAR mRNA levels in contrast to those of L-FABP which were increased (Fig. 4).

4. Discussion

Retinoids are vitamin A derivatives which exert multiple essential functions in development, differentiation, reproduction and vision. Retinol is metabolized to form retinal and retinoic acids, i.e. all-*trans*-RA and 9-*cis*-RA [17], which are major vitamin A metabolites involved in the regulation of gene expression. This control takes place through the activation of two distinct classes of nuclear receptors: the RA receptors (RARα, β and γ), and retinoid X receptors (RXRα, β and γ) which bind with high affinity all-*trans*-RA and 9-*cis*-RA, respectively [10,11].

Several proteins involved in the lipid metabolism, including ACO [7,8], BFE [9], albumin [18] and Apo A-I [19] are directly modulated by 9-*cis*-RA. In this report, we provide evidence that L-FABP gene expression is also regulated by this vitamin A derivative. The induction is gene-specific (PPARα, PPARβ/FAAR and β-actin mRNA levels are unaffected), rapid (effective 1 h after addition of 9-*cis*-RA) and triggered by a

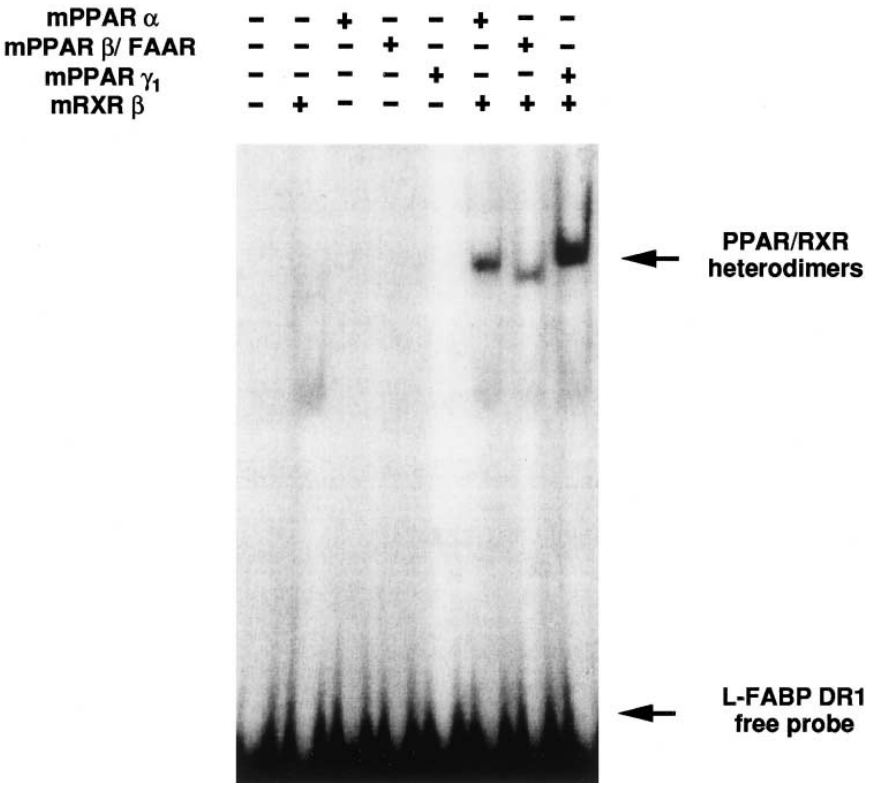

Fig. 3. Gel shift analysis of the L-FABP PPRE in presence of PPARs and RXRβ taken separately or combined. In vitro translated PPARα, PPARβ/FAAR, PPARγ and RXRβ were incubated with the labeled L-FABP PPRE as described in Section 2. The ACO PPRE was taken as control. Lanes: 1, DNA probe only; 2–5, PPARs or RXRβ alone; 6–8, PPAR and RXR combined as indicated at the top of the figure.

low concentration of inducer (10^{-7} M). We have recently reported that the L-FABP gene was up-regulated by LCFA in both liver cell lines [2] and small intestine [20]. It is noteworthy that 9-*cis*-RA enhances the LCFA induced up-regulation of L-FABP gene expression.

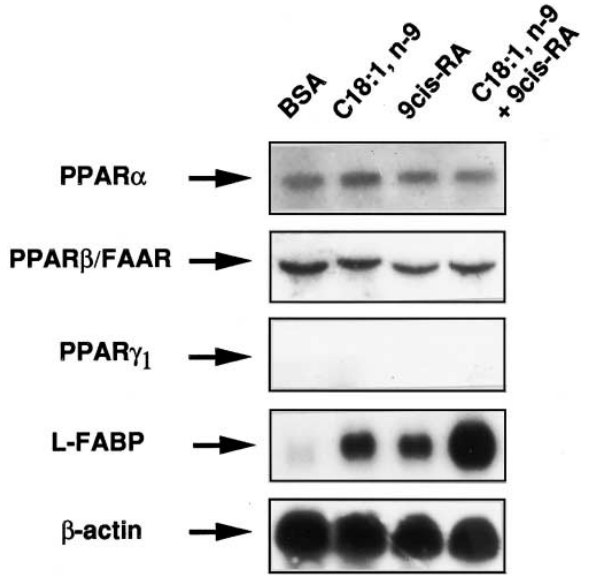

Fig. 4. Effect of oleic acid and/or 9-*cis*-RA on PPAR sub-types expression in FAO cell line. Northern blot analysis was performed as described in Section 2. Cells were cultured for 6 h in presence of 10^{-6} M 9-*cis*-RA in DMSO (0.1% v/v) and/or 8×10^{-5} M oleic acid complexed with 2×10^{-5} M BSA. Control cultures were performed in presence of DMSO and BSA alone.

Recent findings indicate that PPARs bind PPREs as PPAR-RXR heterodimer and that the complex can be activated by both PPAR activator and RXR selective ligands [21]. We demonstrate herein that such a heterodimer can also bind the L-FABP-PPRE at least in vitro. Since 9-*cis*-RA and LCFA activate RXRs and PPARs, respectively, [10,11,22], it is tempting to speculate that, like in the above mentioned study, the co-operative up-regulation of L-FABP expression triggered by these molecules takes place through such a functional heterodimer that directly binds oleic acid [23] and 9-*cis*-RA. The lack of PPARγ expression in FAO cells suggest that PPARα and/or PPARβ/FAAR might be involved in this gene regulation. Transfection studies performed in FAO cells and the use of PPARα knock-out mice [24] will be used to test this hypothesis.

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