Decreased Liver Fatty Acid Binding Capacity and Altered Liver Lipid Distribution in Mice Lacking the Liver Fatty Acid-binding Protein Gene*

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Although liver fatty acid-binding protein (L-FABP) is an important binding site for various hydrophobic ligands in hepatocytes, its in vivo significance is not understood. We have therefore created L-FABP null mice and report here their initial analysis, focusing on the impact of this mutation on hepatic fatty acid binding capacity, lipid composition and expression of other lipid-binding proteins. Gel-filtered cytosol from L-FABP null liver lacked the main fatty acid binding peak in the fraction that normally comprises both L-FABP and sterol carrier protein-2 (SCP-2). The binding capacity for cis-parinaric acid was decreased >80% in this region. Molar ratios of cholesterol/cholesterol ester, cholesteryl ester/triglyceride, and cholesterol/phospholipid were 2- to 3-fold greater, reflecting up to 3-fold absolute increases in specific lipid classes in the order cholesterol > cholesteryl esters > phospholipids. In contrast, the liver pool sizes of nonesterified fatty acids and triglycerides were not altered. However, hepatic deposition of a bolus of intravenously injected [14C]oleate was markedly reduced, showing altered lipid pool turnover. An increase of ~75% of soluble SCP-2 but little or no change of other soluble ( glutathione S-transferase, albumin) and membrane (fatty acid transport protein, CD36, aspartate aminotransferase, caveolin) fatty acid transporters was measured. These results (i) provide for the first time a quantitative assessment of the contribution of L-FABP to cytosolic fatty acid binding capacity, (ii) establish L-FABP as an important determinant of hepatic lipid composition and turnover, and (iii) suggest that SCP-2 contributes to the accumulation of cholesterol in L-FABP null liver.

Liver fatty acid-binding protein (L-FABP), a member of the genetically related cytosolic fatty acid-binding protein (FABP) family (1–3), is found in the liver, intestine, and kidney, but only in liver is it not co-expressed with other members of its family. L-FABP is known to bind fatty acids and various other hydrophobic molecules, although its actual contribution to the lipid-binding capacity of liver cytosol is not known. Given that L-FABP is expressed at very high levels (2–5% of cytosolic protein) in the differentiated hepatocyte (4, 5) and that these levels correlate well with lipid metabolism (2), it can be speculated that L-FABP contributes considerably to hepatic lipid-binding and lipid metabolism. Work with cell-free systems and transfected cells has further strengthened this view. For example, in cell-free preparations L-FABP was shown to stimulate the esterification of oleic acid while inhibiting that of palmitic acid (6). L cells overexpressing L-FABP show increased rates of fatty acid uptake and esterification (7) as well as increased contents of phospholipid and cholesteryl esters (8, 9). HepG2 hepatoma cells expressing an L-FABP antisense RNA showed a dose-dependent reduction of fatty acid uptake (10). Furthermore, overexpression of L-FABP in McA-RH7777 hepatoma cells incubated with palmitic acid decreased the synthesis and secretion of triglycerides while increasing beta oxidation and the secretion of apolipoprotein B100 (11). Thus, the various in vitro systems have allowed researchers to propose specific functions of L-FABP in vivo.

However, in vitro studies of FABPs have inherent limitations. The only firmly established function of FABPs is the reversible binding of hydrophobic ligands, and these proteins do not exhibit any enzymatic function or energy requirement. This suggests that these proteins play passive (facilitative) roles that, almost by definition, are strongly dependent on the cellular context. One context of the highly expressed L-FABP is the highly differentiated hepatocyte, a cell type featuring an intense lipid metabolism that is not easily modeled in cell-free systems or transfected cells. Thus, an in vivo approach is probably needed to elucidate the physiologically relevant roles of this protein. Within this context, a deletional approach is likely to be more revealing than an overexpression approach, because L-FABP is extremely highly expressed even under basal conditions. It is these considerations that have brought us to believe that the in vitro assays need to be complemented by targeted deletion of the L-FABP gene in vivo to reveal its function(s) and mode(s) of action. We have therefore decided to create L-FABP null mice by homologous recombination in embryonic stem cells, and the present paper reports their initial analysis. We have focused this analysis on the impact of this mutation on lipid composition, lipid binding, and the expression of other known fatty acid-binding proteins.

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Lever DNA was purified by standard procedures (18) and used as a template for long PCR to amplify an 8-kb fragment from wild type and a 2.5-kb fragment from L-FABP null DNA (see Fig. 1A). Cycling conditions were 96 °C, 30 s; 52 °C (94 °C, 30 s; 63 °C, 30 s; 68 °C, 8 min). One primer (5′-cttcaacgctcagggattgag) corresponded to a sequence located immediately 3′ of the short arm homology region (i.e. outside of the recombination construct), the other primer (5′-ctctggactgagacttgcctc) was specific for the long homology arm. The long PCR products (Fig. 1B) were further verified by nested PCR for a 157-bp fragment of exon 2, using primers 5′-cgagcatctccagaag and 5′-tccagctgttcctgac at an annealing temperature of 60 °C (Fig. 1C). With the same exon 2 primers, absence of exon 2 was also directly confirmed by PCR with genomic DNA (not shown). After verification of the targeted gene deletion, another PCR assay was designed for routine single-tube genotyping of tail biopsies. Primers 5′-caaggctgctgatctgc and 5′-ccagctgttcctgac amplify 123 bp from exon 2 of the wild type allele, and 5′-agagcttgagcaagtg and 5′-ggctttgcgttgctgc amplify 227 bp from the neomycin resistance marker into the 3′ flank of both alleles. An annealing temperature of 68 °C was used.

**RT-PCR**—Total liver RNA was isolated with the TRIzol reagent from Invitrogen (Carlsbad, CA), and reverse transcription was performed with random hexamer primers and Moloney murine leukemia virus reverse transcriptase (Invitrogen) according to a standard protocol (18). Aliquots of the RT reaction were used for PCR, using L-FABP primers (5′-etctgacacaactttc and 5′-agcctgtaaacccttgcagtcg, amplifying 404 bp) and hypoxanthine phosphoribosyl transferase (HPRT) primers (5′-gtctggctggaagaccct and 5′-ggaatctggcagcttcgctgc, amplifying 584 bp) at an annealing temperature of 62 °C.

**Animals**—Chimeric mice were bred with C57Bl/6 mice, and the resulting heterozygous offspring were interbred to produce the L-FABP null (−/−) and wild type (+/+ ) littermate control mice used for this study. Mice used for the present study were female and 13–15 months of age, except for those used for the in vivo labeling experiment that were from the next generation and 5 months of age. Mice were fed a pelletTeklad Rodent Diet (W86004) obtained from Harlan Teklad Diets (Madison, WI). The animals were maintained in a temperature-controlled (25 °C) facility on a 12-h light/dark cycle and were allowed free access to food and water. The experimental protocols for the use of laboratory animals were approved by the University Lab Animal Care Committee.

**Animal Sacrifice and Tissue Collection**—For determination of serum parameters and liver fatty acid binding capacity and lipid distribution, female mice (13-15 months old), were fasted for 12 h, weighted, and anesthetized with Avertin. Blood was collected via cardiac puncture and immediately processed to serum. The animals were euthanized by cervical dislocation, and tissues of interest were removed, flash-frozen with dry ice, and stored at −80 °C for further analysis. The tissues were dissected and weighed. A small portion of liver was used immediately for histological analysis. The remainder of the liver was divided into small portions, flash-frozen with dry ice, and stored at −80 °C for further analysis.

**Serum Parameters**—Serum metabolites were determined with kits (triglycerides, Sigma #336; glucose, Sigma #315; nonesterified fatty acids, half micro kit from Roche Applied Science).

**Liver Tissue Homogenization and Fractionation**—All procedures were performed on ice or at 4 °C. 0.1 g of fresh, minced mouse liver was homogenized in 0.5 ml of phosphate-buffered saline (PBS, pH 7.4) containing protease inhibitor mixture (Sigma, St. Louis, MO) by 20 strokes in a Potter-Elvehjem homogenizer. After centrifugation at 105,000 × g for 90 min, the resulting supernatant was obtained immediately for further analysis. The supernatant was divided into two portions, one for protein determination (using a bicinchoninic acid protein assay (Bio-Rad, Richmond, CA) (19). SDS-PAGE—16.5% SDS-PAGE was performed using the system of Schagger and von Jagow (20), with minor modifications (21). Protein samples were reduced with 2-mercaptoethanol before loading. The gels were stained with 0.1% Coomassie Brilliant Blue R-250, and the stained proteins were quantified by densitometry, utilizing a single-chip charge-coupled device video camera FluorChemimgager and accompanying FluorChem image analysis software (version 2.0) from Alpha Innotech (San Leandro, CA).

**Western Analysis**—Proteins separated by SDS-PAGE were transferred to nitrocellulose membranes with a Miniprotein II transfer apparatus (Bio-Rad) at 40 V gel constant voltage and 4 °C for 2 h. After transfer, the nitrocellulose membranes were rinsed in 10 mM Tris (pH 8.0), 150 mM NaCl, 0.05% Tween 20 (TBST) and blocked by incubation.
in TBST plus 3% gelatin for 30 min at room temperature. The membranes were then washed 3 × 5 min with TBST and incubated with the primary antibody (1:1000 dilution in 10 ml Tris, pH 8.0, 150 mM NaCl (TBS), 1% gelatin) for several hours at room temperature with gentle shaking. Then the membranes were washed 2 × 5 min with TBST, 2 × 5 min with TBS, and incubated with alkaline phosphatase-conjugated secondary antibody (1:3000 dilution in TBS/1% gelatin) for 2 h at room temperature with gentle shaking. The membranes were then washed 2 × 5 min with TBST, 2 × 5 min with TBS, and 1 × 5 min with alkaline phosphatase buffer (100 mM Tris, pH 9.0, 100 mM NaCl, 5 mM MgCl₂).

Color development was initiated by the addition of alkaline phosphatase substrate (5-bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium, Sigma, St. Louis, MO) and stopped by washing the membranes with distilled water. Membrane photography and protein quantification were accomplished utilizing the imaging system described above. For the quantification of L-FABP, SCP-2, SCP-x, albumin, and glutathione S-transferase, standard curves were produced with the appropriate purified proteins that had been processed under identical conditions.

**Gel Permeation Chromatography of Liver**

The biologic activity (units) was assessed by gel permeation (5-bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium) coupled with a Model 2210 single-channel recorder (Amersham Biosciences, Piscataway, NJ). Absorbance (280 nm) was monitored using a Model 0.8% gel.

**Determination of Fatty Acid Binding Capacity and Binding Parameters** —The assay has been previously described (22). Briefly, in a final volume of 2 ml the incubation mixture contained an aliquot of Superdex 75 column fraction III (15 μg of protein/ml) plus 2 ml of 10 mM potassium phosphate, pH 7.4. The protein sample was titrated with small amounts of cis-parinaric acid using a 100 μL stock solution prepared in 10 mM NaOH. Upon addition of cis-parinaric acid, the mixture was allowed to equilibrate at 24 °C for 5 min prior to spectroscopic analysis. Fluorescence intensities were measured in a 1-cm quartz cuvette utilizing a PC1 photon-counting spectrofluorometer (ISS Instruments, Champaign, IL). For each cis-parinaric acid concentration, a control fluorescence intensity was measured in the absence of protein and subtracted. cis-Parinaric acid was added at 324 nm, whereas fluorescence emission was monitored at 410 nm. Excitation and emission monochromator bandwidths were 4 nm. To avoid the inner filter artifact, absorbance at the wavelength of excitation was maintained at ≤0.15 absorbance units. The dissociation constant, Kᵣ, and the binding stoichiometry, n, were calculated as described previously (22).

**Lipid Quantification** —All glassware was washed with sulfuric acid/chromate and rinsed several times with doubly distilled water prior to use. Lipid analysis was performed as previously described (9, 21). Each homogenate (5 mg of protein) and lipid standard sample (see below) was extracted two times with a total of 10 ml of hexane:2-propanol (5:1, v/v), i.e. 5 ml per extraction. The organic phases were collected after centrifugation (1500 rpm, 4 °C) and combined, then dried under N₂ resuspended in 100 μl of chloroform, and spotted onto silica gel G thin-layer chromatography plates. After running the plates with petroleum ether/diethyl ether/methanol/acetic acid (180:14:4:1, v/v), the separated lipids were visualized in an iodine chamber and scraped into acid-washed glass test tubes. Lipid content was determined by the method of Marzo et al. (23). To this end, each TLC scraping was extracted twice with 2 ml of chloroform/methanol/hydrochloric acid (100:50:0.375, v/v) per sample (i.e. 4 ml total). The TLC scropings were removed by centrifugation at 1000 rpm, 4 °C, 10 min. The two extracts from each sample were dried, vortexed with 2 ml of 2 M HClO₄, and centrifuged (1500 rpm, 4 °C, 10 min). The organic phase was dried under N₂ and the residue was resuspended in 1 ml of sulfuric acid and incubated at 200 °C for 15 min in screw-cap glass test tubes. After removing debris by centrifugation at 1000 rpm, 4 °C, 10 min, absorbance was measured at 375 nm using a Lambda 2 UV-visible spectrophotometer (PerkinElmer Life Sciences, Shelton, CT). The standard curve was generated using 5, 10, 20, 50, and 100 μl of 100 μCi/ml 14C fatty acid-free bovine serum albumin) was injected evenly over 1 min into the vena cava inferior. 10 min after start of injection, liver was taken and blood was drawn by cardiac puncture for measurement of fatty acid levels. Liver pieces (50–80 mg) were quickly rinsed with cold isotonic saline, blotted dry, weighed, and dissolved in hyamine for scintillation counting. Tissue radioactivities (per milligram of liver) were corrected for serum fatty acid levels to represent true tissue deposition. Depositions are given in arbitrary units versus 

**Results**

**Creation of L-FABP Null Mice** —The entire L-FABP gene was deleted by homologous recombination (Fig. 1A) in embryonic mice. L-FABP null mice were anesthetized with Avertin, the abdomen was opened, and 0.1 ml of [14C]oleate (5 μCi/ml in 0.9% NaCl/0.1% fatty acid-free bovine serum albumin) was injected evenly over 1 min into the vena cava inferior. 10 min after start of injection, liver was taken and blood was drawn by cardiac puncture for measurement of fatty acid levels. Liver pieces (50–80 mg) were quickly rinsed with cold isotonic saline, blotted dry, weighed, and dissolved in hyamine for scintillation counting. Tissue radioactivities (per milligram of liver) were corrected for serum fatty acid levels to represent true tissue deposition. Depositions are given in arbitrary units versus 

**Statistical Analysis** —Data are presented as the mean ± S.E. with n and p indicated under “Results.” Statistical analysis was performed using the unpaired Student’s t test (GraphPad Prism, San Diego, CA).
stem cells. The gene deletion was verified by long PCR (Fig. 1B), specificity of this reaction was confirmed by nested PCR (Fig. 1C, lanes 2–4), and specificity of the nested primers was in turn confirmed on genomic DNA (Fig. 1C, lanes 5–7). As expected from these results, RT-PCR failed to amplify L-FABP cDNA from total RNA of L-FABP (−/−) livers, whereas the control HPRT cDNA was readily amplified (Fig. 1D, lanes 2 and 3); in contrast, L-FABP cDNA was easily amplified from wild type RNA (Fig. 1D, lanes 4 and 5). To confirm the absence of L-FABP (14.2 kDa) on the protein level, Western blotting followed by quantitative densitometry was performed. The results (see Fig. 3A below) showed that L-FABP was present at 18.1 ± 3.0 ng/µg in homogenates and at 45.2 ± 3.8 ng/µg in 105,000 × g supernatants prepared from wild type livers. These data confirm earlier findings that L-FABP constitutes 2–3% of cytosolic proteins and is largely soluble (4, 5). In contrast, immunoreactivity was nondetectable in liver homogenates and 105,000 × g supernatants from the L-FABP (−/−) mice (Fig. 3A). This result demonstrates not only the absence of L-FABP but also of any potential close relatives that previously might have gone undetected. Thus, the L-FABP gene and its product are eliminated in the L-FABP (−/−) mice.

**General Characterization**—L-FABP null mice did not show any obvious abnormalities in appearance, behavior, sex ratio, or fertility. Under the standard chow, the body weight of L-FABP (−/−) littermates (37.4 ± 3.8 g) for the present study did not differ significantly from that of their wild type (+/+) littermates (37.4 ± 3.8 g). No significant alterations were noticed in the fasting serum levels of triglycerides (1.01 ± 0.17 mM (−/−) versus 0.92 ± 0.15 mM (+/+)) and free fatty acids (0.93 ± 0.11 mM (−/−) versus 0.81 ± 0.18 mM (+/+)), but a small reduction (p < 0.05) in glucose levels was seen (6.06 ± 0.58 mM (−/−) versus 9.07 ± 0.61 mM (+/+) ). Liver weights of FABP (−/−) mice were normal (1.59 ± 0.44 g (−/−) versus 1.52 ± 0.39 g (+/+)). Gross histology of L-FABP (−/−) livers was also essentially normal.

**Protein Distribution**—Because L-FABP is normally one of the most abundant proteins in liver cytosol, its absence might visibly alter the protein pattern. When equal amounts of protein were loaded onto SDS-polyacrylamide gels, a conspicuous decrease of band intensity was noticed in the 14-kDa region of both homogenate (Fig. 2A) and 105,000 × g supernatant (Fig.

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**Fig. 2. Altered protein pattern of L-FABP null (−/−) versus wild type (+/+) livers.** Homogenates (50 µg/lane; A and B) and 105,000 × g supernatants (10 µg/lane; C and D) from L-FABP (+/+) and L-FABP (−/−) livers were analyzed by SDS-PAGE. Each lane represents a different mouse. Gels were stained with Coomassie Blue (A and C), and the integrated density at 14.2 kDa was determined (B and D). a.u., arbitrary units; ***, p < 0.001 for −/− (n = 6) versus +/+ (n = 4).

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**Fig. 3. Levels of L-FABP and other intracellular fatty acid-binding proteins in L-FABP wild type (+/+) and null (−/−) livers.** Liver homogenates and 105,000 × g supernatants (50 µg/lane) were separated by SDS-PAGE and blotted onto nitrocellulose as described under “Experimental Procedures.” The membranes were incubated with rabbit polyclonal antiserum raised against L-FABP (A), albumin (B), glutathione S-transferase (C), SCP-2 (D), or SCP-x (E). Specific protein contents were determined from standard curves prepared with pure proteins in parallel. The data represent the mean ± S.E. (*, p < 0.05; **, p < 0.01; *** p < 0.001 for −/− versus +/+). N.D., not detectable.

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G. Martin and F. Schroeder, unpublished result.
liver (−/−) versus 19.4 ± 1.8 μg/g liver (+/+). This increase was even more pronounced (1.8-fold, p < 0.05) when expressed per microgram of homogenate protein (1.00 ± 0.10 ng/μg (−/−) versus 0.57 ± 0.19 ng/μg (+/+)) (Fig. 3D). Concomitantly, SCP-2 levels in 105,000 × g supernatants were also significantly (1.6-fold, p < 0.05) increased (1.01 ± 0.41 ng/μg supernatant protein (−/−) versus 1.88 ± 0.44 ng/μg (+/+) (Fig. 3D). Despite these genotypic differences, SCP-2 was enriched similarly (3-fold) in the 105,000 × g supernatant over homogenate (Fig. 3D).

In contrast to the increased levels of SCP-2, levels of its precursor protein, SCP-x, were dramatically reduced (p < 0.01) in liver of L-FABP (−/−) mice (5.8 ± 0.8 μg of SCP-x/μg of liver (−/−) versus 12.4 ± 1.1 μg/μg (+/+)). As in the case of SCP-2, the difference was more striking (4-fold, p < 0.001) when expressed per microgram of homogenate protein (0.08 ng of SCP-x/μg of homogenate protein (−/−) versus 0.33 ± 0.05 ng of SCP-x/μg of homogenate protein (+/+) (Fig. 3E). SCP-x was not detected in 105,000 × g supernatant (Fig. 3E).

Importantly, the total increase in SCP-2 levels in livers from L-FABP (−/−) mice appeared to match the decrease in SCP-x levels. Thus, total (i.e. SCP-2 plus SCP-x) values (33.0 ± 2.0 μg of protein/g of liver) were not significantly different from those in wild type mice (30.8 ± 1.6 μg of protein/g of liver) (Fig. 3F). However, because SCP-x was absent from the 105,000 × g supernatant fraction, the increase in SCP-2 levels in livers from L-FABP (−/−) mice resulted in an increase in total (SCP-2 plus SCP-x) in 105,000 × g supernatant from L-FABP (−/−) mice (Fig. 3F).

In summary, livers of L-FABP (−/−) mice showed an increase of SCP-2 levels and a matching decrease of SCP-x levels, whereas albumin and glutathione S-transferase were not significantly altered. However, the increment of SCP-2 levels by 1.13 ng of SCP-2/μg of supernatant protein was not nearly as large as the decrement of L-FABP levels (by nearly 50 ng/μg of supernatant protein).

**Potential Compensation by Plasma Membrane-associated Fatty Acid-binding Proteins**—A number of membrane-bound proteins are known to be involved in cellular lipid transport and metabolism. These include the caveolins, fatty acid transport protein (FATP), fatty acid translocase (FAT/CD) (36), and aspartate aminotransferase (glutamic-oxalacetic transaminase) (28–31). Western blotting was performed in homogenates of L-FABP (−/−) and wild type livers to determine whether L-FABP gene ablation might result in compensatory up-regulation of these proteins. No significant changes in the levels of caveolin, fatty acid transport protein, and aspartate aminotransferase were found, but a slight (20%, p < 0.05) increase in fatty acid translocase levels was noticed (Table I).

**Gel Permeation Chromatography**—The absence of major compensation by other fatty acid-binding proteins predicts a significantly reduced fatty acid binding of L-FABP null cytosol. To investigate this possibility in more detail, cytosols (105,000 × g supernatants) from wild type and L-FABP (−/−) livers were incubated with [3H]oleic acid and loaded onto a Superdex 75 gel filtration column. The eluted fractions were examined for radioactivity, total protein, and presence of L-FABP, SCP-2, and albumin. Radioactivity added to wild type cytosol resolved into four distinct regions designated as Fractions I-IV (Fig. 4A). Highest levels of total protein (Fig. 4A, closed circles) and albumin (Fig. 4C) were detected along with significant levels of [3H]oleic acid in Fractions I and II (Fig. 4A, open circles). Fraction III contained the least amount of total protein (Fig. 4A, closed circles) but the largest amount of [3H]oleic acid (Fig. 4A, open circles). Western blot analysis of Fraction III showed the presence of both L-FABP and SCP-2 (Fig. 4C). In the gel filtration medium, L-FABP migrated with an apparent molecular mass of 10 kDa; its hydrodynamic volume was smaller than the hydrodynamic volume of the 12.4-kDa column calibration protein, cytochrome c. Amino acid analysis and SDS-PAGE confirmed that this protein was intact L-FABP (data not shown). Thus, Fraction III from the gel permeation column comprised the soluble fatty acid-binding proteins that appear in liver cytosol (primarily L-FABP; less so SCP-2).

The gel permeation chromatographic profile of L-FABP (−/−) mouse liver 105,000 × g supernatant proteins (Fig. 4B) differed markedly from that of L-FABP (+/+ ) mice (Fig. 4A) in that [3H]oleic acid binding of Fraction III was reduced by >95%. Western blot analysis confirmed that L-FABP was absent throughout (Fig. 4C) and that SCP-2 was present in Fraction III (Fig. 4C).

**Relative Contribution of L-FABP to the Maximal Fatty Acid Binding Capacity of Fraction III-soluble Proteins**—Because it is not obtained under equilibrium conditions, the radioactivity profile of a gel filtration experiment may not correctly reflect binding capacity of the eluted fractions. To measure the binding capacity of column fraction III, a fluorescent ligand, saturation-binding assay was performed with cis-parinaric acid. This fatty acid shows low quantum yield and fluorescence intensity in aqueous environment (22) but high quantum yields upon binding to proteins such as L-FABP (22) or SCP-2 (25). Aliquots of fraction III (Fig. 4), equivalent to ∼0.2 μM L-FABP in the wild type fraction III, were therefore titrated with increasing amounts of cis-parinaric acid. Wild type Fraction III showed a biphasic binding curve (Fig. 5A), consistent with the known presence of two cis-parinaric acid ligand-binding sites in L-FABP (22, 32). Mathematical analysis revealed the presence of a high affinity (Kd1 = 130 ± 17 nM) and a low affinity (Kd2 = 691 ± 111 nM) binding site (Table II). When the assay was repeated with pure recombinant L-FABP, a similar biphasic titration curve (Fig. 5B) and similar binding constants were obtained (Table II).

In contrast, column Fraction III from L-FABP (−/−) mice exhibited a markedly (>80%) reduced cis-parinaric acid binding capacity and yielded a monophasic binding curve (Fig. 5A). Mathematical analysis of this curve revealed the presence of a single low affinity-binding site with a Kd of 2.78 ± 0.56 μM (Table II) and thus a considerably lower affinity for fatty acids than SCP-2 (Kd near 0.2 μM) (25). Thus, although SCP-2 was present in fraction III (see above), the residual fatty acid binding of this fraction seems to be largely caused by less specific proteins.

In summary, the above saturation binding experiments demonstrate for the first time that L-FABP constitutes the majority (80–95%) of fatty acid binding capacity of all low molecular weight cytosolic proteins. The small increase in SCP-2 noted in

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**Table I**

| Concentrations of known membrane transport proteins in L-FABP null (−/−) and wild type (+/+ ) mouse liver |
|---------------------------------------------------------------|
| Caveolin | FATP | FAT | AAT |
| (+/+ ) | 2.65 ± 0.44 | 1.49 ± 0.04 | 1.59 ± 0.06 | 6.76 ± 0.34 |
| (−/−) | 2.47 ± 0.30 | 1.33 ± 0.06* | 1.91 ± 0.12* | 6.54 ± 0.29 |

* p < 0.05 for −/− versus +/+. Expression levels (integrated density values) are given in arbitrary units (×10−6).
liver of L-FABP (−/−) mice (see preceding sections) did not compensate for the loss of L-FABP in terms of fatty acid binding capacity. On this basis, it was predicted that liver fatty acid and esterified lipid levels might be altered in L-FABP gene-ablated mice.

Effect of L-FABP Gene Ablation on Liver Nonesterified Fatty Acid Pool Size—Because of its abundance, high fatty acid binding affinity (22), and ability to enhance fatty acid uptake in transfected cells (7, 10, 33, 34), L-FABP might be expected to increase the nonesterified fatty acid pool size. However, as shown in Fig. 6D, there was no statistically significant difference in the amount of nonesterified fatty acid between wild type (24.6 ± 3.0 nmol/mg of protein) and L-FABP (−/−) (26.6 ± 2.5) livers.

Effect of L-FABP Gene Ablation on Fatty Acids Esterified to Glycerides: Phospholipids and Triglycerides—L-FABP is known to stimulate microsomal fatty acid esterification to glycerides (4, 35–37) in vitro and in transfected cells overexpressing L-FABP (5, 7, 9, 33). Therefore, deletion of the L-FABP gene might be expected to reduce the cellular levels of these compounds in vivo. To investigate this possibility, lipid was extracted from mouse liver homogenates, resolved into individual lipid classes, and quantified. Contrary to expectations, L-FABP (−/−) liver contained about 35% (p < 0.05) more phospholipids (63.8 ± 4.1 nmol/mg of protein) than wild type liver (47.5 ± 3.2 nmol/mg of protein) (Fig. 6F). In contrast, the amount of triglycerides was not significantly altered (Fig. 6E).

Effect of L-FABP Gene Ablation on Fatty Acids Esterified to Cholesterol—L-FABP has been shown to stimulate microsomal fatty acid esterification to cholesterol (38) in vitro and in transfected cells overexpressing L-FABP (8, 39). Therefore, deletion of the L-FABP gene might reduce the cellular levels of cholesterol and cholesterol esters. To test this possibility, lipid was extracted from mouse liver homogenates, resolved into individual lipid classes, and cholesterol and cholesteryl esters were quantified. Contrary to expectations, L-FABP (−/−) livers contained about 63% (p < 0.001) more cholesteryl esters (21.5 ± 1.2 nmol/mg of protein) than wild type livers (13.2 ± 0.2 nmol of cholesteryl esters/mg of protein) (Fig. 6C). The increase was even more marked (3-fold, p < 0.001) for nonesterified cholesterol (17.2 ± 1.5 nmol/mg of protein (−/−) versus 5.3 ± 0.8 nmol/mg of protein (+/+) ) (Fig. 6B).

Effect of L-FABP Gene Ablation on Overall Distribution of Liver Lipid Classes—The above data were used to calculate the change in total cholesterol contents and to describe the change of cholesterol contents in molar terms. Total cholesterol (cholesterol plus cholesteryl esters) increased 2-fold (p < 0.001) in livers of L-FABP (−/−) mice (38.7 ± 2.1 nmol/mg of protein) as compared with wild type mice (18.5 ± 0.8 nmol/mg of protein, Fig. 7A). The nonesterified cholesterol/cholesterol ester molar ratio increased 2-fold (p < 0.01) in the L-FABP (−/−) livers (0.87 ± 0.10 mol/mol) when compared with the wild type mice (0.40 ± 0.07 mol/mol) (Fig. 7B). The cholesterol ester/triglyceride molar ratio increased 66% (p < 0.05) in L-FABP (−/−) liver (0.48 ± 0.04 mol/mol) as compared with the wild type liver (0.29 ± 0.05 mol/mol) (Fig. 7C). Thus, absence of L-FABP favored esterification of fatty acids with cholesterol more than that of glyceride. Finally, L-FABP gene ablation caused a dramatic 2-fold increase (p < 0.05) of the cholesterol/phospholipid molar ratio (0.25 ± 0.04 mol/mol (−/−) versus 0.11 ± 0.01 mol/mol (+/+) ) (Fig. 7D). As a result, the quantitative distribution of lipid classes in mouse liver was changed from the order, phospholipids and triglycerides > nonesterified fatty acids > cholesteryl esters > cholesterol (wild type), to the order, phospholipid > triglyceride > nonesterified fatty acids > cholesteryl esters > cholesterol (knockout).
FIG. 5. Dramatically reduced fatty acid binding capacity in column fraction III from L-FABP null (−/−) liver (see Fig. 4 for position of Fraction III). A, 1-μg aliquots of 105,000 × g supernatants from L-FABP (−/−) (filled circles) and L-FABP (+/+)(empty circles) were fractionated by Superdex 75 gel filtration chromatography as in Fig. 4, but in the absence of added oleic acid. A sample (15 μg of protein/ml) from fraction III was examined for cis-parinaric acid binding ability as described under “Experimental Procedures.” The fluorescence intensity data were used to determine the dissociation constant, \( K_d \), and the binding stoichiometry, \( n \) (inset). These data represent the mean ± S.E. \( (n = 4 \text{ for } +/+; n = 6 \text{ for } −/−) \). B, purified, recombinant L-FABP (0.2 μM) was examined for cis-parinaric acid binding ability using the same fluorescent ligand-binding assay as under A. The fluorescence intensity data were analyzed to determine \( K_d \) and \( n \) (inset). The data represent the mean ± S.E. of four independent measurements.

TABLE II

| Parameter | Peak III | Recombinant L-FABP |
|-----------|----------|---------------------|
| \( K_{d1} \) (μM) | 0.130 ± 0.017 | ND* | 0.068 ± 0.008 |
| \( K_{d2} \) (μM) | 0.691 ± 0.111 | ND | 0.386 ± 0.067 |
| \( K_{dNS} \) (μM) | 2.78 ± 0.56 | ND | 0.008 ± 0.008 |
| \( n_1 \) (mole/mole) | 1.69 ± 0.28 | 0.59 ± 0.15 | 1.48 ± 0.21 |
| \( n_2 \) (mole/mole) | 0.65 ± 0.09 | ND | 0.58 ± 0.13 |

* ND, not detectable.

Effect of L-FABP Gene Ablation on Deposition of Blood-borne Long-chain Fatty Acids—Finally, we asked whether L-FABP influences the hepatic deposition of long-chain fatty acids or their metabolites. A bolus of [14C]oleic acid was injected intravenously, and tissue radioactivity was measured 10 min later. This was first done with mice that had been fasted overnight. Under this predominantly oxidative condition, the tissue radioactivity level was low in both wild type and L-FABP null liver, likely because [14C]CO₂ (the main radioactive product of oxidation) is not retained. Nevertheless, a small \( (p < 0.05) \) reduction was seen in L-FABP null versus wild type liver (Fig. 8).

However, the effect of the mutation was much more visible when this method was applied to fed mice. Under this predominantly lipogenic condition, retention of blood-borne fatty acid by both wild type and L-FABP null livers was high compared with fasting, but livers of L-FABP null mice showed a reduction of almost 50% compared with wild type mice (Fig. 8).

DISCUSSION

Although L-FABP is a well-studied protein, its physiological roles remain unclear. The ligand-binding properties and x-ray structure (32, 40), isoforms (35), and nutritional and pharmacological regulation (2) of L-FABP are known in great detail. In addition, cell-free systems and transfection studies with L-FABP cDNA have provided hints as to its functions (2, 7, 9, 10, 14, 33, 39, 41–43). However, no in vivo function can be considered as established, and we believe that a satisfactory understanding of this protein requires an analysis of its role in the native cellular context. The results presented herein are the first step to such an analysis, in which we have focused on the measurement of relevant liver components to describe the system and provide a framework for future functional studies.

The results of the present report add important new findings on the role of L-FABP in vivo and raise new interesting questions. First, the data presented here provided, for the first time, a quantitative assessment of the contribution of a fatty acid-binding protein to cytosolic fatty acid binding capacity in vivo. This assessment was facilitated by the fortuitous circumstance that no quantitatively significant compensation by other proteins occurred. Second, lack of L-FABP exerted an important influence on hepatic lipid composition. Some of these changes were quite unexpected, and, as discussed below, it remains to be elucidated whether they are direct or indirect consequences of L-FABP deficiency. Third, although the fatty acid-binding capacity of L-FABP null liver cytosol was reduced to very low levels, the specific increase of another lipid-binding protein, SCP-2, was observed, and, as discussed below, this may be relevant for the observed changes in lipid composition. Fourth, lack of L-FABP altered the turnover of the hepatic lipid pool.
The present data formally establish that L-FABP constitutes at least 80% of fatty acid-binding capacity of all low molecular weight proteins of cytosol (Fraction III of gel permeation chromatography). This finding automatically excludes the possibility of any significant compensatory expression of other members of the FABP gene family. With respect to larger molecular weight regions, changes in fatty acid binding capacity due to deletion of the L-FABP gene cannot be formally excluded yet; however, we found that the major known fatty acid-binding proteins present in this region, glutathione S-transferase and serum albumin, were not changed. Moreover, little or no change was found in the levels of several plasma membrane fatty acid transporters.

The absence of L-FABP may conceivably alter cellular lipid composition in several ways. First, absence of L-FABP might change lipid composition simply because of the absence of a quantitatively important lipid-binding site. Second, because lipid binding to L-FABP is reversible and thus contributes to cellular lipid fluxes, absence of L-FABP can be expected to alter the rates of both lipid synthesis and breakdown and establish a new steady state. Finally, secondary changes of other protein levels may affect the lipid profile. Thus, the absence of L-FABP may affect cellular lipid composition in complex ways that potentially vary under different physiological conditions. Here we have analyzed the main liver lipid classes of L-FABP null liver of fasted mice.

On one hand, we found that the hepatocytes maintained normal levels of total lipid, nonesterified fatty acid, and triglyceride. We note, however, that turnover of at least one of these pools must be changed, because the hepatic deposition of a pulse of blood-borne fatty acid was much lower in L-FABP null versus wild type mice under lipogenic conditions. Although the one or more responsible pathways remain to be identified, reduced influx or esterification rates of fatty acids are more likely explanations than increased secretion of newly esterified triglyceride because of the short time frame (10 min) of the experiment. Clearly, the impacts of the L-FABP gene deletion on the rates of fatty acid influx, esterification, hydrolysis, and oxidation deserve more detailed investigation.

On the other hand, a moderate increase of the phospholipid level in L-FABP null liver was seen, but this change was opposite to what was expected based on the increased phospholipid levels previously found in transfected cells overexpressing L-FABP (8, 9). The most striking finding of the present investigation was a dramatic increase of hepatic cellular cholesterol levels in L-FABP null mice. Cholesterol esters were also increased, although somewhat less. Again, the opposite effect of L-FABP gene deletion was predicted, because overexpression of L-FABP in cell culture has previously been shown to increase the cholesterol pool as well as the cholesterol ester level (8, 9). This difference in cholesterol ester levels is more likely related to the change in cholesterol, rather than to alterations of free fatty acids, given that the free fatty acid pool was not changed.

The one or more mechanisms causing these conspicuous alterations, especially of cholesterol homeostasis, remain to be elucidated. The first possibility to be considered is that the absence of L-FABP is directly responsible. Fluorescence and light scattering direct binding assays indicate that L-FABP binds cholesterol and a fluorescent sterol (dehydroergosterol) with low affinity (i.e. submicromolar \( K_d \) values) (38, 44, 45). Although L-FABP poorly competes for binding, extracting, or transferring sterol from model membranes (46–48), it nevertheless enhances transfer of sterol in vitro from purified plasma membrane vesicles, albeit not as strongly as sterol carrier protein-2 (46). Thus, L-FABP might affect cholesterol levels directly, although its potential cholesterol binding ability would not yet explain the direction (increase rather than decrease) of change observed in L-FABP null liver. We note that increased L-FABP gene expression seen in SCP-2/SCP-x knockout livers does not cause reduced unesterified cholesterol levels (49). Furthermore, although cholesterol ester levels are reduced in SCP null liver (in line with the increase seen in L-FABP null liver) (49), they are increased in L cells transfected with L-FABP (8), in contradiction to the increase we see in L-FABP null liver. Thus, absence of L-FABP \( \text{per se} \) does not provide a straightforward explanation for the increased cholesterol/cholesterol ester levels in L-FABP null liver. A second mechanism for the increased cholesterol in L-FABP null liver might be provided by our observation made here that the level of SCP-2 is also increased. This increase in SCP-2 was clearly not sufficient to prevent the dramatic reduction in total fatty acid binding capacity of L-FABP-deficient cytosol. However, it might have been sufficient to cause the observed alterations in cholesterol homeostasis. The ability of SCP-2 to bind cholesterol is well established (52). Overexpression of SCP-2 in L cells inhibited cholesterol efflux and increased the intracellular cholesterol pool (53), and adenoviral overexpression of SCP-2 was previously found to increase hepatic cholesterol contents (54). Conversely, liver cholesterol ester levels are reduced by 50% in SCP-2/SCP-x null mice (49) along with hypersecretion of cholesterol in bile (17, 50, 51). The findings in the latter two
models are especially relevant, because they were obtained in vivo, although at least the second model (SCP null mice) is complicated by the concomitant alteration of L-FABP gene expression (49) mentioned above. A third formal explanation for the increased cholesterol contents in the L-FABP null liver is the decrease in the levels of SCP-x that we have found. However, this seems to be not likely, because the cholesterol content of SCP-x/SCP-2 null liver was not increased (49). Moreover, overexpression of full-length SCP-x in L cells led not only to higher (rather than lower) cholesterol contents but also to increased levels of 13-kDa SCP-2 (13). In summary, although the mechanism underlying the increased cholesterol content in L-FABP null liver remains to be resolved, the available data are more in support of a direct role of the increased content of SCP-2 as opposed to the absence of L-FABP or the reduction of SCP-X. In any case, the foregoing discussion suggests that an important strategy to conclusively resolve the relative contributions of these three proteins to hepatocellular cholesterol levels and the other shifts in lipid distribution will be the creation and analysis of L-FABP/SCP double-knockout mice.

Apart from their potential contributions to cholesterol metabolism, the reciprocal alterations in the levels of SCP-2 and SCP-x in the L-FABP null liver raise the interesting question as to what causes them. SCP-2 is encoded by the same gene as SCP-X. In any case, the foregoing discussion suggests that an important strategy to conclusively resolve the relative contributions of these three proteins to hepatocellular cholesterol levels and the other shifts in lipid distribution will be the creation and analysis of L-FABP/SCP double-knockout mice. Apart from their potential contributions to cholesterol metabolism, the reciprocal alterations in the levels of SCP-2 and SCP-x in the L-FABP null liver raise the interesting question as to what causes them. SCP-2 is encoded by the same gene as SCP-X. In any case, the foregoing discussion suggests that an important strategy to conclusively resolve the relative contributions of these three proteins to hepatocellular cholesterol levels and the other shifts in lipid distribution will be the creation and analysis of L-FABP/SCP double-knockout mice.

Further experiments are needed to understand the mechanism and the functional consequences of the dramatic change in hepatocellular lipid distribution in the L-FABP null mice.

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Decreased Liver Fatty Acid Binding Capacity and Altered Liver Lipid Distribution in Mice Lacking the Liver Fatty Acid-binding Protein Gene

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