Differential involvement of peroxisome-proliferator-activated receptors α and δ in fibrate and fatty-acid-mediated inductions of the gene encoding liver fatty-acid-binding protein in the liver and the small intestine

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Liver fatty-acid-binding protein (L-FABP) is a cytoplasmic polypeptide that binds with strong affinity especially to long-chain fatty acids (LCFAs). It is highly expressed in both the liver and small intestine, where it is thought to have an essential role in the control of the cellular fatty acid (FA) flux. Because expression of the gene encoding L-FABP is increased by both fibrate hypolipidaemic drugs and LCFAs, it seems to be under the control of transcription factors, termed peroxisome-proliferator-activated receptors (PPARs), activated by fibrate or FAs. However, the precise molecular mechanism by which these regulations take place remain to be fully substantiated. Using transfection assays, we found that the different PPAR subtypes (α , γ and δ) are able to mediate the up-regulation by FAs of the gene encoding L-FABP *in vitro*. Through analysis of LCFA- and

fibrate-mediated effects on L-FABP mRNA levels in wild-type and PPAR α -null mice, we have found that PPAR α in the intestine does not constitute a dominant regulator of L-FABP gene expression, in contrast with what is known in the liver. Only the PPAR δ / α agonist GW2433 is able to up-regulate the gene encoding L-FABP in the intestine of PPAR α -null mice. These findings demonstrate that PPAR δ can act as a fibrate/FA-activated receptor in tissues in which it is highly expressed and that L-FABP is a PPAR δ target gene in the small intestine. We propose that PPAR δ contributes to metabolic adaptation of the small intestine to changes in the lipid content of the diet.

Key words: lipid homeostasis, PPAR α -null mice, PPAR δ .

INTRODUCTION

The liver fatty-acid-binding protein (L-FABP) is a 14 kDa cytoplasmic protein that binds long-chain fatty acids (LCFAs) with high affinity [1]. The putative functions assigned to L-FABP include the desorption of LCFAs from the plasma membrane to the cytoplasm, the promotion of intracellular fatty acid (FA) diffusion, the targeting of FAs to different metabolic pathways and protection against the cytotoxic effects of free FA (reviewed in [2]). Roles in signal transduction pathways and gene regulation have also been reported [3–5]. The involvement of L-FABP in the metabolic adaptation to changes in lipid content of the diet can therefore be envisaged in tissues in which it is expressed at substantial levels (i.e. small intestine and liver). Drugs that affect either lipid metabolism or the lipid content of the diet modulate L-FABP gene expression in these tissues. Indeed, it has been shown that both fibrate hypolipidaemic drugs and LCFAs enhance the transcription rate of the gene encoding L-FABP [6–9]. It is well established that fibrates and LCFAs regulate gene expression through the activation of nuclear receptors termed peroxisome-proliferator-activated receptors (PPARs) [10]. PPARs bind as heterodimers with the retinoid X receptor

(RXR) to a specific DNA sequence termed the peroxisome-proliferator-responsive element (PPRE) located upstream of target genes that are involved mainly in lipid metabolism [11]. A PPRE has been identified in the proximal 5' flanking region (position –67/–55) of the gene encoding L-FABP in rodents [12,13]. *In vitro* this PPRE^{L-FABP} sequence permits the efficient binding of complexes between the different PPAR subtypes (α , γ and δ) and RXRs [14,15]. Moreover, it seems to be functional *in vivo* because transgenic mice expressing only the short L-FABP promoter (–132/+21) sequence, in which the PPRE is found, are able to reproduce the fibrate-mediated induction of the gene for L-FABP in the liver [13]. The targeted disruption of the gene encoding PPAR α fully abolishes the hepatic induction by fibrates of the gene for L-FABP [16]. This last result demonstrates that PPAR α is crucial for the fibrate action in this organ. In contrast, although LCFAs and their metabolites have been reported to be activators of PPARs [17–19], the molecular mechanism by which they up-regulate L-FABP gene expression remains to be established. Moreover, no information is available for the small intestine, where the gene encoding L-FABP is also highly expressed and is thought to be involved in FA absorption. PPAR δ is especially highly expressed in enterocytes, in contrast

Abbreviations used: ACS, acyl-CoA synthase; A-FABP, adipocyte fatty-acid-binding protein; CAT, chloramphenicol acetyltransferase; DEHP, di-2-ethylhexyl phthalate; FA, fatty acid; FAT/CD36, fatty-acid translocase; L-FABP, liver fatty-acid-binding protein; LCFA, long-chain fatty acid; NaT, sodium taurocholate; PPAR, peroxisome-proliferator-activated receptor; PPRE, peroxisome-proliferator-responsive element; PUFA, polyunsaturated fatty acid; RXR, retinoid X receptor; tk, herpesvirus thymidine kinase.

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with hepatocytes, where the dominant subtype is PPAR α [20]. These differences in tissue expression raise the question of the respective roles of these two PPAR subtypes in the regulation of the gene for L-FABP in the small intestine and the liver.

To explore the molecular mechanism involved in the cellular trafficking of LCFAs in both the small intestine and liver, the contribution of PPAR α and PPAR δ to the regulation of L-FABP gene expression was studied *in vitro* by transfection assays and *in vivo* with the help of PPAR α -deficient mouse model [16]. In contrast with results from the liver, our results for the small intestine demonstrate that PPAR α is not required there for the fibrate/FA-mediated inductions of the gene encoding L-FABP, which seems to be a PPAR δ target gene in this organ.

MATERIALS AND METHODS

Animals and experimental treatments

French guidelines for the use and care of laboratory animals were followed. The male homozygous PPAR α -deficient mice of C57BL/6 genetic background were used [16]. Age-matched wild-type male C57BL/6 mice, purchased from CERJ (Le Genest, France) were used as PPAR α ^{+/+} controls. Animals were housed individually in a controlled environment (constant temperature and humidity, darkness from 08:00 h to 20:00 h). They were fed *ad libitum* with a standard chow containing 8% (w/v) lipid in total calories (UAR A-04; Usine d'alimentation rationnelle, Epinay sur Orge, France).

To study the involvement of PPAR α in fibrate-mediated induction of the gene encoding L-FABP, C57BL/6 wild-type and PPAR α -deficient mice were fed for 7 days with a laboratory chow containing 0.5% (w/v) bezafibrate. This synthetic hypo-lipidaemic agent was chosen because it elicits the transcription of the gene for L-FABP in the small intestine [7]. The up-regulation of L-FABP expression by FAs was also checked in PPAR α ^{+/+} and PPAR α ^{-/-} mice either force-fed for 5 days with 0.2 ml of sunflower oil to provide 16% of total calories in lipids (compared with 8% in the control diet) or with a laboratory chow containing 30% sunflower oil (in total calories). In the force-feeding experiment, controls received in the same manner 0.2 ml of 0.15 M NaCl. Sunflower oil is characterized by its high content of linoleic acid.

In adult C57BL/6, the length of the small intestine was approx. 35 cm. To study accurately the expression of L-FABP and PPARs along the gut, the small intestine was divided into seven segments of 5 ± 0.3 cm from the pylorus to the ileocaecal valvula. It is usually stated that the jejunum starts only after Treitz's ligament. Because it starts approx. 7 cm from the pylorus in the strain that we used, the first 10 cm (segments 1 and 2) were arbitrarily considered to be the duodenum, the next 15 cm (segments 3–5) the jejunum and the last 10 cm (segments 6 and 7) the ileum. After the animals had been killed, intestinal and hepatic samples were snap-frozen in liquid nitrogen and stored at –80 °C until required for RNA extractions.

The direct effect of the non-metabolizable LCFA α -bromopalmitate on L-FABP gene expression was checked with the use of the distal ileum model [7]. Anaesthetized PPAR α ^{+/+} and PPAR α ^{-/-} mice were laparotomized; they then received, by direct infusion into the distal part of the ileum (segment 7), 0.2 ml of α -bromopalmitate (10 μ M) emulsified with sodium taurocholate (α -bromopalmitate-to-NaT ratio 1:2). Animals were quickly sutured and kept in individual cages. Controls were treated with the vehicle alone (20 μ M NaT) by the same protocol. At 1 day after surgery, the entire ileum was removed, flushed with cold 0.15 M NaCl and a 5 cm segment was taken 1 cm above the caecum. The mucosa was scraped off with a spatula,

frozen immediately in liquid nitrogen and stored at –80 °C until used for RNA extraction.

Organ culture of intestinal explants

PPAR α ^{+/+} and PPAR α ^{-/-} mice were fasted overnight and duodeno-jejunal explants (i.e. segments 2 and 3) and ileal explants (segment 7) were prepared as described previously [21]. Explants were cultured at 37 °C under an oxygenated atmosphere in DMEM (Dulbecco's modified Eagle's medium) containing 10% (v/v) NCTC-135, 10% (v/v) fetal calf serum, 0.1% Fungizone, 0.1 mg/ml gentamicin (all from Gibco BRL) and 0.7 i.u./ml ribonuclease inhibitor from human placenta (Boehringer). After a 4 h preculture period, explants were cultured for 20 h in the presence of 50 μ M bezafibrate, 1.5 μ M PPAR α agonist (GW7647) or 1.5 μ M PPAR δ agonist (GW2433) (Glaxo Wellcome, Research Triangle Park, NC, U.S.A.). Control cultures received vehicle only [0.15% (v/v) DMSO]. The jars containing the explants were re-oxygenated [95% O₂, air/CO₂ (19:1)] for 30 min several times during the culture. At the end of the culture, the explants were immediately frozen in liquid nitrogen and stored at –80 °C until required for RNA extraction.

Northern blotting

Total RNA species were extracted from liver and intestinal mucosa by the method of Chomczynski and Sacchi [22]. They were denatured, subjected to electrophoresis on a 1% (w/v) agarose gel and transferred to Gene Screen membranes (NEN) as described previously [6]. Rat L-FABP [23], mouse PPAR α [10], PPAR δ [24] and β -actin [25] were used as probes. They were labelled with [α ³²P]dCTP (3000 Ci/mmol; Amersham) by a megaprime kit (Amersham). A 24-residue oligonucleotide specific for rat 18 S rRNA was used as probe to ensure that equivalent quantities of RNA were loaded and transferred. This oligonucleotide was 5' end-labelled with T₄ polynucleotide kinase and [γ ³²P]ATP (3000 Ci/mmol; Amersham).

Recombinant plasmids

The large promoter pL-FABP(–4000/+21)-tk-CAT (in which CAT stands for chloramphenicol acetyltransferase) was constructed by cloning the *Bam*HI/*Hind*III-digested fragment of pLFhG4 plasmid [26] into pBL-tk-CAT8⁺. The short promoter pL-FABP(–326/+21)-tk-CAT was generated by *Hind*III/*Nsi*I digestion of the full-length L-FABP(–4000/+21) promoter. The pL-FABP(–4000/+21)-CAT plasmid was obtained by *Bam*HI/*Bgl*II deletion of the herpesvirus thymidine kinase (tk) promoter from pL-FABP(–4000/+21)-tk-CAT. Mutations in the PPRL-FABP(–67/–55) sequence were generated with the QuickChange[®] mutagenesis kit (Stratagene). The following oligonucleotides were used: 5'-CGA CAA TCA CTG AGC GGC CGC CTA TAT TTG AGG AGG AAG AAG CCC-3' (PPRL-FABP in bold; mutations are underlined) and 5'-GGG CTT CTT CCT CCT CAA ATA TAG GCG GCC GCT CAG TGA TTG TCG-3'. Each of the mutated bases is either poorly represented or lacking in the native functional PPRLs [27]. The different DNA constructs were analysed before use (T7 sequencing kit; Pharmacia) to confirm the sequence and orientation of the insert.

The unpublished DNA sequence of the L-FABP promoter (nt –4000 to –597) was sequenced with the dideoxynucleotide procedure. The sequencing was undertaken from both sides of the promoter sequence, with specific oligonucleotides (28–30-mers) chosen every 250 nt along the progressively known sequence of the promoter.

Figure 1 Analysis of the rat L-FABP promoter

(A) The induction of L-FABP gene expression by LCFAs is mediated by PPARs. Caco-2 cells were transiently co-transfected with the gene reporter construct and pSG5-PPAR α , δ or γ . Cells were treated as described in the Materials and Methods section with 320 μ M linoleic acid 24 h before harvesting (open bars). Control cultures (solid bars) had only the vehicle added [i.e. 0.15% (v/v) DMSO or 40 μ M BSA respectively]. Transcriptional activity is expressed as relative CAT activity normalized to β -galactosidase activity; results are means $\pm$ S.E.M., $n = 3$. * $P < 0.05$; ** $P < 0.01$, *** $P < 0.001$. (B) The proximal PPRE sequence is functional and sufficient to reproduce the mediated induction of the gene encoding L-FABP in the presence of activated PPARs. Caco-2 cells were co-transfected with different constructs of the L-FABP promoter and mPPAR α , mPPAR δ and mPPAR γ expression vectors activated by selective pharmacological agonists. Lane 1, the large -4000/+21 L-FABP construct containing the proximal PPREs; lane 2, the -326/+21 L-FABP construct resulting from 5' end deletion of the large promoter; lane 3, large construct -4000/+21 L-FABP in which the PPRE(-67/-55) sequence was mutated (mutation underlined). Cells were treated with 25 nM PPAR α (GW7647), 25 nM PPAR γ (GW7845) or 1.5 μ M PPAR δ/α (GW2433) agonist for 24 h. Results are expressed as fold induction relative to cells treated with the vehicle alone and are means $\pm$ S.E.M., $n = 3$.

Cell culture and transfection assays

Transient transfections were performed in undifferentiated human enterocyte-like Caco-2 cells (passages 53–60). They were cultured in 60 mm dishes at 37 $^{\circ}$ C under a humidified atmosphere [air/CO₂ (19:1)] in DMEM supplemented with 10% (v/v) fetal calf serum, 4 mM L-glutamine, 1% non-essential amino acids, 50 mg/ml streptomycin and 200 i.u./ml penicillin. At 1 day before transfection, Caco-2 cells were treated with 0.5% trypsin/0.25 g/l EDTA, then replated in six-well plates. They were refed with medium supplemented with 10% (v/v) delipidated fetal calf serum (Sigma) 4 h before transfection. Cells were typically co-transfected with 4 μ g of the reporter pL-FABP-CAT or pL-FABP-tk-CAT gene constructs and, where indicated, with 0.5 μ g of effector pSG5 plasmids expressing full-length cDNA species for mouse PPAR α [10], PPAR δ [24], PPAR γ 1 [28] or pSG5 alone. Plasmid CMV β -Gal (cytomegalovirus promoter β -galactosidase) (1 μ g) was included as an internal control for transfection efficiency. Transfection experiments were performed with the calcium phosphate method [29]. Experiments were conducted with 25 nM selective PPAR α (GW7647) and PPAR γ (GW7845) agonists and 1.5 μ M dual agonist for PPAR α and PPAR δ (GW2433) (i.e. 5-fold the EC₅₀ values of these compounds). Control cultures were performed with the vehicle alone [0.1% (v/v) DMSO]. Cells were harvested 24 h after induction. Cellular extracts were prepared and assayed for β -galactosidase (β -Gal) and CAT activities [29]. All measurements were made in triplicate.

Statistical methods

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

RESULTS

Linoleic acid induces the transcription of L-FABP gene promoter-reporter constructs

To demonstrate that the LCFA-regulated transcription of the gene encoding L-FABP requires PPARs, Caco-2 cells were transiently transfected with the large fragment (-4000/+21 bp) of rat L-FABP promoter cloned upstream of a tk-CAT reporter gene in the presence or absence of linoleic acid and expression vectors for mouse PPAR α , PPAR δ or PPAR γ . The efficiency of transfections was assessed by co-transfection with the β -galactosidase expression vector and the assay of enzymic activity. The addition to the medium of 320 μ M linoleic acid led to a weak but significant increase of the CAT activity (Figure 1A), suggesting that Caco-2 cells express low levels of endogenous PPARs. In contrast with PPAR δ and PPAR γ , transfection with the PPAR α expression vector alone resulted in a strong stimulation of the reporter gene (Figure 1A). Similar results have been already reported for HeLa, CV1, NIH 3T3 and HepG2 cell lines [14,30,31]. It was probably due to the presence of specific endogenous activators for PPAR α in the Caco-2 cells and/or the culture medium. Linoleic acid induced the transcriptional activity of the gene reporter system in the presence of the three PPAR expression vectors, the weakest effect being found with the γ subtype (Figure 1A). A higher transcriptional activation was found in cells co-transfected with both PPAR and RXR β after addition to the culture medium of 320 μ M linoleic acid and 1 μ M 9-*cis*-retinoic acid (results not shown). This observation is consistent with studies *in vitro* showing that only heterodimers between PPAR α , PPAR δ or PPAR γ and RXR β are able to bind specifically the PPRE already identified [14,15]. Taken together, these results strongly suggest that the L-FABP promoter responds to fibrates and LCFAs through PPAR-RXR heterodimers. It is

Figure 2 Effect of PPAR α gene disruption on the L-FABP (left panel) and PPAR δ (right panel) mRNA levels along the cephalocaudal axis of the small intestine in mice

The small intestines were divided into seven segments of approx. 5 cm from the pylorus to the ileo-caecal valvula; the mucosa of each segment was scraped off. Symbols: ●, PPAR $\alpha^{+/+}$; ○, PPAR $\alpha^{-/-}$. Values are expressed relative to the segment from age-matched PPAR $\alpha^{+/+}$ mice expressing the higher mRNA levels of L-FABP or PPAR δ respectively. Results are means $\pm$ S.E.M. The curves were derived from 3 independent experiments. Abbreviation: ud, undetectable.

Figure 3 Effect of bezafibrate treatment on L-FABP mRNA levels in the liver and small intestine from PPAR $\alpha^{+/+}$ and PPAR $\alpha^{-/-}$ mice

In the study *in vivo*, animals were fed for 7 days with a laboratory chow containing 0.5% (w/v) bezafibrate (B). Controls (C) received a standard chow. Northern blot analysis of L-FABP mRNA levels in the liver and the proximal intestine (segments 1 and 2) was performed with 30 μ g of total RNA. The bar graph representation was derived from four independent experiments and shows means $\pm$ S.E.M. * $P < 0.05$, *** $P < 0.001$. The study *in vitro* was performed in duodeno-jejunal explants (segments 2 and 3) from PPAR $\alpha^{-/-}$ and PPAR $\alpha^{+/+}$ mice, cultured for 20 h in presence of 50 μ M bezafibrate (B). Control cultures (C) were performed with vehicle alone (0.15% DMSO). L-FABP mRNA levels were analysed by Northern blotting (20 μ g of total RNA). To demonstrate the specificity of the response, blots were reprobed with mouse β -actin cDNA. The results were normalized to 18 S rRNA to control for differences in RNA loading. PPAR $\alpha^{+/+}$ and PPAR $\alpha^{-/-}$ control mice were arbitrarily assigned the value 1. Filled bars, controls; open bars, bezafibrate-treated mice.

noteworthy that the large fragment L-FABP promoter was also functional in the context of the natural promoter. Indeed, when the cells were transfected with PPAR α expression vector in the

presence of 50 μ M bezafibrate, a similar CAT activity induction (1.6-fold) was found with the (−4000/+21)CAT and (−4000/+21)tk-CAT constructs.

Unique functional PPRE directs the gene encoding L-FABP

More than one functional PPRE has been identified in a few PPAR/RXR target genes [11]. The significance of such a redundant system is currently unclear. It might support a PPAR isotype selectivity. Indeed, a specific PPAR δ -responsive element has recently been described [32]. To explore whether similar features are found in the L-FABP promoter, the unpublished DNA sequence from -4000 to -597 was sequenced and analysed (GenBank accession number AF329653). A second PPRE-like sequence (5'-AGAGCTaTGGCCTTGTTATC; bold letters indicate identity with the ideal PPRE sequence; italics indicate the flanking sequence [27]) located at position -1616/-1597 bp upstream of the transcription site was identified by a computer search (Mat Inspector V2.2). To explore the functional role of this distal PPRE sequence, different constructs of L-FABP promoter containing either native or mutated proximal PPRE sequence were obtained by mutation or 5'-deletion of the long promoter (-4000/+21). In the presence of the pharmacological agonists GW7647, GW2433 or GW7845, which preferentially activate PPAR α , PPAR δ/α and PPAR γ respectively, a significant transactivation was found when the reporter gene was driven by the native large construct. A similar induction was also found with the native short promoter (-326/+21). In contrast, transactivation was fully abolished when the PPRE sequence located at position -67/-55 was mutated (Figure 1B). These results demonstrate that the rat gene encoding L-FABP contains a unique functional PPRE located in the proximal part of the promoter. Moreover, this experiment yields a better characterization of the responsiveness of the gene encoding L-FABP to the different PPARs by the use of selective agonists. As shown in Figure 1(B), all PPAR subtypes activated the reporter gene, in good agreement with the ability of various PPARs to bind the proximal PPRE^{L-FABP} *in vitro* [14,15]. The strongest transactivation occurred with PPAR δ activated by GW2433.

PPAR α is not required for bezafibrate or FA inductions of the gene encoding L-FABP in the small intestine

The magnitude of induction of PPAR-responsive genes is influenced not only by the PPAR ligand specificity but also by the ratio of the expression of the three PPAR isoforms. In the liver, the PPAR α subtype is the most highly expressed. In contrast, it is found at low levels in the small intestine, where the PPAR δ isoform is predominant and PPAR γ is undetectable [20]. To determine the relative contributions of PPAR α and PPAR δ subtypes in the fibrate- and LCFA-mediated regulation of the gene encoding L-FABP *in vivo*, PPAR α -null (PPAR α ^{-/-}) mice were subjected to treatment with bezafibrate or high-fat diets, enriched in linoleic acid. In the small intestine, L-FABP mRNA levels were not significantly changed by disruption of the gene encoding PPAR α , with the exception of the proximal duodenum, where the concentration was lower in PPAR α -null mice (Figure 2). After reaching a maximum of expression in the distal duodenum, L-FABP decreased progressively, falling to undetectable levels in the distal ileum (segment 7). This pattern of expression is in agreement with previously published results [9]. In both PPAR α ^{+/+} and PPAR α ^{-/-} mice, the highest PPAR δ mRNA levels occurred in the duodenum, decreased progressively in the jejunum, and were slightly increased in the distal ileum (Figure 2). Bezafibrate treatment or high-fat diet did not modify the PPAR δ mRNA levels along the small intestine in control or PPAR α -null mice (results not shown).

In agreement with previous reports [6,7], significant increases in both intestinal and hepatic L-FABP mRNA levels were

Figure 4 Effect of a chronic administration of sunflower oil on the L-FABP mRNA levels in the liver (left panel) and small intestine (right panels) from PPAR α ^{+/+} and PPAR α ^{-/-} mice

Mice were fed *ad libitum* with a standard chow containing 8% of total calories in lipids. Mice subjected to the high-fat diet containing 16% total calories in lipids were daily force-fed in the morning for 5 days with 0.2 ml of sunflower oil (SFO). The controls (C) received in the same manner 0.2 ml of 0.15 M NaCl. Mice exposed to the very-high-fat diet were fed for 5 days with a laboratory chow containing 30% of total calories in sunflower oil. Northern blots were performed with 30 μ g of total RNA. The effects of the dietary lipids on L-FABP mRNA levels were studied throughout the small intestine (from the pylorus to the ileo-caecal valvula). For the gut, the bar graphs represent the means of areas under the curves corresponding to the L-FABP expression. The results were normalized to 18 S rRNA to control for differences in RNA loading. PPAR α ^{+/+} and PPAR α ^{-/-} control mice were arbitrarily assigned the value 1. Black bars, controls; white bars, high-fat diet containing 16% of lipids; grey bars, very-high-fat diet containing 30% of lipids. Results are means $\pm$ S.E.M. for three or four independent experiments. * P < 0.025; ** P < 0.01.

found in PPAR α ^{+/+} mice subjected to bezafibrate treatment (Figure 3). As expected, and in agreement with Lee et al. [16], the fibrate-mediated induction of the gene encoding L-FABP was fully abolished in the liver of PPAR α -null mice. Surprisingly, the bezafibrate-mediated induction of the gene for L-FABP was maintained in the small intestine in PPAR α ^{-/-} mice (Figure 3). To verify this, the experiment was reproduced *ex vivo* with duodeno-jejunal explants (i.e. intestinal segments 2 and 3). After 20 h of culture in presence of bezafibrate, 2.2-fold and 1.9-fold increases in L-FABP mRNA levels were found in PPAR α ^{+/+} and PPAR α ^{-/-} mice respectively (Figure 3). Thus, in contrast with the liver, PPAR α expression is not absolutely required for induction of the gene encoding L-FABP by fibrates in the small intestine.

Similar results were found in mice subjected to high-fat diets containing either 16% or 30% lipids in total calories. As shown in Figure 4, the increase in L-FABP mRNA levels in the liver of sunflower oil-fed mice was ablated in PPAR α -null mice. In contrast, as found with bezafibrate treatment, disruption of the gene encoding PPAR α did not suppress the well-known lipid-mediated up-regulation of L-FABP gene expression in the small intestine [9]. To confirm this last result, infusions into terminal ileum were performed. In this model *in vivo*, because the expression of the gene encoding L-FABP is undetectable in the distal intestine (Figure 2), it can be highly induced when

Figure 5 Ileal infusion of a LCFA triggers the appearance of L-FABP mRNA in $\text{PPAR}\alpha^{-/-}$ mice

α -Bromopalmitate (α -BrPal) ($10 \mu\text{mol}$) complexed with NaT in a 1:2 ratio was infused directly into the distal ileum of $\text{PPAR}\alpha^{+/+}$ and $\text{PPAR}\alpha^{-/-}$ mice (segment 7). Controls received vehicle only (NaT) in the same manner. Total RNA species were extracted from ileal mucosa 24 h after surgery and analysed by Northern blotting. Shown is a typical Northern blot performed with $30 \mu\text{g}$ of total RNA. Results were normalized to 18 S rRNA to control for differences in RNA loading.

adequate stimuli are directly infused into the ileal lumen [7,9]. The use of the non-metabolizable FA, α -bromopalmitate, which is a good inducer of L-FABP expression in this model, triggered the appearance of a strong L-FABP mRNA signal in both controls and $\text{PPAR}\alpha$ -null mice (Figure 5).

$\text{PPAR}\delta/\alpha$ agonist GW2433 triggers the induction of L-FABP mRNA in intestine from $\text{PPAR}\alpha$ -null mice

Taken together, our results indicate that $\text{PPAR}\alpha$ is not required for the bezafibrate- and LCFA-mediated inductions of the gene encoding L-FABP in the small intestine. Because $\text{PPAR}\delta$ is highly expressed in this tissue (Figure 2), and might bind to and activate the $\text{PPRE}^{\text{L-FABP}}$ sequence ([14,15], and Figure 1), we hypothesized that the gene encoding L-FABP might be under the control of $\text{PPAR}\delta$ in the small intestine. To examine this possibility, we compared the effect of the selective $\text{PPAR}\alpha$ agonist GW7647 and the dual $\text{PPAR}\delta/\alpha$ agonist GW2433 [33] on L-FABP expression by using intestinal explants from wild-type and $\text{PPAR}\alpha$ -deficient mice. This *ex vivo* experiment allowed us to check the direct effect of a $\text{PPAR}\delta$ agonist on the gene

encoding L-FABP. Indeed, the dual $\text{PPAR}\delta/\alpha$ agonist GW2433 became a selective $\text{PPAR}\delta$ agonist in explants from mice in which $\text{PPAR}\alpha$ was lacking. As shown in Figure 6(A), in duodeno-jejunal explants from wild-type mice, both the selective $\text{PPAR}\alpha$ agonists GW7647 and GW2433 were able to increase the L-FABP mRNA levels. In contrast, and in spite of the high concentration of GW7647 used ($1.5 \mu\text{M}$; $\text{EC}_{50} = 5 \text{ nM}$), the inductive effect was found only with GW2433 ($1.5 \mu\text{M}$; $\text{EC}_{50} = 300 \text{ nM}$) in explants from $\text{PPAR}\alpha$ -null mice (Figure 6). To visualize this regulation better, the experiment was reproduced with ileal explants in which the L-FABP mRNA level was undetectable in the basal condition of culture. Only GW2433 was able to elicit a strong L-FABP mRNA signal in both $\text{PPAR}\alpha^{+/+}$ and $\text{PPAR}\alpha^{-/-}$ mice (Figure 6B).

DISCUSSION

We have demonstrated previously that the gene encoding L-FABP is regulated transcriptionally both by bezafibrate and LCFAs in the liver and small intestine [6–9]. The transfection results reported here provide the first functional evidence that this regulation involves direct interaction between the PPARs and a unique PPRE sequence located in the proximal part of the promoter: notably, transactivation of a CAT reporter gene is lost when the PPRE sequence (–67/–55) of the L-FABP promoter (–4000/+21) is mutated. Moreover, the short promoter fragment (–326/+21) is sufficient to reproduce fully the induction of the reporter gene in the presence of the three PPAR subtypes activated by specific pharmacological agonists.

$\text{PPAR}\alpha$ and $\text{PPAR}\delta$ are expressed predominantly in the liver and the small intestine respectively. To explore their respective contributions to the regulation of the gene encoding L-FABP *in vivo*, we used $\text{PPAR}\alpha$ -null mice. In the liver, in which L-FABP and $\text{PPAR}\alpha$ are tightly co-localized in a periportal-to-perivenous gradient along the acinus in wild-type rodents [20,34], disruption of the gene encoding $\text{PPAR}\alpha$ leads to a total loss of both bezafibrate- and FA-mediated induction of the gene encoding L-FABP (Figures 3 and 4). Surprisingly, the increase in L-FABP mRNA levels, found classically in wild-type mice subjected to bezafibrate treatment or a high-fat diet, is maintained in the small intestine in spite of disruption of the gene for $\text{PPAR}\alpha$ (Figures 3–5). These results are the first demonstration that the regulation of L-FABP gene expression by fibrates and LCFAs

Figure 6 Effects of agonists for $\text{PPAR}\alpha$ (GW7647) and $\text{PPAR}\delta$ (GW2433) on L-FABP mRNA levels in organ cultures of duodeno-jejunal (A) and ileal (B) explants from $\text{PPAR}\alpha^{+/+}$ and $\text{PPAR}\alpha^{-/-}$ mice

Explants were cultured for 20 h in the presence of $1.5 \mu\text{M}$ GW7647 or GW2433 as described in the Materials and methods section. Control cultures (C) were performed with vehicle alone [0.1% (v/v) DMSO]. A Northern blot representative of two separate experiments is shown.

might be independent of PPAR α gene expression in the gut. The activation of mPPAR δ by polyunsaturated fatty acid (PUFA) and bezafibrate, and its exclusive expression in the small intestine in PPAR α -null mice, suggest strongly that the gene encoding L-FABP is under the control of PPAR δ in this tissue. To verify this hypothesis, the use of a selective delta agonist was required. So far, the best synthetic ligands for PPAR δ are GW2433 [33] and L165041 [35]. Unfortunately, these compounds also exhibit significant PPAR α and PPAR γ activity respectively [33,35]. Nevertheless, the dual PPAR δ/α agonist GW2433 was chosen because it becomes a selective PPAR δ agonist in PPAR α -null mice. The fact that GW2433-mediated induction of L-FABP expression is maintained in intestinal explants when PPAR α is absent strongly suggests the involvement of PPAR δ in this regulation. This new finding demonstrates that (1) PPAR δ can act as fibrates/FA-activated receptor in tissues in which it is abundantly expressed and (2) L-FABP is a PPAR δ target gene in the small intestine. Interestingly, a similar observation has been reported in the kidney, where the PPAR α and PPAR δ subtypes are also co-expressed. In this organ, the induction of acyl-CoA oxidase gene expression by the peroxisome proliferator di-2-ethylhexyl phthalate (DEHP) was maintained in PPAR α -deficient mice [36]. The small intestine and the kidney exhibit complementary physiological functions because they are implicated in the absorption and reabsorption of nutrients respectively.

The biological functions of PPAR α and PPAR γ are relatively well understood in rodents. PPAR α is expressed abundantly in the liver, where it promotes lipid catabolism and peroxisome proliferation [11]. PPAR γ , which is expressed predominantly in adipose tissue, regulates adipocyte differentiation and insulin sensitivity [37,38]. In contrast, relatively little is known about the physiological function of PPAR δ . PPAR δ is found mainly in tissues characterized by a high lipid metabolism, such as the adipose tissue, heart, skeletal muscles, developing brain and small intestine [20]. In white adipose tissue, PPAR δ induces the expression of early genes implicated in the initial steps of the adipocyte differentiation process, e.g. the fatty-acid translocase (FAT/CD36), adipocyte fatty-acid-binding protein (A-FABP) and acyl-CoA synthases (ACSS) [24]. In brain, ACS-2 might be a PPAR δ target gene [39]. FAT/CD36 and A-FABP are plasma membrane and cytoplasmic lipid-binding proteins respectively, which bind LCFAs. They are believed to have a crucial role in the cellular uptake and trafficking of LCFAs. ACSs are intracellular enzymes that promote the synthesis of the acyl-CoA esters of FAs. By maintaining of a low concentration of LCFAs in the cytoplasm, ACSs create a concentration gradient that promotes LCFA entry into the cell. This unidirectional flux is reinforced by the lack of diffusion of acyl-CoA esters through the plasma membrane. PPAR δ might therefore have a role in the modulation of LCFA flux within cells through a co-ordinate regulation of genes involved in both cellular LCFA uptake (FAT/CD36, A-FABP) and FA activation (ACSS). The intestinal results presented here support this hypothesis.

In the small intestine, the strongest expression of PPAR δ is found at the major site of lipid absorption (i.e. the duodeno-jejunal segment). Moreover, PPAR δ up-regulates the expression of L-FABP, a lipid-binding protein found abundantly in the enterocyte. From transfection studies in different cell lines, it seems that L-FABP increases cellular LCFA uptake and trafficking [40,41]. L-FABP is also thought to have an indirect but essential role in the co-ordinated regulation of FA-responsive genes, through the modulation of the intracellular concentration of free LCFAs. These putative functions are supported by two observations. First, L-FABP exhibits a strong affinity for PUFA [1], which are natural ligands for the PPARs [17–19]. Secondly,

a linear correlation between L-FABP levels and PPAR α activation was reported in nuclei from hepatocytes [4]. Thus L-FABP seems to be a cytosolic–nuclear shuttle protein implicated in the ligand activation of PPAR α and therefore in the transcription of PPAR α target genes in the liver. Because mouse PPAR α and PPAR δ have overlapping binding characteristics for PUFA, a similar mechanism could be envisaged in the small intestine. This function would be important in an organ whose physiological role is to ensure the efficient absorption of nutrients. Interestingly, several putative FA target genes expressed in the enterocyte are lipid-binding proteins (FAT/CD36, fatty-acid transport protein and L-FABP), or FA activators (ACSS). Therefore PPAR δ -mediated regulation of the gene encoding L-FABP might contribute to the regulation of intestinal absorption in response to changes in the lipid content of the diet through the co-ordinated regulation of genes involved in lipid transport and metabolism. A PPAR δ -null mouse model was generated [42]. It might contribute to the establishment of the definitive proof for a specific function of PPAR δ in the small intestine.

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