Liver Fatty Acid-binding Protein Gene Ablation Inhibits Branched-chain Fatty Acid Metabolism in Cultured Primary Hepatocytes

Barbara P. Atshaves‡, Avery M. McIntosh‡, Olga I. Lyuksyutova§, Warren Zipfel‡, Watt W. Webb§, and Friedhelm Schroeder‡¶

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The role of liver fatty acid-binding protein (L-FABP) in the uptake, transport, mitochondrial oxidation, and esterification of normal straight-chain fatty acids has been studied extensively, almost nothing is known regarding the function of L-FABP in peroxisomal oxidation and metabolism of branched-chain fatty acids. Therefore, phytanic acid (most common dietary branched-chain fatty acid) was chosen to address these issues in cultured primary hepatocytes isolated from livers of L-FABP gene-ablated (−/−) and wild type (+/+ ) mice. These studies provided three new insights: First, L-FABP gene ablation reduced maximal, but not initial, uptake of phytanic acid 3.2-fold. Initial uptake of phytanic acid uptake was unaltered apparently due to concomitant 5.3-, 1.6-, and 1.4-fold up-regulation of plasma membrane fatty acid transporter/translocase proteins (glutamic-oxaloacetic transaminase, fatty acid transport protein, and fatty acid translocase, respectively). Second, L-FABP gene ablation inhibited phytanic acid peroxisomal oxidation and microsomal esterification. These effects were consistent with reduced cytoplasmic fatty acid transport as evidenced by multiphoton fluorescence photobleaching recovery, where L-FABP gene ablation reduced the cytoplasmic, but not membrane, diffusional component of NBD-stearic acid movement 2-fold. Third, lipid analysis of the L-FABP gene-ablated hepatocytes revealed an altered fatty acid phenotype. Free fatty acid and triglyceride levels were decreased 1.9- and 1.6-fold, respectively. In summary, results with cultured primary hepatocytes isolated from L-FABP (+/+ ) and L-FABP (−/−) mice demonstrated for the first time a physiological role of L-FABP in the uptake and metabolism of branched-chain fatty acids.

The role of liver fatty acid-binding protein (L-FABP) in the uptake, transport, intracellular transport, and esterification of normal straight-chain fatty acids (LCFA) has been examined extensively in vitro (reviewed in Refs. 1–3), in transfected cells (reviewed in Ref. 1), and cultured hepatocytes (4, 5). Whereas recent data with L-FABP gene-ablated female mice show that loss of L-FABP greatly reduces the binding capacity of liver cytosol for straight-chain fatty acids and fatty acyl-CoA's (6, 7), specific effects of L-FABP gene ablation on liver uptake of straight-chain fatty acid and liver lipid distribution are complex, apparently depending on feeding status, gender, and age of the mice (6–9).

Although relatively little is known about the role of L-FABP in the oxidation of straight-chain fatty acids, which occurs primarily in mitochondria (reviewed in Refs. 1–3), recent studies with L-FABP gene-ablated mice suggest that L-FABP may affect liver oxidation of straight-chain fatty acids under high fatty acid load. Under fed conditions, serum free fatty acid levels were found to be low, and β-hydroxybutyrate levels were unaltered in L-FABP (−/−) male or female mice, suggesting that L-FABP may not play a role in fatty acid oxidation (8, 9). However, under starvation conditions, serum free fatty acid levels were highly elevated, and serum β-hydroxybutyrate levels were reduced. These findings led to the conclusion that under fasting conditions L-FABP gene ablation reduces fatty acid oxidation (8, 9). However, other in vitro studies measuring fatty acid oxidation and β-hydroxybutyrate production in liver homogenates showed that L-FABP gene ablation had no effect on oxidation of straight-chain, radiolabeled palmitic acid at high levels (1 mM) (9). In contrast, when fatty acid oxidation and β-hydroxybutyrate production were measured with hepatocyte suspensions freshly isolated from female mice, L-FABP gene ablation reduced oxidation of high levels (1 mM) of straight-chain palmitic acid by about 30% (9). Whereas the above results appear contradictory, the intact hepatocyte and in vivo data suggest that L-FABP gene ablation does not affect oxidation of straight-chain fatty acids under normal fed conditions when serum fatty acid levels are low but may do so when serum fatty acids are high as in starvation.

In contrast to the above studies with straight-chain fatty acids, almost nothing is known regarding potential roles of L-FABP in the uptake, oxidation, and esterification of branched-chain fatty acids. The most common dietary branched-chain fatty acid, phytanic acid, is produced by ruminants in the gut by bacterial cleavage of the side chain of chlorophyll to yield phytol, followed by conversion to phytanic acid (reviewed in Ref. 10). Consequently, levels of phytanic acid in dairy products (butter, margarine, and cheese) are high, up azol; PL, phospholipid; Tricine, N-(2-hydroxy-1,1-bis(hydroxymethyl)-ethyl)glycine.
to 500 mg of phytanic acid/100 g of wet weight (11), with average human daily consumption — 50–100 mg/day (11, 12). Although this level of phytic acid is readily metabolized in normal humans, patients with peroxisomal disorders such as Refsum’s disease and other genetic mutations involving peroxisomes (the site for branched-chain fatty acid oxidation) are compromised in the ability to metabolize phytol (11, 12). Since accumulation of excess branched-chain fatty acids is toxic, especially in individuals with peroxisomal disorders, it is essential that branched-chain fatty acids (phytanic acid) be transported to peroxisomes for oxidation therein. However, almost nothing is known regarding extraperoxisomal factors that may influence phytic acid oxidation.

Several correlative studies suggest a role for L-FABP in transporting branched-chain fatty acids to the peroxisome and, thus, influencing peroxisomal oxidation of branched-chain fatty acids. (i) L-FABP is the predominant fatty acid binding and transport protein in liver cytosol (reviewed in Refs. 1, 4, and 5). Whereas L-FABP is not found within peroxisomes and only small amounts are associated with mitochondria, L-FABP levels within liver hepatocyte cytosol are high, 100–300 μM (reviewed in Refs. 1, 6, and 7). Because of this high localization and concentration of L-FABP in cytosol, this protein can function to stimulate fatty acid transport to peroxisomes or mitochondria for fatty acyl-CoA synthetase-mediated conversion to fatty acyl-CoAs, stimulate translocation into peroxisomes and mitochondria by distinct pathways and subsequent oxidation within these organelles, and/or direct transport of fatty acyl-CoAs from other sites to peroxisomes and mitochondria for subsequent oxidation (13). (ii) L-FABP has two fatty acid binding sites. The high affinity site binds the branched-chain phytanic acid with Kd values ranging from 15 to 30 nM (14, 15), similar to those exhibited for normal straight-chain fatty acids (e.g. palmitic acid, oleic acid, etc.; Kd values of 8–60 nM) (16–18). (iii) L-FABP expression is up-regulated by peroxisomal proliferators, including phytanic acid (19, 20). L-FABP has also been suggested to selectively cooperate with peroxisome proliferator-activated receptor α to enhance peroxisomal fatty acid oxidation (21). Although these and other correlative data (22, 23) suggest a role for L-FABP in peroxisomal oxidation of branched-chain fatty acid, this possibility remains to be proven.

The objective of the present investigation was to directly examine the effect of L-FABP gene ablation on uptake, oxidation, and esterification of a branched-chain fatty acid (phytanic acid). The data presented herein with cultured primary hepatocytes isolated from male L-FABP (−/−) mice yielded fundamental new insights indicating for the first time that L-FABP gene-ablation (i) reduces maximal but not initial uptake of phytanic acid; (ii) Reduces cytoplasmic fatty acid transport, independent of the contribution of membrane fatty acid diffusion; (iii) inhibits peroxisomal oxidation of phytic acid; and (iv) reduces phytic acid esterification.

EXPERIMENTAL PROCEDURES

Materials—(2,3-3H)Phytanic acid (50 Ci/nmol) was prepared by Moravek Biochemicals, Inc. (Brea, CA). [9,10-3H]Palmitic acid (36 Ci/mmol) was from PerkinElmer Life Sciences. Silica Gel G plates were from Analtech (Newark, DE); Silica Gel 60 TLC plates were from VWR Scientific Inc. (West Chester, PA). Lipid standards were from Nu-Chek Prep, Inc. (Elysian, MN). Phospholipid standards were from Avanti (Alabaster, AL). Lab-Tek chambered cover glass slides were from Nunc (Minneapolis, MN). Goats anti-human apolipoprotein B was purchased from Research Diagnostics (Flanders, NJ). Rabbit polyclonal anti-caveolin-1 was purchased from BD Transduction Laboratories (Lexington, KY). Rabbit polyclonal anti-fatty acid transport protein (FATP) was a generous gift from Dr. J. Schaffer (Department of Internal Medicine, Washington University School of Medicine, St. Louis, MO). Goat anti-human apolipoprotein B was purchased from Biodiagnostic International (Saco, ME). Goat anti-human albumin was purchased from Miles-Yeda (Rehovot, Israel). All reagents and solvents used were of the highest grade available and were cell culture-tested.

Animals—L-FABP null mice (L-FABP+/+) were generated by targeted disruption of the L-FABP gene through homologous recombination (6, 7). Wild type littermates with no disruption (L-FABP+/+) were designated as controls. Mice were kept under constant light-dark cycles and had access to food and water ad libitum. Protocols defining animal care were approved by the Institutional Animal Care and Use Committee at Illinois Institute of Technology. Experiments were conducted on male mice ranging in age from 2 to 4 months (25–35 g) or with hepatocytes derived from male mice of the same age and weight range (i.e. 2–4 months and 25–35 g). One week prior to the start of experiments, mice were switched to a modified AIN-76A rodent diet (5% of calories from fat, number D11243, Research Diets, Inc., New Brunswick, NJ), chosen because it is essentially phytoestrogen-free (25, 26). Genotype was verified on all animals before hepatocyte preparation.

Hepatocyte Isolation—Hepatocytes from L-FABP+/+ and L-FABP−/− mice were isolated as described earlier (27). Briefly, mice were euthanized by CO2 asphyxiation, and the livers were removed and perfused first with Buffer A (10 mM Hepes, pH 7.4, in calcium/magnesium-free Hanks’ balanced salt solution (HBSS), gentamycin sulfate (100 μg/ml), and 0.5 mM EGTA) for 10 min (3 ml/min), followed by Buffer B (Buffer A without EGTA, supplemented with 5 mM CaCl2, and 0.2 mg/ml collagenase B) for an additional 10 min. Hepatocytes were released into the collagenase solution by removing the liver capsule and gently shaking. Released hepatocytes were washed three times with ice-cold Dulbecco’s modified Eagle’s medium containing 5% fetal bovine serum and pelleted at 50 × g. Resuspended hepatocytes were plated on collagen-coated dishes (5 × 10⁶ cells/5 mm dish). After 24 h, hepatocytes were transferred into serum-free medium (28), and the medium was changed every 48 h.

Hepatocyte Viability and Function—The following assays were used to monitor hepatocyte viability and function. First, hepatocyte viability was monitored qualitatively using trypan blue (Sigma). Regardless of whether hepatocytes were obtained from livers of L-FABP+/+ or L-FABP−/− mice, >90% of hepatocytes excluded trypan blue. Second, hepatocyte viability was also monitored quantitatively with a LIVE/DEAD® viability/cytotoxicity kit used two dyes: (i) membrane-permeant calcine AM, which, after cleavage by intracellular esterases, yielded a green cytoplasmic fluorescence (with a blank nucleus) in live cells and (ii) ethidium homodimer-1, a membrane-impermeant fluorophore that penetrated membrane-compromised (dead) cells to label nucleic acids in the nucleus. Hepatocytes were incubated with the fluorophores for 30 min, washed, and imaged on a MRC-1024MP laser-scanning confocal microscope (Bio-Rad) equipped with an Axiovert 135 microscope and > 63 Plan-Fluor oil immersion objective, numerical aperture 1.45 (Zeiss). Both dyes were excited simultaneously through a 1% neutral density filter by the 488-nm laser line of a krypton-argon laser (Bio-Rad). Fluorescence emission was detected through two separate filters: calcine (green fluorescence through a 525–565-nm band pass filter) and ethidium homodimer-1 (red fluorescence through a 585–630-nm band pass filter). In order to limit artifacts associated with potential toxicity and/or saturation of live/dead dyes in the assay, dye concentrations were individually titrated over a broad dye range to obtain an optimal dye dual staining: 0.1 μM calcine AM, 0.8 μM ethidium homodimer-1. Examination of multiple chambers of cultured hepatocytes with this dual stain assay revealed that cell viability was typically also >90%. Third, hepatocyte morphology was monitored as previously described (29). Briefly, primary hepatocytes were cultured in serum-free medium supplemented with 4 μM and seeded at 4 μg/ml. Cells were cultured on collagen-chambered slides (3 × 10⁶ cells/slide) and, after incubation with both dyes, imaged on an MRC-1024 MP laser-scanning confocal microscope (Bio-Rad) and MetaMorph Image Analysis (Advanced Scientific Imaging, Meraux, LA) software. Morphologic examination of transmission micrographs of cultured primary hepatocytes isolated from L-FABP+/+ and L-FABP−/− mice indicated the presence of multinucleated cells...
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with canalicular structures characteristic of liver cells. Fourth, function of cultured primary hepatocytes was monitored by ability to synthesize L-FABP in wild type L-FABP+/+ mice. Hepatocytes from L-FABP+/+ mice were cultured on several 35-mm dishes to check for L-FABP expression by Western blot analysis during all a 5-day period (24). Levels of L-FABP in cultured hepatocytes from L-FABP+/+ mice were constant up to 3 days in culture but significantly decreased 5 days postisolation. Based on these results where several parameters of primary hepatocyte culture viability and function were examined, all experiments performed herein were with hepatocytes maintained in culture 2–3 days.

Western Blotting to Determine Hepatocyte Levels of Plasma Membrane Fatty Acid Transport and Intracellular Fatty Acid Transport Proteins—Cell homogenates of hepatocytes isolated from L-FABP−/− and L-FABP+/+ mice were analyzed by Western blot analysis to determine whether L-FABP gene ablation altered the levels of two other classes of proteins involved in fatty acid uptake: plasma membrane fatty acid transport/translocase proteins (FATP, CD-36/FAT, and caveolin-1) and intracellular fatty acid transport proteins (L-FABP, SCP-2, SCP-x, ACBP, caveolin-1, and GOT). Hepatocyte homogenates (0.2–5 µg) were run on Tricine gels (12%) before transferring to 0.45-µm nitrocellulose paper (Sigma) by electroblotting in a continuous buffer system at 0.8 mA/cm² for 1.5 h. After transfer, blots were treated as described previously (31) using affinity-purified antisera against (i) monocarboxylic fatty acid transport proteins (FATP, CD-36/FAT, and caveolin-1) and (ii) intracellular fatty acid and fatty acyl-CoA transport proteins that play a role in fatty acid metabolism (L-FABP, SCP-2, SCP-x, ACBP, caveolin-1, and GOT). Proteins were quantified by densitometric analysis after image acquisition using a single chip CCD (charge-coupled device) video camera and a computer work station (IS-500 system from Alpha Innotech, San Leandro, CA). Image files were analyzed (mean 8-bit gray scale density) using NIH Image (available by anonymous FTP).

Lipid Mass Determination—All glassware was washed with sulfuric acid-chromatric before use. In order to determine the effect of L-FABP gene ablation on lipid content, lipids were extracted from liver or hepatocytes from the livers of L-FABP−/− mice with n-hexane-2-propanol 3:2 (v/v) (32) and immediately stored under an atmosphere of N₂ to limit oxidation (33). Protein content was determined by the method of Bradford (30) from the diluted protein extract residue digested overnight in 0.2 M KOH. Individual lipid phases (phosholipids and neutral lipids such as cholesterol, free fatty acid, monoacylglyceride, diacylglyceride, triacylglyceride, and cholesteryl esters) were resolved using silica gel TLC plates developed in acetic acid (90:7:3:2:0.5) (32). Lipid classes were identified by comparison for nonradiolabeled lipids above. Mass (nmol/mg of cell protein) of individual lipid classes was determined as described (32, 35, 36). Protein extraction residue digested overnight in 0.2 M KOH. Individual lipid phases (phosholipids and neutral lipids such as cholesterol, free fatty acid, monoacylglyceride, diacylglyceride, triacylglyceride, and cholesteryl esters) were resolved using silica gel G TLC plates developed in the following solvent system: petroleum ether/diethyl ether/methanol/ acetic acid (90:7:2:0.5) (32). Lipid classes were identified by comparison with known standards. Spots on the TLC plate were visualized by iodine, scraped, and quantitated by the method of Marzo et al. (34). Mass (nmol/mg of cell protein) of individual lipid classes was determined as described earlier (32, 35, 36).

Uptake of [2,3-³H]Phytanic Acid and [9,10-³H]Palmitic Acid by Cultured Hepatocytes Isolated from L-FABP−/− and L-FABP+/+—The uptake of radiolabeled branched-chain fatty acids including phytanic acid and straight-chain fatty acids such as palmitic acid was determined as described earlier (31, 32). Hepatocytes were cultured in serum-free medium supplemented with BSA (4 µg) and then incubated with phytanic acid or palmitic acid (50 nM) containing trace levels of [2,3-³H]phytanic acid or [9,10-³H]palmitic acid (1.5 µCi/nmol). At intervals from 1 to 24 h, medium was removed and saved, cells were harvested, and pulse widths near the anterior and posterior probe were combined with the saved medium. In order to equate the amounts of phytanic acid using in the present study with amounts consumed daily by humans, the following estimations were made. In humans, daily dietary intake of phytol and its metabolite phytanic acid is on the order of 50–100 mg/day (12). In terms of mg of phytol/g of body weight, the approximate value for human consumption would be 1.4 µg of phytol/g of body weight/day for a 70-kg man. The amount of phytanic acid used in the current study was on the order of 0.05 µg/35-mm dish, or 50-fold less than that consumed daily with a diet supplemented with dairy products. Whereas these values are well below levels exhibited by phytol toxicity and are not normally a problem with healthy individuals, toxicity becomes an issue when peroxisomal disorders (such as Refsum’s disease) result in an inability to metabolize phytol (12, 39).

Oxidation of [2,3-³H]Phytanic Acid and [9,10-³H]Palmitic Acid in Cultured Hepatocytes Isolated from L-FABP−/− and L-FABP+/+—The oxidation of radiolabeled branched-chain fatty acids (phytanic acid) and straight-chain fatty acids (palmitic acid) was determined as described earlier (31, 37, 38). Oxidation was measured as the release of water-soluble triitated fatty acid oxidation products into the culture medium after removing the lipids according to the Folch method (40) as described earlier (37, 38, 41).

Extension of [2,3-³H]Phytanic Acid and [9,10-³H]Palmitic Acid into the Intracellular Fatty Acid Transport/Diffusion by Multiphoton Fluorescence Photobleaching Recovery (MPFPR) of NBD-Stearic Acid—The effective diffusion coefficient (Dₑ) of NBD-stearic acid is an established method for determining intracellular fatty acid transport/diffusion (1, 42–45). To determine whether L-FABP gene ablation altered the intracellular transport/diffusion of fatty acid, the effective diffusion coefficient of NBD-stearic acid was determined in cultured hepatocytes by MPFPR. In contrast to single photon excitation (used in conventional and confocal fluorescence microscopy), multiphoton excitation permits 100-fold deeper optical sectioning as well as markedly reduced photobleaching and cell toxicity. Furthermore, MPFPR microscopy allows measurement of diffusion coefficients for a protein-containing only a very small portion of the confocal volume (10–20%) rather than bleaching the entire thickness of the cell (70–20 µm). These features of MPFPR give a more accurate determination of intracellular diffusion coefficients than data obtained using single photon FPR techniques.

In brief, hepatocytes were cultured at 37 °C and 5% CO₂ on chambered glass slides in serum-free medium supplemented with BSA (4 µg) and then incubated with NBD-stearic acid (0.5–4 µg) for 30 min to establish equilibrium distribution. Hepatocytes were washed once with phosphate-buffered saline and then imaged by multiphoton laser-scanning microscopy. Intracellular diffusion was measured using a 80 MHz mode locked Ti:Sapphire laser (Spectra-Physics, Mountain View, CA) and used to generate a 900 MHz probe pulse width near the anterior and posterior probe. The bleaching pulse and the subsequent probe excitation was accomplished through the use of a KDP® Pockels cell (Conoptics, Danbury, CT), pulse generator, and synchronization electronics. Scanning, beam parking, and image acquisition were achieved with a Bio-Rad MRC-600 confocal.
laser scanning microscopy system utilizing a GaAsP photomultiplier (Hamamatsu Corp., Bridgewater, NJ), both mounted on an upright microscope equipped with a ×60 (0.9 numerical aperture) water immersion objective (Olympus, Melville, NY). An intracellular region of the cell was selected from the scanned image with subsequent positioning of the laser beam by the x-y scanner. The region formed by the focal volume of the excitation beam was photobleached for either 500 or 800 μs. The power and length of the bleach pulse was chosen in order to obtain ~15–30% bleach depth. The laser intensity was attenuated, and the fluorescence recovery was monitored for 340 ms. Bleach and recovery data were collected and averaged by a SR430 Multichannel Scaler/Averager (Stanford Research Systems, Sunnyvale, CA). The recovery data were fitted to a multimode component form of the equation that Brown et al. (46) derived for describing the fluorescence intensity over time,

\[
F(t) = \sum_{n=0}^{\infty} \frac{(-\beta)^n}{n!} \left( 1 + \frac{b}{m} + \frac{4bDt}{m\omega_0^2} \right)^n \left[ \sqrt{1 + \frac{n}{m} \frac{4bDt}{m\omega_0^2}} \right]^{-1}
\]

(Eq. 1)

where the following is true,

\[
\omega_0 = 0.325 \sqrt{2}\lambda \sqrt{mN\lambda^{(2\beta)}}^{-1}
\]

(Eq. 2)

for numerical aperture of >0.7 as well as the following.

\[
\omega_n = 0.266 \sqrt{2}\lambda \sqrt{m\sin^2(\arcsin(\lambda/\omega_n))}^{-1}
\]

(Eq. 3)

Statistics—All values were expressed as the mean ± S.E. with n and P indicated under “Results.” Statistical analyses were performed using Student’s t test (GraphPad Prism, San Diego, CA). Values with p < 0.05 were considered statistically significant.

RESULTS

Cultured Primary Hepatocytes—In order to determine the effect of L-FABP gene ablation on phytanic acid uptake in hepatocytes, it was important to check hepatocyte viability, morphology, and ability to express key proteins to avoid potential complications due to loss of plasma membrane fatty acid transporters and/or intracellular L-FABP protein expression resulting from treatment of cells with proteases including collagenase (reviewed in Ref. 1). Therefore, the hepatocytes were grown in cell culture for 1–5 days during which time viability, morphology, and secretion of albumin and apoB were monitored as described under “Experimental Procedures.” Whereas L-FABP was not detectable in cultured primary hepatocytes isolated from L-FABP−/− mice (Fig. 1A), primary hepatocytes isolated from wild type L-FABP+/+ mice exhibited constant L-FABP levels over the first 3 days in culture, decreasing significantly 5 days postisolation as indicated under “Experimental Procedures.” Consequently, all data were obtained with primary hepatocytes maintained in culture for only 2–3 days.

Effect of L-FABP Gene Ablation on Maximal Uptake and Specificity of Branched-chain Fatty Acid Uptake in Cultured Primary Hepatocytes Isolated from Male Mice—Since L-FABP binds branched-chain phytanic acid with high affinity (14), the possibility that L-FABP gene ablation decreases phytanic acid uptake was examined in cultured primary hepatocytes isolated from wild type L-FABP+/+ and L-FABP−/− mice. Based on the curves presented in Fig. 2, the data were fitted as described under “Experimental Procedures” to obtain the half-time and maximal uptake (Fmax) of [2,3-3H]phytanic acid in hepatocytes isolated from L-FABP+/+ and L-FABP−/− mice (Table I). The effect of L-FABP gene ablation was to decrease both the half-time and the Fmax of [2,3-3H]phytanic acid in hepatocytes isolated from L-FABP+/+ and L-FABP−/− mice (Table I). The effect of L-FABP gene ablation was to decrease both the half-time and the Fmax of [2,3-3H]phytanic acid in primary hepatocytes by 2.6- and 3.2-fold, respectively (Table I, p < 0.008, n = 4). To determine whether the effect of L-FABP gene ablation on uptake of branched-chain fatty acid was specific, the half-time and maximal uptake of straight-chain fatty acid, [9,10-3H]palmitic acid was compared under similar conditions (Fig. 2, inset, open circles). The half-time and Fmax of straight-chain [9,10-3H]palmitic acid were 3.1-fold faster and 2.7-fold higher than that of [2,3-3H]phytanic acid (Table I, p < 0.0001, n = 3, as compared with hepatocytes derived from L-FABP+/+ mice).

3H]palmitic acid were 3.1-fold faster and 2.7-fold higher than that of [2,3-3H]phytanic acid (Table I, p < 0.02, m = 3). L-FABP gene ablation did not significantly affect the half-time of straight-chain [9,10-3H]palmitic acid uptake and only reduced the Fmax of [9,10-3H]palmitic acid uptake by 40% (Table I, p < 0.02, m = 3). These data suggest that L-FABP gene ablation specifically altered the half-time and maximal uptake of the branched-chain phytanic acid while not affecting or only slightly affecting these parameters for straight-chain palmitic acid uptake.

Effect of L-FABP Gene Ablation on Expression of Plasma Membrane Fatty Acid Transport/Translocase Proteins in Cultured Primary Hepatocytes Isolated from Male Mice—Because L-FABP gene ablation did not appear to significantly affect the early phase of either phytanic or palmitic acid uptake (Fig. 2), the possibility that plasma membrane fatty acid transport/translocase proteins were concomitantly up-regulated to en-
Fig. 2. Total uptake of [2,3-3H]phytanic acid and [9,10-3H]palmitic acid in cultured primary hepatocytes derived from L-FABP+/+ and L-FABP−/− mice. Total uptake of [2,3-3H]phytanic acid and [9,10-3H]palmitic acid (inset) was determined in cultured primary hepatocytes derived from L-FABP+/+ (closed circles) and L-FABP−/− (open circles) mice as described under “Experimental Procedures.” Values represent mean ± S.E. *p < 0.01, n = 3–6, as compared with hepatocytes derived from L-FABP+/+ mice.

Fatty acid uptake and oxidation were determined as described under “Experimental Procedures.” Values reflect the mean ± S.E. Units for $F_{\text{max}}$ and $t_{\text{1/2}}$ are in pmol/mg protein and h$^{-1}$, respectively.

| Fatty acid | L-FABP genotype | Fatty acid uptake | Fatty acid oxidation |
|-----------|-----------------|-------------------|---------------------|
|           |                 | $F_{\text{max}}$ | $t_{\text{1/2}}$ | $F_{\text{max}}$ | $t_{\text{1/2}}$ |
| Phytanic acid | +/+          | 127.4 ± 6.0      | 13.9 ± 1.1 | 28.5 ± 1.0       | 7.5 ± 0.6         |
| -/+        | 40.2 ± 1.3*    | 5.2 ± 0.4        | 67.7 ± 13.6*     | 6.4 ± 2.9*        |
| Palmitic acid | +/+          | 372.8 ± 68      | 4.4 ± 2.2b | 49.4 ± 8.5       | 4.4 ± 1.9         |
| -/+        | 216.1 ± 27     | 3.2 ± 1.0        | 216.1 ± 27       | 3.2 ± 1.0         |

* Significance p < 0.008, n = 3, as compared with hepatocytes derived from wild type L-FABP+/+ mice.

b Significance p < 0.02, n = 3, as compared with palmitic acid uptake or oxidation.

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Increased expression was insufficient to overcome the loss of L-FABP in the overall uptake process.

Effect of L-FABP Gene Ablation on Targeting, Selectivity, and Retention of Branched-chain Fatty Acids in the Unesterified Fatty Acid Pool of Cultured Primary Hepatocytes Isolated from Male Mice—Since L-FABP gene ablation decreased the maximal phytanic acid uptake in cultured primary hepatocytes 3.2-fold, targeting, specificity, and retention of [2,3-3H]phytanic acid in the unesterified fatty acid pool was examined. Whereas [2,3-3H]phytanic acid was rapidly taken into the unesterified fatty acid pool with a specific activity of 3.4 pmol/mg by 1 h of incubation, thereafter, [2,3-3H]phytanic acid was slowly lost from the unesterified fatty acid pool such that activity was detectable for as long as 5 h (Fig. 3A, open bars). L-FABP gene ablation reduced the specific activity of [2,3-3H]phytanic acid by 1.8-fold at 1 h of incubation and accelerated the loss of [2,3-3H]phytanic acid from the unesterified fatty acid pool by 2-fold (Fig. 3A, closed bars). To examine the specificity of these effects of L-FABP, the appearance and retention of [9,10-3H]palmitic acid in the unesterified fatty acid pool was examined under similar conditions (Fig. 3B, open bar). [9,10-3H]Palmitic acid was targeted to the unesterified fatty acid pool such that the specific activity was 1.9 pmol/mg by 1 h incubation (Fig. 3B, open bar), 1.8-fold lower than that of [2,3-3H]phytanic acid (Fig. 3A, open bar). Further, [9,10-3H]palmitic acid was much more rapidly removed from the unesterified long chain fatty acid pool such that after 1 h [9,10-3H]palmitic acid was no longer detectable (Fig. 3B, open bar). L-FABP gene ablation reduced the retention time of [9,10-3H]palmitic acid in the unesterified fatty acid pool so quickly that it was not detectable at any time point examined (Fig. 3B).

In summary, [2,3-3H]phytanic acid retention in the unesterified fatty acid pool was significantly longer than [9,10-3H]palmitic acid. Whereas the differences observed could reflect the fact that phytanic acid is metabolized more slowly than palmitic acid, the absence of L-FABP can also affect release and/or intracellular transport of fatty acids from the plasma membrane to target organelles.

L-FABP Gene Ablation Reduced the Intracellular Transport/Diffusion of Fatty Acid in Cultured Primary Hepatocytes Isolated from Male Mice—To determine whether L-FABP gene ablation reduced the intracellular transport/diffusion of fatty acid, the cytoplasmic and membrane diffusional components of NBD-stearic acid were resolved by MPFPR. Once the NBD-stearic uptake achieved equilibrium, an area in the cytoplasm
of the culture primary hepatocyte was selected and subjected to a very brief (500-µs) bleaching pulse that destroyed the NBD-stea-ric acid at the focal point of multiphoton excitation. In order to avoid artificial complexities associated with MPFPR, the bleach depth was kept to less than 30% (46). Immediately thereafter, the transport/diffusion of NBD-stea-ric acid from nonbleached areas into the bleached area was measured as recovery from photobleaching as a function of time (Fig. 4A). The NBD-stea-ric acid photobleaching recovery curves from multiple cultured hepatocytes were analyzed as under “Experimental Procedures.” Two-component diffusional coefficients (cytoplasmic and membrane) were resolved describing fast and slow NBD-stea-ric acid diffusion pools, respectively. In hepa-tocytes from wild type L-FABP+/+ mice, the cytoplasmic dif- fusional component of NBD-stea-ric acid (Fig. 4B, inset) was 15-fold faster than that of NBD-stea-ric acid in intracellular membranes (Fig. 4B). Whereas both the cytoplasm and mem-brane cellular diffusional components were observed to de-crease in the hepatocytes derived from livers of L-FABP−/− mice, a significant decrease was observed only with the cy-toplasmic component (2-fold, p < 0.001, n = 29–42, 13.1 × 10−8± 2 × 10−8 cm2/s versus 6.6 × 10−8± 0.8 × 10−8 cm2/s) (Fig. 4B). These results for the first time resolved the cytoplasmic from membrane diffusional components to demonstrate that L-FABP gene ablation selectively reduced the cytoplasmic, but not membrane, component of intracellular fatty acid transport/diffusion.

**Effect of L-FABP Gene Ablation on Expression of Intracellular Fatty Acid Transport Proteins in Cultured Primary Hepatocytes Isolated from Male Mice**—To assure that the reduced cytoplasmic transport/diffusion of NBD-stea-ric acid in hepatocytes isolated from livers of L-FABP−/− mice was associated with loss of L-FABP rather than concomitant down-regulation of other intracellular fatty acid or fatty acyl-CoA-binding pro-teins, Western blot analysis of cultured primary hepatocytes was performed to determine levels of several cytosolic proteins involved in fatty acid diffusion (e.g. SCP-2 (45) and SCP-x (47)) and fatty acid metabolism (SCP-x (47) and ACBP (48)). Levels of the intracellular fatty acid and fatty acyl-CoA transport proteins SCP-2 (Fig. 1B), SCP-x (Fig. 1C), and ACBP (Fig. 1F) were increased 5.1-, 1.6-, and 1.5-fold (p < 0.02, n = 3), respecti-vely, in hepatocytes derived from the L-FABP gene-ablated mice. The fact that these proteins were up-regulated, rather than reduced, suggests that they did not account for the re-duced intracellular fatty acid transport/diffusion observed in hepatocytes from livers of L-FABP−/− mice. Instead, the con-comitant up-regulation of the other cytoplasmic/intracellular proteins involved in fatty acid transport and metabolism appeared to partially compensate loss of L-FABP. Thus, the 2-fold reduction in cytoplasmic fatty acid transport/diffusion in hepa-tocytes from livers of L-FABP−/− mice may actually under represent the direct effect of L-FABP on fatty acid transport/ diffusion.

**L-FABP Gene Ablation Preferentially Reduced Oxidation of Branched-chain Fatty Acids in Cultured Primary Hepatocytes Isolated from Male Mice**—Since L-FABP gene ablation reduced the maximal uptake of phytanic acid as well as the size of the total unesterified fatty acid pool, the effect of L-FABP gene ablation on phytanic acid oxidation was examined in cultured primary hepatocytes. L-FABP gene ablation differentially in-hibited LCFA oxidation, depending on the type of fatty acid examined. L-FABP gene ablation reduced [2,3-3H]phytanic acid oxidation at nearly all time points (Fig. 5, open circles) such that the half-time and maximal oxidation of [2,3-3H]phy-tanic acid were reduced 3.4- and 5.8-fold, respectively (Table I). To determine whether L-FABP gene ablation specifically re-duced oxidation of fatty acids oxidized in peroxisomes (i.e.
In addition to being oxidized, both—Targeting to Esterified Lipids in Cultured Primary Hepatocytes, respectively (Fig. 6A by 2.8-, 1.5-, 1.3-, 1.4-, and 1.8-fold at 1, 3, 6, 15, and 24 h, (49)). The mitochondrial oxidation of [9,10-3H]palmitic acid exhibited 4-fold shorter half-time and 2.4-fold higher maximal oxidation of the straight-chain [9,10-3H]palmitic acid (Table I). L-FABP gene ablation did not significantly affect the either the half-time or maximal oxidation of the straight-chain [9,10-3H]palmitic acid. Taken together, these data suggest that L-FABP gene ablation significantly reduced branched-chain [2,3-3H]phytanic acid oxidation, but not straight-chain [9,10-3H]palmitic acid oxidation, in cultured primary hepatocytes.

L-FABP Gene Ablation Reduced Branched-chain Fatty Acid Targeting to Esterified Lipids in Cultured Primary Hepatocytes Isolated from Male Mice—in addition to being oxidized, both [2,3-3H]phytanic acid and [9,10-3H]palmitic acid can be esterified to more complex lipids such as phospholipids, and neutral lipids such as diacylglycerides, triacylglycerides, and cholesteryl esters. In hepatocytes isolated from livers of wild type L-FABP+/+ mice, incorporation of [2,3-3H]phytanic acid into total esterified lipids was 10–15-fold less over 24 h (Fig. 6A, closed circles) than observed with [9,10-3H]palmitic acid (Fig. 6a, inset, closed circles). L-FABP gene ablation further reduced the targeting of [2,3-3H]phytanic acid to total esterified lipids by 2.8-, 1.5-, 1.3-, 1.4-, and 1.8-fold at 1, 3, 6, 15, and 24 h, respectively (Fig. 6A, inset, <i>p</i> < 0.05, <i>n</i> = 3–6). This effect was also observed with [9,10-3H]palmitic acid whose targeting to total esterified lipids decreased up to 2-fold (<i>p</i> < 0.008, <i>n</i> = 3–6) in L-FABP−/− hepatocytes (Fig. 6A).

Resolution of esterified lipids into individual lipid classes showed that the reduced incorporation of [2,3-3H]phytanic acid into the total esterified lipids of L-FABP gene-ablated mice was reflected in phospholipids (Fig. 6B), neutral lipids (Fig. 6C), triacylglycerides (Fig. 7A), cholesteryl esters (Fig. 7B), and diacylglycerides (Fig. 7C) in the following order: diacylglycerides > cholesteryl esters > triacylglycerides, phospholipids. In contrast, L-FABP reduced incorporation of the normal straight-chain [9,10-3H]palmitic acid only into the neutral lipids (Fig. 6C, inset) but not phospholipids (Fig. 6B, inset). Within the neutral lipid fraction, L-FABP gene ablation decreased incorporation of [9,10-3H]palmitic acid into triacylglycerides (Fig. 7A, inset) and diacylglycerides (Fig. 7C, inset) but not cholesteryl esters (Fig. 7B, inset).

**Effect of L-FABP Gene Ablation on Lipid Levels in Cultured Primary Hepatocytes and Liver Homogenates of Male Mice—**Lipids were extracted, and mass was determined as described under “Experimental Procedures” (Fig. 8) in order to determine whether alterations in incorporation of radiolabeled fatty acids into esterified lipids of cultured primary hepatocytes isolated from livers of L-FABP−/− mice were consistent with lipid mass. L-FABP gene ablation reduced the mass of neutral lipids, triglycerides, and unesterified fatty acids by 1.5-, 1.6-, and 1.9-fold, respectively (Fig. 8). The mass of total liver lipids from livers of L-FABP−/− versus L-FABP+/+ mice was also determined to determine whether values reflected the reduced esterification of radiolabeled fatty acids and reduced esterified lipid mass in cultured primary hepatocytes. The total lipid mass (Fig. 9A) was reduced 1.2-fold in the livers of L-FABP gene-ablated mice, reflected in the decreased mass of triglycerides (1.5-fold, <i>p</i> < 0.04) (Fig. 9E) and unesterified fatty acids (1.3-fold, <i>p</i> < 0.03) (Fig. 9D). In contrast, the masses of cholesterol (Fig. 9B), cholesteryl esters (Fig. 9C), and phospholipids (Fig. 9F) were not significantly altered.

In summary, the effects of L-FABP gene ablation on reduced esterification of radiolabeled fatty acids into total lipids, especially triglycerides, was consistent with reduced mass of triglycerides and unesterified fatty acids in both cultured primary hepatocytes and livers from L-FABP−/− mice.

**DISCUSSION**

Although the role of L-FABP in straight-chain fatty acid metabolism has been studied extensively for several decades (reviewed in Refs. 1–3 and 50), almost nothing is known regarding the effect of L-FABP on the uptake, oxidation, and esterification of branched-chain fatty acids. To begin to address these issues in a more physiological context, cultured primary hepatocytes were isolated from livers of male wild type L-FABP+/+ and L-FABP−/− mice, aged 2–4 months. These mice were recently developed independently by two separate groups (see Refs. 6, 7, and 9 and Ref. 8). The data present the following new insights summarized schematically in Fig. 10.

First, L-FABP gene ablation specifically inhibited the peroxisomal oxidation of branched-chain fatty acid, while not affecting or only slightly affecting that of mitochondrial straight-chain fatty acid oxidation in cultured primary hepatocytes. Several contributory factors were considered as follows. (i) Plasma membrane fatty acid translocation across the plasma membrane is dependent on fatty acid structure (51–53). As...
shown herein, the half-time of branched-chain fatty acid uptake was 3-fold slower than that of straight-chain fatty acid. These data suggest that, although the specificity of the individual fatty acid transporter proteins is not known, these proteins may exhibit differential selectivity for branched-chain versus straight-chain fatty acid. (ii) L-FABP gene ablation resulted in concomitant, but differential, up-regulation of several plasma membrane transport proteins in cultured hepatocytes. Up-regulation of some, but not all, of these plasma membrane fatty acid transporters was also observed in a sex- and age-dependent manner in liver homogenates from L-FABP−/− mice. The RNA encoding FAT/CD36 (n = 3), but not FATP (n = 3), was slightly up-regulated in liver homogenates from 2.5–3.5-month-old male mice, whereas GOT was not examined (8). Western blotting of liver homogenates from old female mice aged 13–15 months detected 1.2-fold increased level of FAT/CD36 (p < 0.05, n = 4–6) but not the other plasma membrane fatty acid transport proteins, GOT, FATP, or caveolin (6). Since intracellular fatty acid-binding proteins and/or fatty acyl-CoA synthetase are thought to interact with plasma membrane fatty acid translocase/transport proteins to facilitate fatty acid uptake (54, 55), L-FABP may selectively interact with plasma membrane transport proteins facilitating branched-chain fatty acid uptake much more than straight-chain fatty acid. (iii) L-FABP gene ablation may selectively inhibit peroxisomal branched-chain fatty acid oxidation, because the latter system has lower capacity and is more easily inhibited by excess branched-chain fatty acid as compared with mitochondrial oxidation of straight-chain fatty acid (31). Consistent with this possibility, in L-FABP−/− hepatocytes the half-time of perox-
L-FABP Ablation Inhibits Branched-chain Fatty Acid Metabolism

Isomolar oxidation of branched-chain fatty acid was very similar to that of mitochondrial straight-chain fatty acid oxidation (Table I). However, in L-FABP+/− hepatocytes, the expression of L-FABP increased the half-time and maximal oxidation for branched-chain much more than that of straight-chain fatty acid (Table I). (iv) Since the binding affinities of L-FABP for straight-chain and branched-chain fatty acid are similar (16), this is unlikely to account for the much slower uptake or lower maximal uptake of branched-chain versus straight-chain fatty acid in wild type hepatocytes. This was confirmed by the fact that loss of L-FABP did not abolish the difference in uptake of branched versus straight-chain fatty acid. (v) Differential effects of L-FABP on intracellular transport/diffusion of branched versus straight-chain fatty acid were unlikely to account for differential effects of L-FABP gene ablation on the uptake and oxidation of these fatty acids. Previous work with transfected cells overexpressing L-FABP and with liver hepatocytes from wild type mice (1, 5, 38), together with the data presented herein, determined that L-FABP enhances intracellular fatty acid transport/diffusion, essential for bringing fatty acid/acyl-CoAs to peroxisomes and also to mitochondria. L-FABP gene ablation significantly reduced the intracellular transport/diffusion of a straight-chain fluorescent fatty acid analogue, NBD-stearic acid. Although a fluorescent labeled phytanic acid is unavailable, the fact that L-FABP exhibits similar affinity for branched and straight-chain fatty acid (16) suggests that L-FABP most likely similarly affects branched-chain and straight-chain fatty acid transport/diffusion within hepatocytes.

Second, in contrast to the effects of L-FABP on branched-chain fatty acid oxidation, L-FABP gene ablation had no effect on oxidation of the straight-chain palmitic acid by cultured primary hepatocytes isolated from 2–4-month-old male mice. This was despite the fact that L-FABP binds straight-chain fatty acids such as palmitic acid and the branched-chain phytanic acid with similar high affinity (16, 56). However, it is important to note that, in contrast to branched-chain fatty acid oxidation that occurs in peroxisomes, straight-chain fatty oxidation occurs primarily in mitochondria (49). Furthermore, comparison of other recent studies suggests that the effect of L-FABP gene ablation on straight-chain fatty acid oxidation may be dependent on fatty acid load. When cultured primary hepatocytes were incubated with low levels of palmitic acid, similar to that of phytanic acid above, L-FABP gene ablation did not alter oxidation of the straight-chain palmitic acid. Based on these observations, it was predicted that overexpression of L-FABP should also not alter the oxidation of the straight-chain palmitic acid when present at a low level. Indeed, L-FABP overexpression in cultured L-cell fibroblasts did not significantly alter palmitic acid oxidation when palmitic acid was supplied at a low level in the culture medium (38). In contrast, at very high palmitic acid concentration (1 mM), oxidation of palmitic acid and β-hydroxybutyrate production were decreased by about 30% in hepatocytes isolated from 2–3-month-old female mice (9). The potential significance of high fatty acid load on influencing the effect of L-FABP on straight-chain fatty acid oxidation was recently demonstrated in starved mice, wherein serum unesterified fatty acid levels were increased as much as 3-fold (>1 mM) compared with the fed state (about 0.3 mM), and the concentration of serum β-hydroxybutyrate was significantly decreased (8, 9). However, it must be noted that comparisons between these studies are complicated by the fact that livers of male rats (reviewed in Ref. 57) and wild type L-FABP+/+ mice (not shown) express significantly lower levels of L-FABP protein than age-matched female rats and mice. Furthermore, starvation significantly reduces liver L-FABP expression (reviewed in Ref. 57). Taken together, these data suggest that L-FABP expression has no effect on straight-chain fatty acid oxidation when such fatty acids are present at low level, whereas at high fatty acid load, L-FABP gene ablation significantly reduces the oxidation of straight-chain fatty acid.

Third, to determine whether the reduced oxidation of branched-chain fatty acids in cultured primary hepatocytes isolated from L-FABP gene-ablated male mice was due to reduced maximal uptake of phytanic acid uptake, the maximal uptake of the branched-chain [2,3-3H]phytanic acid was examined. Indeed, L-FABP gene ablation significantly reduced the maximal uptake of the saturated branched-chain [2,3-3H]phytanic acid by cultured primary hepatocytes isolated from 2–4-month-old, fed male mice. L-FABP gene ablation also significantly reduced the hepatocyte maximal uptake of the non-branched-chain fatty acids: saturated [9,10-3H]palmitic acid (data presented herein) and unsaturated [3H]oleic acid (8). Importantly, the uptake of the straight-chain, unsaturated [3H]oleic acid by liver in vivo is reduced similarly in aged, male L-FABP−/− mice fed ad libitum. After injection of a tracer bolus of the unsaturated non-branched-chain [3H]oleic acid, hepatic deposition of [3H]oleic acid was decreased nearly 2-fold (6). The fact that L-FABP gene ablation reduced the maximal uptake of fatty acids in cultured hepatocytes and liver suggests that the opposite result should be observed in cells overexpressing L-FABP. Indeed, L-FABP overexpression enhances maximal fatty acid uptake in transfected L-cell fibroblasts (38) and transfected hepatoma cells (58). These findings with cultured hepatocytes and livers of intact L-FABP−/− mice establish for the first time in a more physiological context that L-FABP

Fig. 8. Lipid mass and distribution in cultured primary hepatocytes derived from L-FABP+/+ and L-FABP−/− mice. Lipids were extracted from cultured primary hepatocytes isolated from wild type L-FABP+/+ (open bars) and L-FABP−/− (closed bars) mice, followed by resolution into individual lipid fractions as described under “Experimental Procedures.” Values represent means ± S.E. *, significance p < 0.05, n = 3–4, as compared with hepatocytes derived from L-FABP+/+ mice. A, TL, total lipid; PL, phospholipids; NL, neutral lipids; B, TG, triglycerides; Chol, unesterified cholesterol; CE, cholesteryl ester.

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plays an important role in the maximal uptake of branched-chain fatty acid similar to that observed with nonbranched, saturated and unsaturated fatty acids (Fig. 10).

Fourth, the possibility that L-FABP gene ablation reduced oxidation of branched-chain fatty acid and, under high fatty acid load, oxidation of straight-chain fatty acid by altering intracellular fatty acid transport was examined using MPFPR. The rationale for this possibility was as follows. (i) L-FABP is not a peroxisomal protein, and only small quantities are associated with mitochondrial membranes (59). (ii) L-FABP is known to bind both branched- and straight-chain fatty acids with high affinity (14). This is important because peroxisomal oxidation of branched-chain fatty acids requires transport of the fatty acid in unesterified form to the peroxisomal membrane, where it is converted to fatty acyl-CoA, internalized, and oxidized (reviewed in Ref. 60). (iii) L-FABP binds straight-chain fatty acyl-CoA with high affinity (16). Mitochondrial oxidation occurs in a series of steps where carnitine acyltransferase enzymes sequentially transfer the fatty acid into the mitochondrial matrix for oxidation (61). The recent finding of a long chain acyl-CoA synthetase (ACS5) located on the mitochondrial outer membrane (62, 63) allows both fatty acids and CoA derivatives to be utilized for mitochondrial oxidation.

With MPFPR, it was shown for the first time that L-FABP gene ablation specifically reduced by 2-fold the cytoplasmic, but not membrane, component of fatty acid transport/diffusion in cultured primary hepatocytes. Although earlier studies of NBD-stearic acid diffusion in transfected cells, hepatocytes from drug-treated animals, and hepatocytes of male versus female mice suggested that L-FABP influenced the intracellular diffusion of NBD-stearic acid (1, 42, 43, 45), these studies utilized confocal FPR to obtain an average diffusion coefficient of NBD-stearic acid (i.e. membranes/cytoplasm) and did not resolve the cytoplasmic from the membrane components of NBD-stearic acid diffusion. Resolution of these components is very important, because L-FABP overexpression in L-cells also increases membrane fluidity (64, 65), which in turn would increase NBD-stearic acid diffusion in membranes and thereby complicate interpretation of data obtained by confocal FPR. The reduced cytoplasmatic fatty acid diffusion in cultured primary hepatocytes from L-FABP gene-ablated mice was not due...
to concomitant down-regulation of other soluble fatty acid (SCP-2) and fatty acyl-CoA (SCP-x, ACBP) binding proteins. On the contrary, these proteins were up-regulated 5.1-, 1.6-, and 1.5-fold, respectively ($p < 0.02$, $n = 3$), in cultured primary hepatocytes isolated from 2–4-month-old male L-FABP−/− mice. In summary, the present MPPFR data demonstrate for the first time that L-FABP gene ablation inhibits cytoplasmic LCFA diffusion despite concomitant up-regulation of other fatty acid and fatty acyl-CoA-binding proteins. These data provide the first clear evidence supporting an early hypothesis (reviewed in Refs. 1, 4, and 5) that L-FABP facilitates LCFA transport/diffusion. Since liver fatty acid levels are 10–100-fold higher than fatty acyl-CoAs (reviewed in Refs. 1 and 48) and peroxisomal fatty acid oxidation requires transport of the unesterified form of fatty acid to the peroxisome, it is likely that reduced cytoplasmic transport/diffusion of fatty acid in cultured primary hepatocytes from L-FABP gene-ablated mice contributes to inhibition of branched-chain fatty acid oxidation in peroxisomes (Fig. 10). Taken together, the data suggested that L-FABP may be important for transporting bound branched-chain fatty acid to peroxisomes as well as for transporting bound straight-chain fatty acids or their CoA derivatives to sites of utilization including endoplasmic reticulum and, as indicated in the second point above, at high concentrations for mitochondrial oxidation.

Fifth, cultured primary hepatocytes and livers from L-FABP gene-ablated mice exhibited a pronounced fatty acid phenotype characterized by reduced mass of unesterified fatty acids and triglycerides. These reductions correlated with (i) reduced maximal uptake of both branched- and straight-chain fatty acid; (ii) inhibition of branched-chain fatty acid oxidation (described herein) and, as shown by others, inhibition of straight-chain fatty acid oxidation when fatty acid load was high (9); and (iii) inhibition of branched- and straight-chain fatty acid incorporation into triacylglycerides, diacylglycerides, and cholesterol esters. Statistically significant, but smaller, reductions in fatty acid, triacylglyceride, and total fatty acid mass were also observed in livers from 2–4-month-old male L-FABP−/− mice. Although liver mass of unesterified fatty acid levels also tended to decrease in another study of similarly aged male L-FABP−/− mice, this trend was not statistically significant, possibly due to differences in group size with 10–13 mice/group in the present study and 6–10 mice/group in Ref. 8 or to differences in genetic background in the independently derived strains of L-FABP−/− mice. Nevertheless, liver triacylglyceride content was significantly decreased in fasted, 2–4-month-old male L-FABP−/− mice in both the present investigation and in the latter study (8). Based on these findings, it was predicted that overexpression of L-FABP should exert opposite effects on fatty acid phenotype as L-FABP gene ablation. Indeed, overexpression of L-FABP in transfected i-cells enhanced the esterification of the straight-chain palmitic acid into esterified lipids, especially neutral lipids (38). Furthermore, since the 4-fold up-regulation of L-FABP in SCP-x/SCP-2 gene-ablated male mice resulted in 50% decreased liver level of triacylglycerides and cholesteryl esters (10), these findings suggested that the decreased triacylglyceride and cholesteryl ester levels were not likely to be due to L-FABP up-regulation but instead due to the absence of SCP-x/SCP-2, which also participate in LCFA metabolism. Finally, it should be noted that the reduced fatty acid phenotype in livers of 2–4–month-old, fasted male mice was observed only in young 2–4-month-old male L-FABP−/− mice (described herein) but not in aged 13–15-month-old female L-FABP−/− mice (6, 7). Thus, expression of the fatty acid phenotype appeared to be most prominent in young male mice and reduced or absent in old mice. However, other factors such as gender of the L-FABP−/− mice may contribute to the latter observation.

In summary, the data presented herein with 2–4–month-old male L-FABP+/+ and L-FABP−/− mice for the first time demonstrated the importance of L-FABP in the metabolism of the branched-chain phytanic acid, a significant constituent of the human diet. Under low fatty acid load, L-FABP gene ablation selectively decreased the maximal uptake of branched-chain fatty acid nearly 2-fold more than that of the straight-chain palmitic acid. Decreased maximal fatty acid uptake was observed despite the fact that several plasma membrane fatty acid/transport/translocase proteins were up-regulated 1.4–5-fold in cultured hepatocytes and less so in liver. Concomitantly, oxidation of phytanic acid (oxidized in peroxisomes), but not palmitic acid (oxidized primarily in mitochondria), was reduced nearly 6-fold despite the fact that L-FABP is not a peroxisomal protein and that peroxisomal SCP-x was up-regulated 1.6-fold. SCP-x is the only known 3-ketoacyl-CoA thiolase specifically utilizing branched-chain substrates. It is noteworthy that, under conditions of high fatty acid load (1 mM), L-FABP gene ablation modestly inhibits (30%) palmitic acid oxidation (9). L-FABP gene ablation also decreased the esterification of branched-chain, as well as straight-chain, fatty acid. The reduced oxidation and esterification of phytanic acid correlated with 2-fold slower cytoplasmic fatty acid diffusion, as resolved for the first time by MPPFR of NBD-stearic acid. Consistent with this possibility, recent data from our laboratory show reduced distribution of nonmetabolizable fluorescent fatty acids and nonmetabolizable fluorescent fatty acyl-CoA to nuclei in mock-transfected L-cells (containing no detectable L-FABP) as compared with transfected L-cells overexpressing L-FABP (66, 67). The net result of these effects of L-FABP gene ablation on dynamics of metabolizable fatty acids shown in the present investigation was the demonstration for the first time of a consistent fatty acid phenotype detected in both cultured primary hepatocytes as well as livers from L-FABP−/− mice.

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Liver Fatty Acid-binding Protein Gene Ablation Inhibits Branched-chain Fatty Acid Metabolism in Cultured Primary Hepatocytes
Barbara P. Atshaves, Avery M. McIntosh, Olga I. Lyuksyutova, Warren Zipfel, Watt W. Webb and Friedhelm Schroeder

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