Phytanic acid is ligand and transcriptional activator of murine liver fatty acid binding protein

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Abstract Branched-chain phytanic acid is metabolized in liver peroxisomes. Sterol carrier protein 2/sterol carrier protein x (SCP2/SCPx) knockout mice, which develop a phenotype with a deficiency in phytanic acid degradation, accumulate dramatically high concentrations of this fatty acid in serum (Seedorf at al. 1998. *Genes Dev.* 12: 1189–1201) and liver. Concomitantly, a 6.9-fold induction of liver fatty acid binding protein (L-FABP) expression is observed in comparison to wild-type animals fed standard chow, possibly mediated by the peroxisome proliferator-activated receptor alpha (PPARα). Cytosolic transport of phytanic acid to either peroxisomal membranes or to the nucleus for activation of PPARα may be mediated by L-FABP, which gives rise to the question whether phytanic acid is a transactivator of this protein. Here we show first that phytanic acid binds to recombinant L-FABP with high affinity. Then the increase of the in vivo phytanic acid concentration by phytol feeding to mice results in a 4-fold induction of L-FABP expression in liver, which is in the order of that attained with bezafibrate, a known peroxisome proliferator. Finally to test in vitro whether this induction is conferred by phytanic acid, we cotransfected HepG2 cells with an expression plasmid for murine PPARα and a CAT-reporter gene with 176 bp of the murine L-FABP promoter, containing the peroxisome proliferator responsive element (PPRE). After incubation with phytanic acid, we observed a 3.2-fold induction of CAT expression. These findings, both in vivo and in vitro, demonstrate that phytanic acid is a transscriptional activator of L-FABP expression and that this effect is mediated via PPARα.—Wolfrum, C., P. Ellinghaus, M. Fobker, U. Seedorf, G. Assmann, T. Börchers, and F. Spener. **Phytanic acid is ligand and transcriptional activator of murine liver fatty acid binding protein. *J. Lipid Res.* 1999.** 40: **708–714.**

Supplementary key words peroxisome proliferator-activated receptor • peroxisome proliferation • sterol carrier protein 2 • sterol carrier protein x • isothermal titration calorimetry • SCP2/SCPx-deficient knock out mice • L-FABP

Phytanic acid (3,7,11,15-tetramethylhexadecanoic acid) is a branched-chain, isoprenoid-derived fatty acid which is synthesized after bacterial side-chain cleavage of chlorophyll and subsequent oxidation of the resulting phytol in mammals (1). Enzymes for the conversion of phytol to phytanic acid were detected in liver mitochondria and microsomes (2). Phytanic acid is primarily degraded in peroxisomes by α-oxidation to pristanic acid which is then subjected to six cycles of β-oxidation (3, 4). A deficiency in the α-oxidation pathway leads to an accumulation of phytanic acid in serum and livers of men which is known as Refsum's disease (5). The recent characterization of the sterol carrier protein 2/sterol carrier protein x-knockout mouse also revealed drastically increased serum concentrations of phytanic acid accompanied by peroxisome proliferation and altered hepatic gene expression (6). These findings and the different subcellular localization of phytanic acid formation and degradation site led us to hypothesize that in analogy to long-chain fatty acids, a cytosolic carrier is involved in the transport of phytanic acid to peroxisomes. A likely candidate for a cytosolic carrier function is the 14.4 kDa liver type fatty acid binding protein (L-FABP) which belongs to a family of cytosolic binding proteins for hydrophobic ligands (7).

L-FABP is abundantly expressed in liver, but also in small intestine and kidney. So far, cDNA sequences have been reported for several species including human (8), rat (9), pig (10), and cattle L-FABP (11). The binding properties of L-FABP reveal striking differences to that of other the FABP members, as it binds two molecules of fatty acids (11–13), a result that has been confirmed by the recent elucidation of the crystal structure of the rat L-FABP/oleic acid complex (14). In addition to various long-chain fatty acids, L-FABP binds phytanic acid (15), heme (16, 17), lysophosphatidic acid (11, 18), eicosanoids (19), and hypolipidemic drugs like fibrates (20), the latter known to induce peroxisome proliferation in rodents.

Abbreviations: L-FABP, liver fatty acid binding protein; ILBP, ileal lipid binding protein; TOF-SIMS, time-of-flight secondary-ion mass spectrometry; PPAR, peroxisome proliferator-activated receptor; SCP2, sterol carrier protein 2; SCPx, sterol carrier protein x.

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Peroxisome proliferation is a pleiotropic cellular response to a range of chemical compounds that is accompanied by profound increases in the acitivties of peroxisomal β-oxidation enzymes (21). This phenomenon is restricted to liver and kidney and is only observed in rodents, where it can lead to hepatocarcinogenesis (22, 23). By use of transgenic mice it has been demonstrated that the structural diverse peroxisome proliferators mediate their effects via activation of the transcription factor peroxisome proliferator-activated receptor alpha (PPARα) (24). After dimerization with the retinoid x receptor alpha (RXRα), the ligand-activated heterodimer binds to peroxisome proliferator response elements (PPREs), consisting of direct repeats of the sequence 'TGACCT' (25). Such PPREs have been identified within the promoter regions of various genes involved in lipid metabolism including rat L-FABP (25). Moreover, L-FABP transcription is known to be affected by xenobiotic peroxisome proliferators (26, 27). As phytanic acid has been described earlier to be a peroxisome proliferator (28), we envisaged the phytanic acid accumulating SCP2/SCPx-deficient knockout mouse to be a good in vivo model to study the effect of this fatty acid on L-FABP expression relative to that of the xenobiotic peroxisome proliferator bezafibrate. Then, to investigate whether phytanic acid is a transcriptional activator of L-FABP, we used transactivation assays as a direct experimental in vitro approach by using the HepG2 cell model.

MATERIALS AND METHODS

Materials

Phytanic acid, nonadecanoic acid, and bezafibrate were obtained from Sigma, phytol was from Aldrich. Oligonucleotides were purchased from MWG Biotech; restriction enzymes were from Boehringer Mannheim. All chemicals used were of analytical grade.

Cloning of murine L-FABP cDNA

Two primers derived from rat liver FABP cDNA (5′-GGCAAG TACCAAGTGCAGAGCAA-3′ and 5′-CAGAGGTAACTTAAGTC AGTGCCTG-3′) were used to generate a 309 bp fragment of the murine L-FABP cDNA by polymerase chain reaction (PCR), using 2 μl of the λZAPII mouse liver cDNA library (Stratagene) as template. The fragment was labeled with DIG-dUTP (Boehringer Mannheim) and used as a probe to screen approximately 25,000 plaques of the λZAPII mouse liver cDNA library, yielding about 100 positive clones. Ten clones were isolated, excised in vivo and the DNA was extracted and digested with Eco RI/Xho I. After Southern blotting and ddNTP-sequencing using the BioCycle Sequencing Kit (GATC), two clones were confirmed to contain the full-length murine L-FABP cDNA, including the 5′- and 3′-untranslated regions (**Fig. 1A**). As expected, the deduced amino acid sequence showed high identity to that of other known L-FABP sequences i.e., 94, 84, 81, and 75% to L-FABPs from rat (9), human (8), pig (10), and cattle (29), respectively. The full-length murine L-FABP cDNA was used as template to amplify the coding region by PCR with oligonucleotide primers 5′-GCCCATATGA ACTTCTCCGG-3′ and 5′-NNGGATCCTAAATTCTCTTGCTG-3′ using Pfu polymerase. The primers were designed to incorporate recognition sites (underlined) for Nde I (upstream primer) and Bam HI (downstream primer) flanking the coding region. The 394 bp fragment obtained was cloned into the pCRscript vector (Stratagene). After double-stranded sequencing, the insert was subcloned into the expression vector pET3c (Novagen). The resulting pET-mL-FABP plasmid was used for transformation of *E. coli* strain BL21(DE3)pLysS.

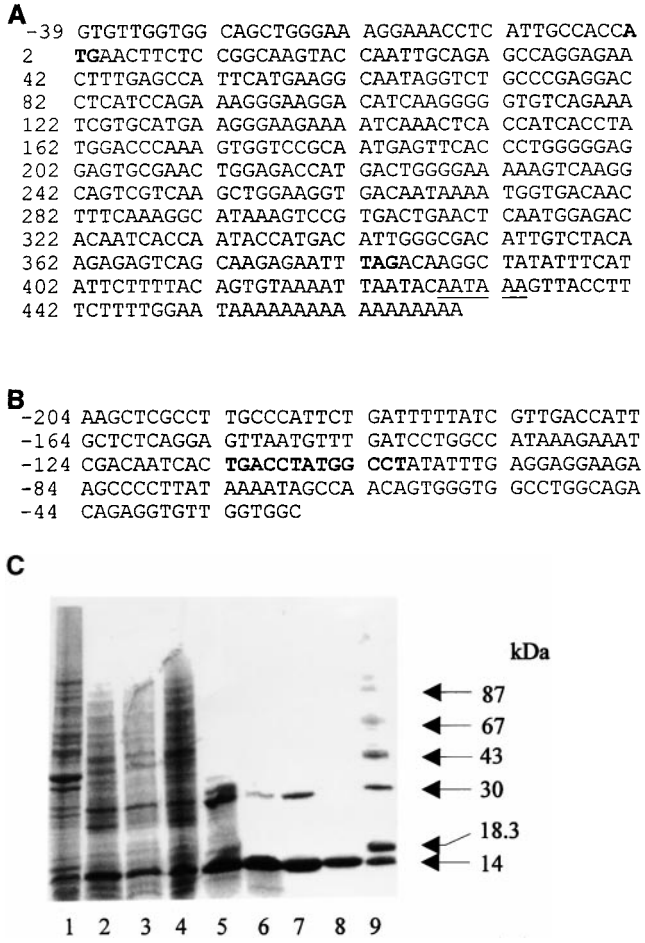

Fig. 1. Characterization of murine L-FABP. A: Sequence of L-FABP cDNA. The start codon at position 1 and the stop codon at position 382 are bold-faced, the polyadenylation signal at position 428 is underlined. The cDNA sequence of murine L-FABP has been deposited in the EMBL Nucleotide Sequence Database under the accession number Y14660. B: Promoter of the murine L-FABP gene. The PPRE at position −114 to −102 is bold-faced. C: Purification of recombinant L-FABP. Lane 1, crude cell extract; lane 2, soluble proteins; lane 3, ammonium sulfate precipitate; lane 4, Sephadex G 25-fraction; lanes 5,6, Q-Sepharose BB-fraction; lane 7,8, pure protein after Superdex 75-polishing; lane 9, low molecular weight standard. The loading buffer for sample 8 contained 1% β-mercaptoethanol affecting the cleavage of dimeric L-FABP (28 kDa, lane 7).

Heterologous expression and purification of murine L-FABP

Growth of *E. coli* cells and expression of murine L-FABP was carried out in Terrific Broth (TB) with 100 μg/ml ampicillin and 35 μg/ml chloramphenicol at 37°C. The expression was induced at an OD_{600} of 0.9–1.0 with 0.5 mm isopropyl-β-d-thiogalactoside. Cells from 6 liter culture volume were harvested 2 h after induction by centrifugation, frozen (1 h at −80°C) and resuspended in a 3-fold volume of buffer A (10 mm Tris/HCl, pH

7.4, 30 mm NaCl) after adding 20 U DNase I (Sigma) for 30 min at 37°C. Cells were disrupted by sonication (3 × 20 s, 0°C, 35 W) and centrifuged (1 h, 4°C, 25,000 *g*) to remove cell debris. DNA was precipitated by adding 1.5% (w/v) streptomycin sulfate and subsequent centrifugation (20 min, 4°C, 25,000 *g*). Ammonium sulfate (25% (w/v)) was added, the solution was stirred for 2 h at 4°C and centrifuged (20 min, 4°C, 25,000 *g*). Soluble proteins were desalted by gel filtration on Sephadex G-25 (5 × 28 cm, 5 ml/min) in buffer A. Fractions containing L-FABP were pooled and chromatographed on Q-Sepharose Big-Beads (5 × 30 cm, 5 ml/min) in buffer A. Under these conditions *E. coli* proteins, but not L-FABP, bound to the column. L-FABP containing fractions were concentrated by ultrafiltration (Amicon). The final purification step was done on a Superdex 75 column (1.6 × 85 cm, 2 ml/min) in buffer A and purity of the protein was checked by SDS-PAGE (30) (Fig. 1B). The protein was delipidated by extracting the aqueous protein containing phase 3 times with 1/3 volume of butanol-1 (12). The suspension was centrifuged for phase separation and the aqueous phase was freed of butanol-1 using a vacuum centrifuge (Speedvac) and concentrated using Centripreps (Amicon).

Antibody preparation and purification

Antibodies were generated in New Zealand White rabbits by injection of 250 μg of recombinant murine L-FABP in complete Freund's adjuvant (Sigma). Rabbits were boostered every 6 weeks with 100 μg of recombinant murine L-FABP in incomplete Freund's adjuvant. Polyclonal anti-murine L-FABP antibodies were isolated by passing serum over an affinity column with murine L-FABP bound to CH-activated Sepharose 4B and subsequent elution with 10 mm citrate, 20 mm phosphate, pH 2.8.

Western blot analysis

Ten μg of liver cytosolic proteins was separated by SDS-PAGE (15% T, 2.7% C) and transferred onto a nitrocellulose membrane (Schleicher & Schuell) by electroblotting (1 h, 90 mA). L-FABP was detected with affinity purified L-FABP antibodies (2.5 μg/ml) and goat anti-rabbit IgGs conjugated to alkaline phosphatase (1:10,000). For visualization, the chemiluminescence substrate CDP-Star (Tropix) was used.

Time-of-flight secondary ion mass spectrometry

Mouse liver (0.1 g) was homogenized in 1 ml PBS, followed by the addition of 0.162 g/l heneicosanoic acid as internal standard and 1 ml of freshly prepared ethanolic KOH (150 g/l). The saponification was performed at 37°C for 1 h. An equal volume of chloroform–methanol 2:1 (v/v) according to the methods of Folch, Lees, and Sloane Stanley (31) was added and the mixture was vortexed for 5 min followed by centrifugation at 800 *g* (5 min, 15°C). The extraction procedure was repeated twice (total of three extraction procedures). The combined organic phase was transferred to clean, tapered glass tubes and thoroughly dried under nitrogen at 40°C. The tubes were allowed to cool to room temperature, 100 μl of n-hexane was added, and the sample was solubilized by placing in a Laboson 200 ultrasound water bath (Bender and Hobein) for 5 min at room temperature. After centrifugation at 800 *g* for 5 min, 2 μl of the sample was applied to a silver target (galvanic-deposited silver layers on a platinum substrate). After evaporation of the solvent, the resulting submonolayer was analyzed by a TOF-SIMS II mass spectrometer (ION-TOF) (32). Samples were bombarded with $^{40}Ar^{+}$, 10^{11} ions/cm^2 (11 keV with typical acquisition times of 100 s. The approximate mass resolution was $M/\Delta M = 3000$, where M is the target ion mass and ΔM is the resolved mass difference at half-width full peak mode. Quantitation was carried out by determining relative sensitivity factors as described previously (33).

Titration calorimetry

For titration calorimetry (34) with an isothermal microcalorimeter (Microcal), protein was equilibrated with buffer C (20 mm KH_2PO_4, pH 7.25, 50 mm KCl, 0.02% NaN_3) on a fast desalting column (Pharmacia). Protein concentration was measured using $E_{280}^{1\%} = 5.1$ which was determined according to Gill and von Hippel (35). The concentration of the protein solution in the cell was 0.09 mm at 37°C. The ligand (77.5 μmol) was dissolved in 500 μl of KOH solution (250 mm) and buffer C was added up to 25 ml. To increase the solubility of phytanic acid and to generate carrier peaks for an improved data acquisition, 1% methanol was added to the buffer as described earlier (36). The ligand solution (3.1 mm) was injected in 30 aliquots of 4 μl each in 3-min intervals. The solution was stored at 4°C for 8 h under nitrogen. Each binding curve was measured at least two times with different solutions of the same ligand, and the ligand solution was also titrated with buffer C without protein, to account for the heat of dilution. The effect of methanol was studied by titrating oleic acid with or without solvent, resulting in similar isotherms. The raw data was processed using the Microcal Origin software and then fitted using a model of two independent binding sites as described in great detail in the manual of the supplier. During fit, all variable parameters like binding stoichiometry, binding enthalpy, and dissociation constants for both binding sites were floated.

Transactivation assay

For transactivation studies, a 176-nt segment of the murine L-FABP promoter (Fig. 1B) was amplified by PCR with primers (5′-GAGCTCGCCTTGCCCATTCTGATT-3′ and 5′-AGATCTCA GCTGACCACAACAGCT-3′) derived from the promoter sequence of rat L-FABP (37) and cloned into the pCAT3-Promoter plasmid (Promega). As a control, an oligonucleotide consisting solely of the murine L-FABP-PPRE (38) was cloned into the same vector. HepG2 cells (ATCC, HB-8065) were grown to 60–70% confluency in T75 culture flasks (Nunc) in Dulbecco's modified Eagle's medium (Biochrom) supplemented with 10% BMS artificial serum, fatty acid-free (Biochrome) and then transfected with the reporter genes (1.5 μg), using pSV-β-Gal (Promega) as internal reference (0.5 μg) and cotransfected with a murine PPARα-expressing plasmid (friendly gift from Dr. P. Holden, Zeneca) by use of the transfection reagent Fugene6 (Boehringer Mannheim). After transfection cells were incubated for 42 h, which is a common time used for incubation (39), with 200 μm phytanic acid or 200 μm bezafibrate in dimethylsulfoxide, whose concentration in the medium was kept below 1%. The resulting CAT and β-Gal expressions were measured with the CAT- and β-GAL-ELISA kits (Boehringer Mannheim), respectively. During the 42-h incubation time, the cells did not reach 100% confluency.

Animal treatment

The construction of SCP2/SCPx-deficient mice (−/−) (strain C57BL6), the feeding experiments with −/− and wild-type mice (+/+) (strain BALB B6) and the preparation of bezafibrate- (0.5%, w/w) and phytol- (0.5 %, w/w) enriched chows has been explained in detail earlier (6). The uptake of standard chow and supplemented chows was monitored and found to be similar for the two strains (6). The standard chow contained only low amounts of phytol (<0.075 mg/g) and phytanic acid (<0.2 mg/g) (6).

RESULTS

Binding of phytanic acid to recombinant murine L-FABP

With the aid of a displacement assay it is has been shown that phytanic acid was able to displace 48% of *cis*-parinaric acid from rat L-FABP when applied in equal amounts, but a binding constant was not given (15). Isothermal titration calorimetry allowed us here to derive individual constants for the affinity of phytanic acid to the two binding sites of L-FABP (K_{d1} = 0.015 μm, K_{d2} = 0.286 μm). Indeed by titrating the murine L-FABP with phytanic acid we obtained data points to which the binding isotherm could only be fitted under the assumption of two binding sites (**Fig. 2**). The binding enthalpies for both binding sites were negative, whereas the second binding site of bovine L-FABP, which was analyzed for comparison, exhibited endothermic binding characteristics. Both proteins bound phytanic acid with similar affinity; however, the overall stoichiometry was slightly higher for bovine L-FABP (1.88) than for murine L-FABP (1.50) (**Table 1**). The validity of the calorimetric method was further strengthend by our data for oleic acid binding to murine L-FABP with K_{d1} = 0.021 ± 0.008 μm and K_{d2} = 0.312 ± 0.073 μm. These values are highly comparable to those determined by the ADIFAB method for the binding of oleic acid to rat L-FABP (40).

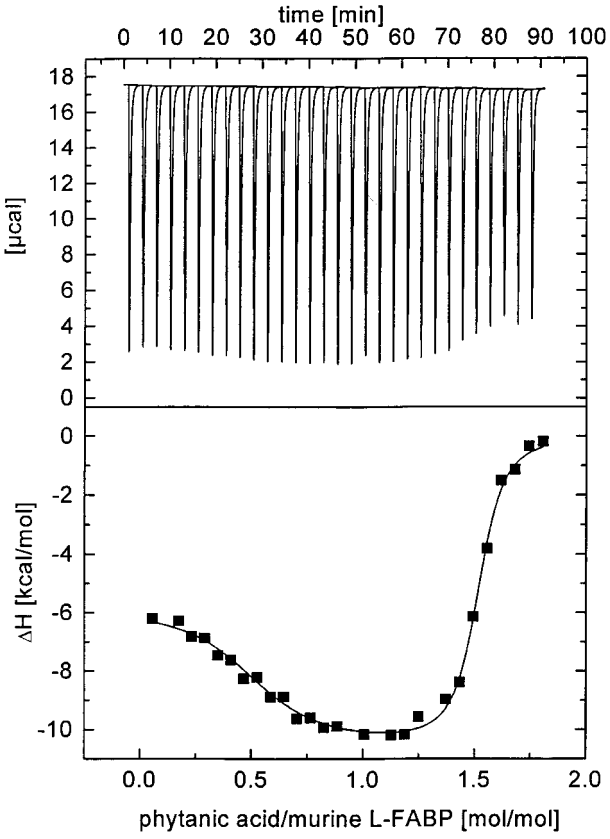

Fig. 2. Binding of phytanic acid to murine L-FABP. The upper panel shows the raw data of a representative experiment, each peak representing the amount of heat generated or consumed during binding of the ligand to recombinant L-FABP. The last peak reflects the carrier peak produced by the heat of dilution (mainly due to methanol). The lower panel shows the corresponding binding isotherm fitted under the assumption of two independent binding sites.

TABLE 1. Thermodynamic data for binding of phytanic acid to recombinant murine and bovine L-FABPs

	n_1	K_{d1}	ΔH_1	n_2	K_{d2}	ΔH_2
		μM	*kJ/mol*		*μM*	*kJ/mol*
Murine L-FABP	0.51 ±0.01	0.015 ±0.001	−24.2 ±0.02	0.99 ±0.01	0.282 ±0.006	−46.3 ±1.9
Bovine L-FABP	1.55 ±0.01	0.028 ±0.001	−11.6 ±0.2	0.33 ±0.03	1.06 ±0.04	17.6 ±0.3

Data were obtained by fitting the binding isotherms with a model of two independent binding sites and are presented as mean ± deviation of two independent experiments.

Induction of murine L-FABP expression by phytanic acid and bezafibrate

It has been demonstrated that the expression of rat L-FABP can be induced on mRNA as well as on protein level by oral administration of L-FABP ligands, e.g., fatty acids and peroxisome proliferators (26, 27). Therefore we addressed the question whether phytanic acid would induce the expression of murine L-FABP in vivo. After feeding mice with the phytanic acid precursor phytol for 4 days, we measured first phytanic acid concentrations in liver homogenates by TOF-SIMS. In control mice fed standard chow, phytanic acid was barely detectable but increased to 0.23 ± 0.03 μg/mg liver protein after feeding the phytol-enriched chow. SCP2/SCPx-deficient mice fed standard chow revealed 6- to 10-fold higher basal phytanic acid serum concentrations (6); in livers of these mice phytanic acid was not detected. However, after feeding the phytol-enriched chow, phytanic acid concentrations dramatically rose to 2.4 ± 0.4 μg/mg liver protein. Then, by applying affinity-purified anti-L-FABP antibodies, we used Western blot analysis to screen the proteins of liver homogenates for L-FABP expression and found that L-FABP concentration in liver of wild-type mice increased 4.1-fold after feeding the phytol-enriched chow (**Fig. 3**). Not in correlation to the liver concentration but to the serum concentration of phytanic acid (6), L-FABP expression in the liver of SCP2/SCPx-deficient mice was already 3.5-fold higher as compared to that of wild-type mice after standard chow feeding and rose to a 6.9-fold increase in livers of knockout mice receiving the phytol-enriched chow, which now correlated with the enhanced phytanic acid concentration in the livers of these animals. These results were consistent with those obtained from Northern blots, which showed the same correlation between serum phytanic acid concentration and L-FABP mRNA expression (data not shown). To compare the induction of L-FABP expression after phytol feeding to that of other peroxisome proliferators, we fed wild-type mice for 7 days with a chow containing 0.5% (w/w) bezafibrate and observed a 4.9-fold increased level in liver. It was noted that the level of bezafibrate-triggered L-FABP expression rose until day 2 and then remained constant up to day 7 at the end of the trial period.

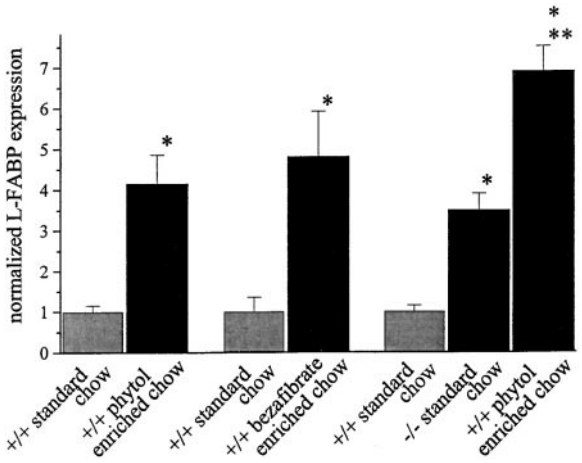

Fig. 3. Western blot analysis of L-FABP expression in liver induced by phytanic acid and bezafibrate. Mouse liver cytosolic proteins of wild type mice (+/+) and SCP2/SCPx-deficient mice (−/−) fed with standard chow, with standard chow supplemented with 0.5% phytol and wild-type mice fed with bezafibrate, respectively, were separated by SDS-PAGE (15% T, 2.7% C) and blotted as described under Materials and Methods. Recombinant murine L-FABP was used as standard. Differences in L-FABP protein expression were quantified by laser densitometry of stained proteins and normalized to the L-FABP content in liver cytosols of wild type (+/+) mice fed standard chow. Each value represents the mean ± SD (n = 3). *$P < 0.006$ vs. +/+ standard chow, **$P < 0.001$ vs. −/− standard chow.

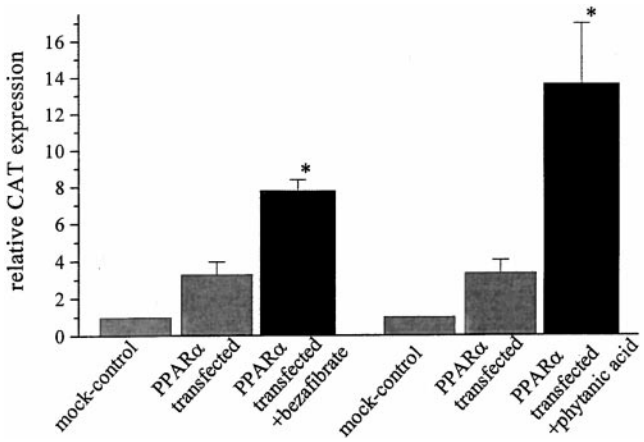

Fig. 4. Transactivation of L-FABP expression by peroxisome proliferators. HepG2 cells grown to 60–70% confluency were cotransfected with the CAT-reporter gene containing the 176-nt segment of the murine L-FABP promoter, the pSV-β-Gal as reference and the expression plasmid for murine PPARα or vector alone (mock control). After transfection cells were incubated with phytanic acid (200 μm) and bezafibrate (200 μm), respectively. Upon incubation for 42 h cells were lysed and CAT and β-Gal expression were determined with respective ELISAs. Each value represents the mean ± SD (n = 8). *$P < 0.005$ vs. murine PPARα transfected cells.

Transactivation studies

As phytanic acid revealed an effect on L-FABP expression in vivo, we then turned to a cell culture model to assess the effect of phytanic acid on gene activation more directly. As the HepG2 cells used for the subsequent transactivation assay expressed only small amounts of endogenous PPARα (41), we cotransfected this cell line with a murine PPARα-expressing plasmid and the reporter plasmid. This led already to a 3.2-fold increase in CAT expression in comparison to HepG2 cells transfected with the vector only as mock control (**Fig. 4**). By titrating out the toxic potential of phytanic acid, we found that concentrations above 300 μm caused the HepG2 cells to die. We therefore applied 200 μm phytanic acid for 42 h and observed an additional 4.2-fold increase in CAT expression (Fig. 4), when transfecting the cells with 176 nt promotor construct, and a comparable 3.9 ± 0.4-fold increase, when transfecting with the vector containing solely the PPRE. Similarly, when HepG2 cells were incubated with 200 μm bezafibrate, a mere 2.4-fold increase was observed for the promoter construct (Fig. 4) and again a comparable 2.1 ± 0.4-fold increase for the construct containing the PPRE only.

DISCUSSION

In order to have DNA probes, recombinant protein, and antibodies available, we cloned and expressed the murine L-FABP. As expected, a high sequence identity of murine L-FABP with other L-FABPs was noted. The deduced amino acid sequence of L-FABP matches a partial amino acid sequence of a 14 kDa selenium binding protein (37) which was identified after two-dimensional gel electrophoresis of ^{75}Se-labeled proteins from mouse liver. With regard to the Asn/Asp heterogeneity often observed at position 105 in mammalian L-FABPs, the sequence of the murine L-FABP cDNA reported here showed a codon for asparagine, in accordance with the finding of asparagine 105 in the partial protein sequence of the murine selenium binding protein (37). The promoter region of murine L-FABP shows high homology to the promoter region of rat L-FABP with a PPRE at the same position (25).

Initiated by our recent findings that SCP2/SCPx-deficient mice not only accumulate phytanic acid, but also reveal an enhanced expression of several hepatic genes including L-FABP (6), we became interested in further actions of this branched chain fatty acid, in particular in its role in gene regulation. Recent work confirmed that phytanic acid is degraded in peroxisomes (3, 42) explaining the observation that patients with defects in peroxisome formation (Zellweger syndrome) or in peroxisomal α-oxidation (Refsum's disease) have abnormally high phytanic acid concentrations in blood and liver (5). Prior to its transport across the peroxisomal membrane into the matrix where the enzymes for α-oxidation are mainly localized, phytanic acid is believed to be activated to its CoA-ester. The corresponding enzyme phytanoyl-CoA ligase was identified at the cytosolic leaflet of the peroxisomal membrane (4), demonstrating the need of a cytosolic carrier for this branched chain fatty acid.

Recent data on the binding of phytanic acid to L-FABP (15) clearly indicated that this protein is a candidate for carrying this fatty acid through the aqueous cytosol to the

peroxisomal membrane. L-FABP is the only member of the family of hydrophobic carrier proteins extensively expressed in liver and furthermore characterized by a rather large binding site able to accommodate ligands such as fatty acids tagged with bulky fluorophores, acyl-CoAs, lysophosphatidic acid, and heme (7, 13, 43). Thus, in analyzing the binding of phytanic acid to murine L-FABP, the calorimetric titration method enabled us to determine definite K_d values for both binding sites of L-FABP. Data could be best fitted assuming the 2:1 stoichiometry typical for L-FABPs; deviations in the occupancy of the high affinity binding sites might be caused by the assumption of two independent binding sites inherent to the curve-fitting procedure. Recent crystallographic data, however, showed that both fatty acids interact during binding (14) and that this may render the assumption of two independent binding sites as too simplistic. The binding affinity of mouse L-FABP for phytanic acid was similar to that for oleic acid and is in support of a role for L-FABP in intracellular transport of phytanic acid as well. Although it is undisputed that SCP2 is found in peroxisomes (44), it should be noted that this protein has also been reported to be localized in the mitochondria and the cytosol (45). In the latter, SCP2 might act as a carrier for phytanic acid as well, yet the affinity for phytanic acid is lower than that of L-FABP for this ligand (15).

As the serum concentrations of phytanic acid in mice measured earlier (6) revealed 50-fold higher values than K_{d1} of the phytanic acid/L-FABP complex, phytanic acid can be considered as a physiological ligand of L-FABP, even taking into consideration a much lower intracellular concentration of phytanic acid than that observed in serum. Possibly due to the detection limit of the TOF-SIMS method, we did not detect phytanic acid in the livers of SCP2/SCPx-deficient and wild-type mice on standard chow; however, the higher concentrations of phytanic acid in livers of SCP2/SCPx-deficient mice after phytol feeding accompanied by higher L-FABP expression levels clearly indicate that phytanic acid induces L-FABP expression dose-dependently. This view is further supported by the finding that the increase of L-FABP expression determined in this work is matched by the increase in phytanic acid serum concentrations reported previously (6). This is the first example that phytanic acid does not only induce proliferation of peroxisomes as reported earlier (6), but also the expression of a gene involved in lipid metabolism which is typical for established peroxisome proliferators such as fibrates. In line with this, we obtained comparative results on L-FABP expression in liver with the chow containing 0.5% (w/w) bezafibrate, the peroxisomal proliferator which also stimulated L-FABP expression in intestinal mucosal cells (27). The binding studies, however, revealed a much lower binding affinity of L-FABP for bezafibrate (K_{d1} = 0.8 μm, K_{d2} = 67.8 μm) (11) than for phytanic acid.

It has been demonstrated with rat L-FABP that peroxisome proliferators like bezafibrate induce the L-FABP expression by activating the peroxisome proliferator-activated receptor PPARα and subsequent binding of the activated receptor to the PPREs identified in the promoter region of rat L-FABP (25). As we were able to show that phytanic acid induces L-FABP expression in mice, we wanted to confirm these findings by showing that phytanic acid directly regulates L-FABP expression in an in vitro model because the complexity of a whole organism makes it difficult to establish a link between phytanic acid serum and liver concentrations and PPARα-activation. For example, it has been demonstrated that PPARα-dependent gene expression is under strict hormonal control (46). Our transactivation assays with HepG2 cells showed a direct effect of phytanic acid on the induction of a CAT reporter gene driven by the murine L-FABP PPRE in HepG2 cells cotransfected with PPARα. In support of this is our recent finding that phytanic acid is also a ligand of murine PPARα (P. Ellinghaus, C. Wolfrum, G. Assmann, F. Spener, and U. Seedorf, unpublished results). Taken together these data demonstrate that phytanic acid is a transcriptional activator of L-FABP and that this effect is mediated via activation of PPARα.

This work was supported by grants from the Deutsche Forschungsgemeinschaft (SFB 310/A4 and Se 459/2-2). We thank Professors J. C. Sacchettini and G. Reinhard (Texas A&M University, College Station, TX) for access to and help with titration calorimetry. This work is part of the Ph.D. thesis of Christian Wolfrum.

Manuscript received 15 October 1998 and in revised form 15 December 1998.

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