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LIVER TYPE FATTY ACID BINDING PROTEIN (L-FABP) GENE ABLATION REDUCES NUCLEAR LIGAND DISTRIBUTION AND PEROXISOME PROLIFERATOR ACTIVATED RECEPTOR- α ACTIVITY IN CULTURED PRIMARY HEPATOCYTES¹

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Abstract

The effect of liver type fatty acid binding protein (L-FABP) gene ablation on the uptake and distribution of long chain fatty acids (LCFA) to the nucleus by real-time laser scanning confocal imaging and peroxisome proliferator activated receptor- α (PPAR α) activity was examined in cultured primary hepatocytes from livers wild-type L-FABP^{+/+} and gene ablated L-FABP^{-/-} mice. Cultured primary hepatocytes from livers of L-FABP^{-/-} mice exhibited: (i) reduced oxidation of palmitic acid, a common dietary long chain fatty acid (LCFA); (ii) reduced expression of fatty acid oxidative enzymes—proteins transcriptionally regulated by PPAR α ; (iii) reduced palmitic acid-induced PPAR α coimmunoprecipitation with coactivator SRC1 concomitant with increased PPAR α coimmunoprecipitation with coinhibitor N-CoR; (iv) reduced palmitic acid-induced PPAR α . Diminished PPAR α activation in L-FABP null hepatocytes was associated with lower uptake of common dietary LCFA (palmitic acid as well as its fluorescent derivative BODIPY FL C₁₆), reduced level of total unesterified LCFA, and real-time redistribution of BODIPY FL C₁₆ from the central nucleoplasm to the nuclear envelope. Taken together, these studies support the hypothesis that L-FABP may facilitate ligand (LCFA)-activated PPAR α transcriptional activity at least in part by increasing total LCFA ligand available to PPAR α for inducing PPAR α -mediated transcription of proteins involved in LCFA metabolism.

Keywords

fluorescence; binding; hepatocyte; nuclei; L-FABP; PPAR α

INTRODUCTION

Transcription of genes encoding proteins involved in fatty acid metabolism can be induced by a family of ligand activated nuclear receptors known as peroxisome proliferator-activated

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receptors (PPARs) [1;2]. Since abnormal regulation of PPAR α , the major PPAR isoform found in liver, is associated with chronic diseases such as diabetes, obesity, and hyperlipidemia, considerable effort extends to understanding how endogenous ligands regulate PPAR α transcriptional control [2;3]. Increasing evidence obtained *in vitro* and with transformed cells in culture shows that LCFAs and/or their CoA thioesters (LCFA-CoAs) are endogenous, high affinity ligands for PPAR α (as exhibited by nM K_d s) whose nucleoplasmic concentrations are within the range of nucleoplasmic PPAR α levels in living cells [4–9]. Indeed, dietary LCFAs are known to enhance PPAR α activated gene expression in transformed cells and animals (rev. in [4;10]).

Since both LCFAs and LCFA-CoAs have high affinities for membranes, it is unclear how after the uptake of LCFAs (and/or activated to LCFA-CoAs) these ligands are transported not only to the nuclear envelope, but into the nucleoplasm, for regulating the activity of nuclear receptors such as PPAR α (rev. in [10]). Both *in vitro* studies, and investigations with transformed cells overexpressing L-FABP, suggest that this cytoplasmic LCFA and LCFA-CoA binding protein may be a likely candidate for directly enhancing the transfer of these ligands into nuclei [7;9; 11;12]. Since L-FABP protein level is itself regulated by PPAR α transcriptional activity, it has further been postulated that L-FABP may mediate its own expression by enhancing LCFA and LCFA-CoA transport into nuclei to facilitate transcriptional activity of PPAR α (rev. in [10]).

Despite these studies, the evidence supporting mechanistic details of this hypothesis in a more physiological setting is lacking. The objective of the present investigation was to use wild-type L-FABP $^{+/+}$ and L-FABP $^{-/-}$ mice, coimmunoprecipitation, transactivation, and confocal imaging of fluorescent LCFA to resolve new details of L-FABP's role in LCFA-mediated regulation of PPAR α in primary hepatocytes.

MATERIALS AND METHODS

Materials

Silica Gel G and Silica Gel 60 thin layer chromatography plates were purchased from Analtech (Newark, DE) and EM Industries, Inc. (Darmstadt, Germany), respectively. The LCFA methyl ester standards (GLC-411 from Nu-Chek Prep, Inc., Elysian, MN) included 3.23% by weight of 8:0 to 22:6 fatty acid methyl esters. Chloroacetaldehyde (~ 50% solution in water), the LCFA standard palmitic acid (16:0), palmitoyl-CoA (16:0-CoA), palmitoleoyl-CoA (16:1-CoA), heptadecanoyl-CoA (17:0), stearoyl-CoA (18:0-CoA), oleoyl-CoA (18:1-CoA), linoleoyl-CoA (18:2-CoA), arachidoyl-CoA (20:0-CoA), arachidonoyl-CoA (20:4-CoA) were from Sigma-Aldrich (St. Louis, MO). Docosahexaenoyl-CoA (22:6-CoA) was generously provided by Dr. Eric Murphy (Univ. of North Dakota). [9,10- ^{3} H]-palmitic acid was purchased from Perkin Elmer Life Sciences. BODIPY FL C₁₆ (4, 4-difluoro-5, 7-dimethyl-4-bora-3a, 4a-diaza-s-indacene-hexadecanoic acid), Nile red, and SYTO 61 dye were purchased from Molecular Probes Inc. (Eugene, OR). Lab-Tek chambered cover glass slides were from Nunc (Naperville, IL). For Western blotting, rabbit polyclonal antiserum to rat L-FABP was prepared as described earlier [13] while goat polyclonal anti-peroxisome proliferator activated receptor- α (PPAR α) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal antiserum to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was obtained from Millipore (Temecula, CA). For coimmunoprecipitation, antibodies to steroid receptor coactivator-1 (SRC-1) or nuclear receptor corepressor (N-CoR) were from Affinity BioReagents (Golden, CO). Protein concentrations were determined by Bradford assay [14]. All reagents and solvents used were of the highest grade available and were cell culture tested.

Animals

All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at Texas A&M University. L-FABP null mice (L-FABP^{-/-}), generated by targeted disruption of the L-FABP gene through homologous recombination, were obtained as described earlier [15]. L-FABP gene ablation was verified by PCR (not shown) and western blotting. All experiments were performed with livers or hepatocytes derived from male N6 backcross generation mice ranging in age from 2–4 months (25–35 g). Wild-type male littermates with no disruption (L-FABP^{+/+}) were designated as controls. Animals were kept under constant light-dark cycles and had access to food and water *ad libitum*. Genotype was verified on all animals before hepatocyte preparation.

Hepatocyte isolation, viability and function

Hepatocytes from L-FABP^{+/+} and L-FABP^{-/-} mice were isolated as described [16;17]. After 24 hours, cells were transferred into serum-free medium with the following composition: Williams' medium E (WME) in 10 mM HEPES, pH 7.4 supplemented with 0.2 mg/ml gentamycin sulfate, 2 mM Glutamine, 0.5 mg/ml BSA/linoleate, 5 µg/ml insulin, 5 µg/ml glucagon, 100 ng/ml epidermal growth factor (EGF), 1 µg/ml somatotropin, 20 ng/ml liver growth factor (LGF), 100 ng/ml prolactin, 2 ng/ml cholera toxin, 10⁻⁶ M ethanolamine, 10⁻⁷ M selenium. Growth factors, hormones, and other supplements were purchased from Sigma-Aldrich (St. Louis, MO), except for EGF, which was obtained from BD Biosciences (San Jose, CA). Hepatocyte viability was monitored routinely using Trypan Blue (Sigma-Aldrich, St. Louis, MO). Preparations showing greater than 90% Trypan blue exclusion were used for all experiments. In addition, a LIVE/DEAD® Viability Cytotoxicity kit from Molecular Probes (Eugene, OR) was employed to further confirm hepatocyte viability. In brief, hepatocytes were plated on chambered slides (3 × 10⁵ cells/slide) and, after incubation with calcein (green fluorescence) and ethidium homodimer-1 (red fluorescence) dyes, were imaged on a MRC-1024MP laser scanning confocal microscope (Bio-Rad, Hercules, CA) equipped with an Axiovert 135 microscope and 63x Plan-Fluor oil immersion objective, numerical aperture 1.45 (Zeiss, Carl Zeiss Inc., Thornwood, NY). Both dyes were simultaneously excited at 488 nm with a Kr-Ar ion laser (Bio-Rad, Hercules, CA) through a 1% neutral density filter. Fluorescence emission was detected through separate filters: calcein (green fluorescence through a 525–555 nm band-pass filter); ethidium homodimer-1 (red fluorescence through a 585–630 nm band-pass filter).

Evaluation of functional cultures of hepatocytes included morphology, ability to synthesize albumin [16], and, in the case of wild-type mice, monitoring L-FABP protein expression in the cultured primary hepatocytes as described [17]. In brief, hepatocytes were plated on several 35 mm dishes (5 × 10⁵ cells/dish) and cultured up to 5 days. The medium was removed each day and replaced with fresh serum-free medium. Removed medium was centrifuged at 5,000×g for 10 min to remove cell debris. Proteins from the hepatocyte cell monolayer and aliquots from the medium over the 5 day period were subjected to SDS-PAGE and Western blotting with antibodies to detect albumin and L-FABP as described [13;18].

Western analysis

Western blotting was performed to determine protein levels of albumin, L-FABP, and PPARα in cultured primary hepatocytes basically as described earlier [17;19]. To ensure equal loading, expression of each protein was normalized to the mean expression of the housekeeping gene GAPDH. Briefly, since each protein of interest and GAPDH were easily resolved by size on the tricine gels, Western blots were cut widthwise, perpendicular to the loading lanes, to yield two blots that were probed against anti-sera to GAPDH or the protein of choice. Protein expression was quantified by densitometric analysis after image acquisition using a single-chip CCD (charge coupled device) video camera and a computer workstation (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).

Fatty acid uptake and oxidation

Fatty acid uptake was determined after incubating cultured primary hepatocytes for 6h with [9,10-³H]-palmitic acid as described earlier [20–22]. Likewise, fatty acid oxidation was determined by measuring oxidation of [9,10-³H]-palmitic acid also after 6 h incubation with cultured primary hepatocytes as described [20–22].

Real-time PCR

Total RNA was isolated and purified from liver and hepatocytes derived from male wild type L-FABP^{+/+} and L-FABP^{-/-} mice using RNeasy mini kit (Qiagen, Valencia, CA) in accordance with the manufacturer's protocol. RNA concentrations were determined spectrophotometrically, and integrity was verified by agarose electrophoresis and ethidium bromide staining. For quantitative real-time PCR, expression patterns of enzymes involved in peroxisomal and mitochondrial β -oxidation were analyzed with an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) using TaqMan® One Step PCR Master Mix Reagent kit, gene specific TaqMan® PCR probes and primers, and the following thermal cycler protocol: 48°C for 30 min, 95°C for 10 min before the first cycle, 95°C for 15 sec, and 60°C for 1 min, repeated 40 times. By using the TaqMan® One Step chemistry, total RNA was reverse-transcribed in the first step of the thermal cycler protocol (48°C for 30 min) prior to amplification. For specific probes and primers, Assay-on-Demand® products for mouse peroxisome proliferator-activated receptor alpha [PPAR α Mm00440939_m1]; acyl CoA oxidase [ACO (Mm00443579_m1)]; carnitine palmitoyl CoA acyltransferase 1 [CPT1 (Mm00550438_m1)]; and short chain acyl (butyryl) CoA dehydrogenase (Mm00431617_m1) were obtained from Applied Biosystems. Experiments were performed in triplicate and analyzed with ABI Prism 7000 SDS software (Applied Biosystems) to determine the threshold cycle (C_T) from each well. Primer concentrations and cycle number were optimized to ensure that reactions were analyzed in the linear phase of amplification. The mRNA expression of PPAR α , ACO, CPT1, and butyryl CoA dehydrogenase in L-FABP^{-/-} samples relative to wild type L-FABP^{+/+} was calculated using the comparative $2^{-\Delta\Delta C_T}$ method after normalization to a housekeeping gene (18 S RNA) as described in User Bulletin 2, ABI Prism 7000 Sequence Detection System (Applied Biosystems) and [23].

Co-immunoprecipitation (CoIP)

The co-immunoprecipitation of native proteins from liver homogenates of wild type and L-FABP gene ablated mice was performed as previously described [4]. Following homogenization of livers, ligands (10 μ M) were added to 1mg of liver homogenate, and proteins were immunoprecipitated with the ProFound™ mammalian co-immunoprecipitation kit (Pierce Biotechnology, Rockford, IL) and a monoclonal PPAR α antibody (Affinity BioReagents, Golden, CO). Eluted proteins were visualized by SDS-PAGE and western blotting with antibodies to PPAR α , steroid receptor coactivator-1 (SRC-1), and nuclear receptor corepressor (N-CoR) (Affinity BioReagents, Golden, CO) [4]. Co-immunoprecipitated protein values were standardized to values obtained for the antibody used to co-immunoprecipitate both proteins, and standardized values were normalized for each ligand sample to the level of interaction observed in the absence of ligands, which was set equal to 1.

Transactivation Assays

COS-7 cells (ATCC, Manassas, VA) were grown in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% FBS (Invitrogen, Carlsbad, CA) at 37°C with 5% CO₂ in a humidified chamber. Cells were transfected with pcDNA3-LFABP [12] for overexpression of L-FABP

using Lipofectamine 2000 (Invitrogen, Carlsbad, NV). Stable clones were selected through G-418 resistance and examined for increased L-FABP expression by Western blot analysis as previously described for other cell lines [24]. A stable cell line with 2–3 fold increased L-FABP expression (data not shown) was chosen for the transactivation experiments. COS-7 cells (control and L-FABP overexpressor) were grown in 24-well culture plates and transfected with 0.4 µg of each full-length mammalian expression vector (pSG5-PPAR α [25] and pSG5-mRXR α [26]) or empty plasmid (pSG5, Stratagene, La Jolla, CA), 0.4 µg of the reporter construct PPRE₃-TK-LUC [27], and 0.03 µg of the internal transfection control plasmid pRL-CMV (Promega Corp, Madison, WI) as previously described [28]. Following transfection incubation, media was replaced with serum-free DMEM (Invitrogen, Carlsbad, CA) for 1 hour, then 10 µM of fatty acid complexed with BSA [29] was added, and cells grown for an additional 16 hours. Firefly luciferase activity, normalized to Renilla luciferase (for transfection efficiency), was determined with the dual-luciferase reporter assay system (Promega, Madison, WI) according to the manufacturer's instructions. Luminescence was measured with a Synergy-2 microtiter plate reader (BioTek Instruments Inc., Winooski, VT).

Total LCFA and acyl chain composition

Lipids were extracted from cultured primary hepatocytes as described earlier [19]. Briefly, lipid extracts were resolved into individual lipid classes and the total LCFAs were identified by comparison to known standards using Silica gel G thin-layer chromatography plates developed in petroleum ether-diethyl ether-methanol-acetic acid 90:7:2:0.5 [24;30]. Prior to further analysis, lipids were stored under an atmosphere of N₂ to limit oxidation. One half of the total unesterified LCFA was used to determine LCFA mass (µg/mg protein) by the method of Marzo while the other half of the total unesterified LCFA was converted to fatty acid methyl esters (FAMES) by acid-catalyzed transesterification. FAMES were extracted into n-hexane and resolved into individual species by gas-liquid chromatography (GLC) on a dual FID GLC-14A (Shimadzu Kyoto, Japan) using a RTX-2330 capillary column (0.32 mm i.d. × 30 m length, Restek, Bellefonte, PA). Injector and detector were set at 260°C with the temperature program of 100°C for 1 min, 10°C/min to 140°C, then 2°C/min to 220°C, hold 1 min, then ramp 10°C/min to 260°C. The peak area data were collected with a SAT/IN analytical-to-digital interface (Waters Inc., Milford, MA) and was converted to peak area using Millenium³² 3.2 software (Waters Inc., Milford, MA). Individual fatty acid methyl ester peaks were identified by comparison to standards (GLC-411 from Nu-Chek Prep, Inc., Elysian, MN). All glassware used for lipid extraction, chromatography, and LCFA analysis was washed with sulfuric acid-chromate before use.

Total LCFA-CoA pool size

LCFA-CoA pool size was determined as described earlier [31]. In brief, cultured primary hepatocytes were suspended in 1 ml of KH₂PO₄ (100 mM, pH 5.3) containing 1.7 pmol heptadecanoyl-CoA (17:0-CoA) as internal standard. The sample was then homogenized two times for 20 seconds each with a probe sonicator (energy level 3) with a Sonic Dismembrator Model 550 (Fisher Scientific, Pittsburgh, PA, U.S.A.). A 50 µl aliquot was used for protein analysis. The LCFA-CoAs were then extracted with a mixture containing 2-propanol (1 ml), saturated (NH₄)₂SO₄ (0.13 ml), acetonitrile (2.0 ml). After vortexing for 5 min and centrifugation at 3000g for 20 minutes, the supernatant was retained, diluted with 100 mM KH₂PO₄ (pH=5.3, 5 ml), and LCFA-CoAs were isolated by solid phase extraction. For this purpose, an oligonucleotide purification cartridge (Applied Biosystems, Foster City, CA) was pre-rinsed with acetonitrile (5.0 ml), gently flushed with air, washed with 25 mM KH₂PO₄ buffer (2.0 ml), flushed with air, and the sample loaded in a polypropylene syringe. The sample was slowly forced through the cartridge, and the cartridge flushed with 25 mM KH₂PO₄ (pH=5.3, 5 ml), dried by flushing with air, and eluted with 60% CH₃CN in 100 mM KH₂PO₄ (0.3 ml). The eluant containing the LCFA-CoA extracts (or standards) was retained

in an amber HPLC vial for formation of fluorescent acyl etheno CoA derivatives with chloroacetaldehyde reagent as described earlier [32]. Chloroacetaldehyde reagent contained 1M chloroacetaldehyde, 0.3M citrate buffer, 1% SDS (pH=4). LCFA-CoAs were reacted with equal volumes of the chloroacetaldehyde reagent at 85°C for 20 minutes, cooled, and stored (< 7 days) at 4°C prior to HPLC analysis. The acyl etheno CoAs were resolved with a LUNA 150 × 2.0 mm 3 µm particle size phenyl-hexyl HPLC column (Phenomenex, Torrance, CA) as described earlier [33].

Direct ligand binding assay

Mouse recombinant PPAR α Δ AB protein was expressed and purified as previously described [4], and for all studies described herein was referred to simply as PPAR α . The direct binding of BODIPY FL C₁₆ labeled fatty acid to PPAR α was examined by measuring the quenching of PPAR α Δ AB aromatic amino acid residues [4]. This method was utilized to avoid innate problems associated with BODIPY micelle formation. Briefly, PPAR α (0.1 µM in 2 ml of PBS, pH 7.4) was titrated with increasing ligand over the concentration range of 0–1000nM. PPAR α aromatic amino acid excitation was carried out at 280nm and emission was measured from 300–400nm. Spectra obtained at 24°C were corrected for background (buffer and ligand only) and maximal intensities measured. The dissociation constant (K_d) was calculated with the ligand binding function of Sigma Plot (SPSS, Chicago, IL) utilizing the one-site saturation feature.

Fluorescent Fatty Acid distribution to the nucleus: Real Time Imaging of Hepatocytes using Laser Scanning Confocal Microscopy (LSCM)

Real-time LSCM experiments were performed with the fluorescent analogues of natural ligands in hepatocytes isolated from L-FABP $^{-/-}$ and L-FABP $^{+/+}$ mice. To assure that the fluorescence signal reflected that of unesterified LCFA, LSCM studies utilized a poorly metabolizable fluorescent LCFA, i.e. BODIPY FL C₁₆ [7]. Cells were cultured in serum-free medium supplemented with BSA (4 µM) and seeded onto chambered cover glass slides at a cell density of 5×10^5 cells/chamber. To confirm nuclear targeting of the fatty acid, hepatocytes were co-incubated with the nuclear stain SYTO 61 (5 µM) before incubation with BODIPY FL C₁₆ (3 µM) for 30 minutes at 37°C. Ligand concentrations were optimized to simultaneously colocalize both nuclear and fatty acid probes with maximal dynamic range and minimal autofluorescence detection. Hepatocytes were washed once with PBS and then imaged at room temperature using laser scanning confocal microscopy (LSCM). The combination of the 488 nm (excitation of BODIPY fluorophore), 568 nm, and 647 nm (excitation of SYTO 61) laser lines of a Krypton-Argon ion laser (Bio-Rad, Hercules, CA) with total laser fiber output power of 11 mW attenuated by a 1% or 3% neutral density filter provided for excitation of the probes. BODIPY and SYTO 61 fluorescence emission bands were collected simultaneously over the ranges 505–540 and 664–696 nm using bandpass filters. Image files were quantitatively analyzed using image analysis software.

In order to examine the localization parameters of the BODIPY probes within the nucleus, the fluorescence originating from the nuclei of hepatocytes was distinguished from the cytoplasm by using the fluorescent probe SYTO 61 whose emission (maximum at 650 nm and imaged over 664–696 nm) does not overlap with the emission of the BODIPY labeled probes (maximum at 511 nm and imaged over 505–540 nm). The SYTO dyes stain nucleic acids with a relatively high quantum yield as compared to its fluorescence in aqueous. However, since the SYTO stains both nuclear and mitochondrial DNA, both were fluorescently labeled. Thus a selection of the nuclei vs. the mitochondrial DNA regions was made by identification of cells with stronger nuclear staining and no overlap of mitochondria with the nuclear envelope. For convenience, a binary mask of the nuclei from the hepatocytes images was created from the SYTO 61 fluorescence (red channel) using thresholding for suppression of the low level

cytoplasm staining (e.g. a black and white image of the thresholded SYTO 61 emission). Thus, the white pixels within the mask represent every pixel in the nucleus wherein SYTO stained the DNA. This was repeated for all of the images involving the BODIPY FL C₁₆ labeled hepatocytes. Subsequently, a logical AND operation was performed using the original image of BODIPY FL C₁₆ fluorescence (green channel) with the corresponding binary image in MetaMorph 4.5 (Advanced Scientific Imaging, Merieux, LA). This process leaves the original intensities unchanged for the colocalized regions of the hepatocyte nuclei while the pixels outside the nuclei were set to 0 (black). The one-dimensional intensity profiles of the fluorescent fatty acids within nuclei were generated using Lasersharp 3.0 (Bio-Rad, Hercules, CA) while the fluorograms were created using FLUOROGRAM_IM as a correlation plot of the red and green intensities ($I_i^r I_i^g$) from each *i*th pixel of a merged image [34]. The colocalization coefficients were calculated as described [34–36]. Two populations were selected according to ranking of the intensity distributions ($I_{red} > I_{green}$ and $I_{red} < I_{green}$; denoted by Region I and II respectively) as identified visually in the fluorogram. From the selections, only the pixels with the corresponding intensities, I_{red} and I_{green} , were shown while all other intensities outside of the selected range were set to zero (black) in the segmented nuclei image. From the marked intensity distributions of BODIPY FL C₁₆, nuclear membrane regions and interior nuclear regions were separable. From the integrated intensities, an approximate proportion of BODIPY FL C₁₆ was calculated for the nuclear membrane envelope as compared to overall nucleus.

In order to validate the aforementioned technique, hepatocytes from L-FABP^{+/+} mice were incubated with BODIPY FL C₁₆ and Nile red. Both probes, BODIPY FL C₁₆ and Nile red, were imaged simultaneously using two channels and bandpass filters covering the two emission regions, 505–540 and 578–618 nm, with excitation wavelengths of 488 and 568 nm, respectively. Using FLUOROGRAM_IM, the pixels belonging to the BODIPY FL C₁₆ (green) that did not colocalize with Nile red (red) were selected and an image was obtained containing only the non-colocalizing pixels. The non-colocalizing pixel intensities remained unchanged while the colocalized pixel intensities were set to 0 (black). A multi-colored look-up table (LUT) was applied to visualize the resulting intensity variations of the fluorescent fatty acid in the hepatocytes nucleus.

Statistics

Values represented the mean ± SE with *n* and *P* indicated as described. Statistical analyses were performed using Student's *t*-test or two-way analysis of variance (ANOVA) (GraphPad Prism, San Diego, CA). Values with *P* < 0.05 were considered statistically significant.

RESULTS

Hepatocyte Culture

In vitro studies with liver homogenates [37;38] and serum [15;39] from L-FABP^{-/-} mice [15;19;39] show that L-FABP enhances hepatic uptake, cytosolic pool size, and oxidation of long chain fatty acid (LCFA). Since LCFA are known to regulate the transcriptional activity of the nuclear receptor peroxisome proliferator activated receptor α (PPARα), it has been hypothesized that L-FABP may regulate the uptake and transport of LCFA ligands to the nucleus for activating PPARα (rev. in [10]). Examining this possibility at the individual hepatocyte level, primary hepatocytes were isolated from livers of L-FABP^{+/+} and L-FABP^{-/-} mice and established in culture. Viability and function were then determined as a function of time in culture. Morphologic examination of transmission micrographs of cultured primary hepatocytes isolated from L-FABP^{-/-} (Fig. 1A) and L-FABP^{+/+} (Fig. 1B) mice indicated the presence of multi-nucleated cells characteristic of liver cells. Hepatocyte viability over the time period of the experiments described herein was confirmed by the following assays: (i) A

fluorescence live/dead stain assay of viability, showed high cellular fluorescence of calcein (green cytoplasmic stain) and dark nuclei, i.e. no red fluorescence due to ethidium bromide homodimer, for live cells (Fig. 1C, majority of cells) while dead cells were characterized by dark cytoplasm (i.e. absence of green fluorescence due to cytoplasmic calcein) and the presence of ethidium bromide homodimer in the nuclei (red nuclei, dead cell shown in lower middle of Fig. 1C). Examination of multiple chambers of cultured hepatocytes with this dual stain assay confirmed that primary hepatocyte viability was typically >90%. (ii) Cultured primary hepatocytes secreted albumin, monitored by Western blot analysis, at a constant level up to and past 5 days in culture for hepatocytes isolated from livers of L-FABP^{-/-} (Fig. 1D) and wild type L-FABP^{+/+} (Fig. 1E) mice. Continued secretion of albumin is a characteristic of viable, long-term primary hepatocytes in culture [40]. (iii) L-FABP expression, determined by Western analysis of multiple blots, was maintained for 3 days in cultured primary hepatocytes from L-FABP^{+/+} mice, but significantly decreased 4–5 days post isolation (Fig. 1F). The housekeeping gene GAPDH was used to ensure equal protein loading as described in Methods. As expected, L-FABP was not detectable in cultured primary hepatocytes isolated from livers of L-FABP^{-/-} mice (Fig. 2B). Based on the results of the viability and functional parameters of primary hepatocytes in culture, all subsequent experiments were performed with hepatocytes maintained in culture 1–2 days.

Effect of L-FABP gene ablation on PPAR α protein and mRNA levels

Ligand-dependent activation of PPAR α elicits transcription of PPAR α itself [45], proteins involved in LCFA uptake (L-FABP) [46], as well as key enzymes/proteins involved in intracellular LCFA oxidation including carnitine palmitoyl CoA acyltransferase (CPT1), short chain acyl (butyryl) CoA dehydrogenase (ACADS), and acyl CoA oxidase (ACOX1) (rev. in [1;2]). Since L-FABP binds the same endogenous ligands as PPAR α (rev. in [4;5;10]), the possibility that loss of L-FABP may influence LCFA metabolism by downregulating expression of PPAR α was examined by Western blotting and real-time PCR in cultured primary hepatocytes. The protein level of PPAR α was decreased 2-fold ($P < 0.001$, $n = 3$) in cultured primary hepatocytes from livers of L-FABP^{-/-} mice (Fig. 2A, D). In addition, L-FABP gene ablation tended to reduce transcription of PPAR α mRNA in cultured primary hepatocytes (Fig. 2E *right panel*)—an effect that achieved statistical significance ($P < 0.015$) in mouse liver (Fig. 2E *left panel*). The fact that levels of PPAR α were down-regulated in hepatocytes derived from L-FABP null mice suggested that L-FABP has a role in PPAR α protein expression.

Next, comparative mRNA levels were determined in cultured primary hepatocytes (Fig. 2E) for key PPAR α -regulated hepatic enzymes [carnitine palmitoyl CoA acyltransferase 1 (CPT1), short chain acyl (butyryl) CoA dehydrogenase (ACADS), acyl CoA oxidase (ACOX1)] in peroxisomal and mitochondrial β -oxidation. The interactive effects of the two factors, L-FABP expression and the group of mRNA levels of PPAR α combined with genes associated with both peroxisomal and mitochondrial β -oxidation enzymes in hepatocytes, were analyzed by two-way analysis of variance (ANOVA). The reductions in the mRNA levels of CPT1, ACADS, ACOX1, and PPAR α were significant ($P < 0.016$) for hepatocytes of L-FABP^{-/-} vs. L-FABP^{+/+} mice. The two-way ANOVA was repeated comparing the interaction between the L-FABP expression and the oxidative factor including only CPT1, ACADS, and ACOX1, which again showed significant differences ($P < 0.023$) for L-FABP^{-/-} vs. L-FABP^{+/+} mouse hepatocytes. The data would suggest that some factor was negatively affecting the level of PPAR α mRNA and protein as well as the mRNAs of several peroxisomal and mitochondrial β -oxidation enzymes whose genes are transcriptionally regulated by PPAR α .

Fatty acid uptake and oxidation

Since L-FABP overexpression in transformed cells (e.g. L-cell fibroblasts, hepatoma cells) enhanced LCFA uptake and oxidation [22;41–44], it was predicted that L-FABP gene ablation

should decrease LCFA uptake and oxidation in cultured primary hepatocytes. Consistent with this expectation, L-FABP gene ablation inhibited both the uptake and oxidation of LCFA (i.e. palmitic acid) in cultured primary hepatocytes (Fig. 3). The uptake of [9,10-³H]-palmitic acid was reduced by 42% ($p < 0.05$) in cultured primary hepatocytes from livers of L-FABP $-/-$ mice (Fig. 3A). Likewise, the oxidation of [9,10-³H]-palmitic acid was reduced by 40% ($p < 0.05$) in cultured primary hepatocytes from livers of L-FABP $-/-$ mice (Fig. 3B). Combining these results that L-FABP gene ablation reduced LCFA oxidation in cultured primary hepatocytes (Fig. 3B) and, conversely, L-FABP overexpression enhanced LCFA oxidation in transformed cells [22], these data were consistent with the idea that reduced LCFA uptake in L-FABP null hepatocytes (Fig. 3A) negatively affected the level of PPAR α mRNA and protein as well as the mRNAs of several peroxisomal and mitochondrial β -oxidation enzymes whose genes are transcriptionally regulated by PPAR α . Thus, L-FABP expression level positively regulated LCFA uptake and oxidation not only in transformed cell lines, but also in the more physiologically relevant system, i.e. primary hepatocytes from gene targeted mice.

Effect of L-FABP gene ablation on coactivator (SRC-1) and corepressor (N-CoR) recruitment to PPAR α : Co-immunoprecipitation

In the absence of added exogenous LCFA (C16:0, palmitic acid), L-FABP gene ablation enhanced PPAR α co-recruitment (co-immunoprecipitation) of the co-activator SRC-1 by 2-fold (Fig. 4A,B) and, even more so (3-fold) that of the co-repressor N-CoR (Fig. 4C,D) from liver homogenates. In the presence of added exogenous LCFA (C16:0, palmitic acid), L-FABP gene ablation inhibited the increase in corecruitment of co-activator SRC-1 (Fig. 4A,B), but increased co-recruitment of co-repressor N-CoR (Fig. 4C,D). Thus, L-FABP gene ablation, especially in the presence of exogenous palmitic acid, markedly influenced PPAR α co-recruitment of co-repressor N-CoR more than co-activator SRC-1—consistent with reduced expression of PPAR α and PPAR α -regulated LCFA oxidative enzymes (Fig. 2).

Effect of L-FABP overexpression on PPAR α transactivation of acyl-CoA oxidase

To determine the effect of L-FABP on PPAR α transactivation, control and L-FABP overexpressing COS-7 cells were transfected with PPAR α (Fig. 5A), RXR α (not shown), PPAR α and RXR α (Fig. 5B), or empty pSG5 plasmid (not shown) and analyzed for transactivation of an acyl-CoA oxidase reporter construct in the absence (open bars) and presence (filled bars) of palmitic acid. Transactivation was measured as percent firefly luciferase activity normalized to *Renilla* luciferase (internal control) where PPAR α activity in control cells in the absence of palmitic acid was arbitrarily set to 100%. In the absence of added exogenous LCFA (C16:0, palmitic acid), L-FABP overexpression inhibited the transactivation activity of PPAR α (Fig. 5A) as well as PPAR α and RXR α together (Fig. 5B). Conversely, added exogenous LCFA (C16:0, palmitic acid), enhanced the transactivation activity of PPAR α (Fig. 5A) as well as PPAR α and RXR α together (Fig. 5B), especially when compared to no added palmitic acid. These effects were specific to PPAR α since neither palmitic acid nor L-FABP overexpression had any effect on RXR α activity alone or basal pSG5 activity (not shown). Although the reason for this decrease is not completely clear, two possibilities may be suggested: (i) As shown below, the presence of L-FABP increases the cellular content and acyl chain composition of the free fatty acids; (ii) Endogenous fatty acids bound to purified L-FABP are comprised not only of palmitic acid (C16:0), but many other species differing in chain length (C16–C22) and unsaturation (saturated, monounsaturated, polyunsaturated) [47]; (iii) Activation of PPAR α is highly dependent on fatty acid chain length and unsaturation (rev. in [4;5]. While these results obtained with COS-7 cells may not completely reflect those in hepatocytes, nevertheless the data were consistent with L-FABP overexpression enhancing the palmitic acid-mediated activation of PPAR α -regulated LCFA oxidative enzymes (Fig. 2E).

Effect of L-FABP gene ablation on total LCFA and LCFA-CoA in cultured primary hepatocytes

Since L-FABP gene ablation reduced the uptake of exogenous LCFA (e.g. C16:0/palmitic acid; Fig. 3), L-FABP may be hypothesized to affect LCFA-activated transcriptional activity of PPAR α in part by altering the total cellular pool of LCFA and LCFA-CoA potentially available for transport/targeting into the nucleus of cultured primary hepatocytes. To examine this possibility, total LCFA and LCFA-CoA levels were determined in cultured primary hepatocytes isolated from livers of wild-type L-FABP $^{+/+}$ and L-FABP $^{-/-}$ mice. L-FABP gene ablation reduced hepatocyte total LCFA content 1.5-fold ($p < 0.01$, $n = 4$) from 108 ± 10 nmol/mg cell protein to 70 ± 4 nmol/mg cell protein (Table 1). In contrast, total LCFA-CoA was increased 2-fold ($p < 0.05$) from 0.097 ± 0.012 to 0.205 ± 0.038 nmol/mg cell protein in cultured primary hepatocytes isolated from L-FABP $^{-/-}$ mice (Table 1). Although, L-FABP gene ablation increased the ratio of total LCFA-CoA/LCFA by >3 -fold, LCFA still comprised $>99\%$ of the total (LCFA-CoA + LCFA) in cultured primary hepatocytes.

Effect of L-FABP gene ablation on acyl composition of LCFAs in cultured primary hepatocytes

Based on the 2–3 fold higher affinity of L-FABP for unsaturated vs saturated fatty acids [48; 49], the possibility that L-FABP gene ablation may alter fatty acyl chain distribution of the LCFA pools was examined in cultured primary hepatocytes. In general, LCFA of cultured primary hepatocytes from L-FABP $^{-/-}$ mice exhibited significantly decreased masses (nmol/mg cell protein) of several of LCFAs (Table 2) including 18:0, 18:1 n-9, 18:2 n-6, 18:3 n-3, 20:4 n-6, and 22:6 n-3. There was no clear pattern of L-FABP gene ablation specifically altering the relative proportions of individual longer-chain vs shorter-chain LCFAs, yet the % composition of fatty acids in the wild type hepatocytes showed a general trend of 18:0 $>$ 16:0 $>$ 18:1 $>$ 18:2 $>$ 20:4 $>$ 22:6 $>$ 22:4 $>$ other LCFA, while the % composition for the L-FABP ablated hepatocytes was 16:0 $>$ 18:0, 18:1 $>$ 18:2 $>$ 22:6 $>$ 20:4 $>$ 22:4 $>$ other LCFA. However, L-FABP gene ablation significantly decreased the masses of polyunsaturated (1.6-fold, $p < 0.01$, $n = 4$), monounsaturated (1.4-fold, $p < 0.02$, $n = 4$), and saturated (1.6-fold, $p < 0.03$, $n = 4$) LCFAs (Table 2).

Affinity of BODIPY FL C₁₆ labeled fatty acid to PPAR α

While the above data indicated that L-FABP gene ablation markedly altered the hepatocyte total LCFA/LCFA-CoA levels, such studies do not directly measure the effect of L-FABP expression on nuclear distribution of these known PPAR α activator ligands in living hepatocytes. Because naturally-occurring LCFAs are rapidly metabolized (esterified, oxidized), the potential of an interaction of PPAR α with LCFA was tested by using BODIPY FL C₁₆ as described in Methods. BODIPY FL C₁₆ binding to PPAR α was measured by spectroscopically monitoring the quenching of PPAR α aromatic amino acid emission as measurement of BODIPY FL emission was complicated by significant fluorescence of these fluorophores in aqueous buffers alone. Saturation binding curves were obtained by plotting the PPAR α aromatic amino acid fluorescence emission intensity change, $F - F_0$, versus increasing concentration of BODIPY FL C₁₆ (Fig. 6) and, as shown by the insets in the respective panels, PPAR α bound the ligand with 1:1 molar stoichiometry. Fitting the binding curve as described in Methods yielded the dissociation constant of $K_d = 8.3 \pm 1.2$ nM for the poorly metabolizable fluorescent LCFA analogue, BODIPY FL C₁₆ (Fig. 6). Since PPAR α 's binding affinity for this ligand was in the same range as those of L-FABP ($K_d = 21$) [9], this suggested that BODIPY FL C₁₆ would be a useful probe for directly visualizing the effect of L-FABP gene ablation on LCFA distribution to the nucleus and nucleoplasm in living primary hepatocytes in culture.

Effect of L-FABP gene ablation on uptake and nuclear targeting of BODIPY FL C₁₆ in cultured primary hepatocytes

The effect of L-FABP gene ablation on LCFA uptake and distribution was determined in real-time by laser scanning confocal microscopy (LSCM) imaging of the fluorescent LCFA analog BODIPY FL C₁₆ in cultured primary hepatocytes. BODIPY FL C₁₆ was taken up into cultured primary hepatocytes and distributed most intensely in the cytoplasm with diffuse staining in the nucleus regardless of whether the cultured primary hepatocytes were derived from L-FABP +/+ (Fig. 7A) or L-FABP^{-/-} (Fig. 7D) mice. Quantitative analysis of multiple hepatocytes (n = 21–46) from L-FABP null mice showed that total uptake of BODIPY FL C₁₆ was reduced 1.3-fold ($p < 0.001$)—consistent with the lower total LCFA in cultured primary hepatocytes from L-FABP^{-/-} mice (Table 1).

To determine whether L-FABP gene ablation selectively reduced targeting of LCFAs to nuclei, cultured primary hepatocytes isolated from L-FABP^{+/+} and L-FABP^{-/-} mice were coincubated with the fluorescent LCFA analog BODIPY FL C₁₆ (Fig. 7A and Fig. 7D, respectively) and the nuclear DNA binding stain SYTO 61 (Fig. 7B and Fig. 7E, respectively). The merged images of BODIPY FL C₁₆ (green) and SYTO 61 (red) showed colocalized BODIPY FL C₁₆ with SYTO 61 appearing as orange/yellow pixels in the nuclei of cultured hepatocytes from livers of wild-type L-FABP^{+/+} mice (Fig. 7C) with a qualitatively similar pattern appearing in nuclei of cultured hepatocytes from livers of L-FABP^{-/-} mice (Fig. 7F). Quantitative intensity analysis from multiple hepatocytes indicated that L-FABP gene ablation reduced the extent of nuclear targeting of BODIPY FL C₁₆ by 30% (Fig. 8A, $p < 0.001$). However, since L-FABP gene ablation also reduced the intensity of BODIPY FL C₁₆ in the cytoplasm by 21% (Fig. 8B, $p < 0.05$), the ratio of BODIPY FL C₁₆ intensities in the nucleus/cytoplasm was not significantly altered (Fig. 8C).

Effect of L-FABP gene ablation on BODIPY FL C₁₆ distribution across the nucleus of cultured primary hepatocytes

In order to determine if L-FABP gene ablation affected the distribution of BODIPY FL C₁₆ within the nucleus (i.e. nucleoplasm vs nuclear envelope/membrane), plots of the intensity profiles across the hepatocyte nuclei were made on the masked images of hepatocyte nuclei. Representative plot of BODIPY FL C₁₆ fluorescent intensity across the nuclei of cultured primary hepatocytes from L-FABP^{+/+} mice illustrated a highly differential targeting of BODIPY FL C₁₆ distribution within the nucleus (Fig. 9A). Higher intensities of the fatty acid appeared on the fringes of the nucleus (nuclear envelope membranes) and decreased dramatically in the more central regions (nucleoplasm) of the nucleus. A fluorogram was created by plotting the intensity of the SYTO 61 (red) channel and intensity of the BODIPY FL C₁₆ (green) of the segmented nuclei of hepatocytes derived from L-FABP^{+/+} mice (Fig. 9B), allowing for a spatial examination of the intensity distribution of BODIPY FL C₁₆ within the nucleus. Although the BODIPY FL C₁₆ highly colocalized with the nuclear label (SYTO 61) across the nucleus, the plots showed broadening or decorrelation—consistent with differential partitioning of the probes across the nucleus. Therefore, an intensity correlation fluorogram of BODIPY FL C₁₆ was divided along the middle of the correlation distribution in order to create two populations of intensities. One population (Region II, Fig. 9B) involved the brighter pixels from higher concentrations of BODIPY FL C₁₆ (high green intensity) as compared to the concentration of SYTO 61 (red intensity). The pixels with intensities within this range were pseudo-colored yellow (Fig. 9C) and revealed that the concentrated regions of BODIPY FL C₁₆ were mostly localized along the nuclear envelope. Subsequently, the original pixels (see Methods) of the colocalized BODIPY FL C₁₆ (green) and SYTO 61 (red) making up this region of the fluorogram were shown (Fig. 9D). For the second population (Region I, Fig. 9B), pixels whose intensities in the fluorogram showed a lower concentration of BODIPY FL C₁₆ (low green intensities) as compared to SYTO 61 (red) were pseudo-colored magenta

(Fig. 9D). Similarly, the original pixels (see Methods) of the colocalized BODIPY FL C₁₆ (green) and SYTO 61 (red) making up Region I of the fluorogram were shown (Fig. 9E). The fluorescent fatty acid as represented by these pixels was seen distributed throughout the hepatocyte nucleoplasm. The mean ratio of intensities of BODIPY FL C₁₆ mostly along the nuclear envelope to that of the nucleoplasm was 3.35 ± 0.05 .

Since it was possible that the SYTO 61 probe may have influenced the distribution, hepatocytes from L-FABP^{+/+} mice were again labeled with BODIPY FL C₁₆ and co-labeled with Nile red—a probe that labels neutral lipids, previously used to delineate cytoplasmic-spatio regions. [50]. The merged image with the 8-bit channel intensities resulting from confocal detected emission of BODIPY FL C₁₆ were pseudo-colored green while those intensities resulting from emission of Nile red were pseudo-colored red, shown Fig. 10A. Since Nile red does not significantly stain the nuclei of living cells (Fig. 10A), good isolation of the fatty acid in the nuclei was obtained. Thus, the green pixel intensities with very low background red intensity (<10) were selected using FLUOROGRAM_IM. The resulting BODIPY FL C₁₆ pixels were pseudo-colored by range of levels of intensity using a color lookup table (LUT) shown along the right side by the color bar (Fig. 10B). From the different colors in Fig. 10B, the intensities at the nucleus boundary/nuclear membrane typically comprised the highest intensity as compared to the nucleoplasm regions. This result obtained by segmentation of the nucleus using a cytoplasmic component marker was similar to those obtained with the segmentation and inversion technique using the nuclear stain, SYTO 61 (Fig. 9).

Analysis of the intensity profile of BODIPY FL C₁₆ across the nuclei of cultured primary hepatocytes from L-FABP^{-/-} mice showed a more highly differential targeting of the fatty acid probe distribution (Fig. 11A) as compared to that in L-FABP^{+/+} hepatocytes (Fig. 9A). Two nuclear populations of BODIPY FL C₁₆ were also resolved in cultured primary hepatocytes from L-FABP null hepatocytes. The selection of higher concentration of BODIPY FL C₁₆ was made (Region II, Fig. 11B). The pixels with intensities within this range were pseudo-colored yellow (Fig. 11C) and again revealed that the concentrated regions of BODIPY FL C₁₆ were mostly localized along the nuclear envelope; also evidenced by the original pixels (see Methods) of the colocalized BODIPY FL C₁₆ (green) and SYTO 61 (red) making up this region of the fluorogram were shown (Fig. 11D). For the second population (Region I, Fig. 11B), pixels whose intensities in the fluorogram showed a lower concentration of BODIPY FL C₁₆ (low green intensities) as compared to SYTO 61 (red) were pseudo-colored magenta (Fig. 11D). Both these and the original pixels (see Methods) of the colocalized BODIPY FL C₁₆ (green) and SYTO 61 (red) making up Region I of the fluorogram were shown (Fig. 11E) show that the fluorescent fatty acid was seen distributed throughout the hepatocyte nucleoplasm. For comparison, the ratio of intensities of BODIPY FL C₁₆ mostly along the nuclear envelope to that of the nucleoplasm was 2.13 ± 0.08 . In hepatocytes from L-FABP^{-/-} mice, this ratio was 37% lower ($p < 0.05$) than that observed for cultured primary hepatocytes from L-FABP^{+/+} mice. In hepatocyte nuclei from L-FABP^{+/+} mice, $23 \pm 1\%$ of the BODIPY FL C₁₆ fluorescence emission observed within the nucleus occurred at the nuclear envelope region with the remaining $77 \pm 1\%$ in the nucleoplasm. In contrast, hepatocyte nuclei from L-FABP^{-/-} mice exhibited $32 \pm 4\%$ of the BODIPY FL C₁₆ fluorescence within the nuclear envelope region with the remaining $68 \pm 4\%$ in the nucleoplasm. Thus, L-FABP gene ablation significantly ($P < 0.05$) decreased the BODIPY FL C₁₆ appearing in the nucleoplasm.

In summary, although L-FABP gene ablation did not significantly alter the relative distribution of BODIPY FL C₁₆ to the nucleus vs cytoplasm, the relative proportion of nuclear BODIPY FL C₁₆ distributed to the nucleoplasm was significantly decreased. Taken together with the fact that L-FABP decreased the total uptake of BODIPY FL C₁₆ (see above) and C16 (palmitic acid, Fig. 3) as well as decreased the total cellular LCFA content (Table 1), these data showed

that L-FABP gene ablation significantly decreased the nuclear content (especially in nucleoplasm) of LCFA available for interaction with PPAR α .

Use of the non-metabolizable BODIPY FL C₁₆ provided a unique opportunity to examine the effect of L-FABP gene ablation on the uptake of the LCFA into the hepatocyte, especially the nucleus, and to examine the potential nuclear distribution of LCFA. Since LCFA-CoAs quantitatively accounted for nearly 100-fold lower mass than LCFAs, the predominant effect of L-FABP gene ablation on the total mass of potential ligands distributed to the nucleus was primarily on LCFA.

DISCUSSION

It has been postulated that L-FABP may play a longer-acting role in regulating hepatic fatty acid oxidation/metabolism by participating in an intricate interplay between both cytoplasmic (e.g. L-FABP) and nuclear receptor (e.g. PPAR α) proteins that bind and are activated by LCFAs and LCFA-CoAs (rev. in [4;5;10;28]). In this scheme it is hypothesized that L-FABP binds and then co-transportes the bound ligands into the nucleus for delivery to PPAR α . The purpose would be to induce numerous genes encoding enzymes/proteins involved in LCFA oxidation and gluconeogenesis. While appealing, this hypothesis is based largely on assays performed *in vitro* with transformed transfected cells overexpressing L-FABP in culture. The present work provides studies with cultured primary hepatocytes from L-FABP gene ablated mice that yield significant new mechanistic insights into this evolving hypothesis.

First, L-FABP gene ablation markedly reduced the level of unesterified LCFA within cultured primary hepatocytes. Although L-FABP gene ablation did not alter the % acyl chain composition of unesterified LCFAs in cultured primary hepatocytes, the total mass of unesterified LCFAs was decreased 35%. The decreased unesterified LCFA mass in L-FABP null hepatocytes correlated with 42% decreased uptake of [9,10-³H]-palmitic acid and 25% decreased uptake of BODIPY FL C₁₆. These changes were consistent with less LCFA ligand available for interaction with/activation of PPAR α in the nucleus/nucleoplasm of cultured primary hepatocytes from L-FABP gene ablated mice. While hepatocyte levels of LCFA-CoA were also altered by L-FABP gene ablation, the total mass of LCFA-CoA represented nearly 100-fold less than that of LCFA—indicating the L-FABP might regulate PPAR α transcriptional activity primarily by altered cellular and nuclear (especially nucleoplasmic) levels LCFAs rather than LCFA-CoAs.

Second, real-time confocal imaging of living cultured primary hepatocytes L-FABP gene ablation decreased modestly, but significantly, reduced the % distribution of a fluorescent LCFA analogue (BODIPY FL C₁₆). Conversely, L-FABP overexpression in cultured L-cell fibroblasts increased the distribution of BODIPY FL C₁₆ and other fluorescent LCFA analogues to the nucleus. Thus, the modest decrease in % BODIPY FL C₁₆ in the nucleoplasm vs cytoplasm, together with the greater decrease in total LCFA mass, indicated that L-FABP gene ablation decreased the total LCFA appearing in the nucleoplasm of cultured primary hepatocytes. Again, reduced nucleoplasmic LCFA was consistent with the reduced activation of PPAR α in cultured primary hepatocytes from L-FABP gene ablated mice.

Third, L-FABP gene ablation significantly decreased the mRNA and protein levels of the LCFA activated nuclear receptor PPAR α in cultured primary hepatocytes. The reduced PPAR α may be explained in part based on the fact that the PPAR α promoter contains a regulatory element to which PPAR α binds and may thereby positively autoregulate its own expression [45;46]. These findings suggest that L-FABP gene ablation may feedback regulate PPAR α expression through transfer of bound endogenous ligand (e.g. LCFA) to PPAR α .

Fourth, L-FABP gene ablation significantly reduced LCFA oxidation concomitant with reduced PPAR α activities both *in vitro* and in living cells. In the presence of exogenous LCFA (palmitic acid), PPAR α co-immunoprecipitation with coactivator (SRC-1) was decreased while that with corepressor (N-CoR) was increased by L-FABP gene ablation. L-FABP overexpression enhanced transactivation of the PPAR α regulated acyl CoA oxidase (ACOX), while L-FABP gene ablation reduced the mRNA levels of key enzymes (CPT1, butyryl ACADS, and ACOX1) known to be involved in peroxisomal and mitochondrial β -oxidation of LCFA (rev. in [1;2]. These reduced PPAR α activities in hepatocytes of L-FABP gene ablated mice concomitantly inhibited palmitic acid oxidation by over 40%.

The possibility that L-FABP may function to mediate LCFA transport into the nucleus for interaction with PPAR α is supported by a variety of studies performed *in vitro*, transfected transformed cells, and immunomaging in fixed cells. A key requirement is that L-FABP must actually enter the nucleus. *In vitro* studies with purified nuclei show that LCFA entry into nuclei is facilitated by L-FABP—suggesting co-transport of L-FABP bound ligand [11]. Immunogold electron microscopy and immunofluorescence confocal microscopy detected L-FABP in nuclei [9;12;51]. While the mechanism whereby L-FABP passes through the nuclear envelope pores is not yet been resolved, one possibility is passive diffusion. The hydrodynamic diameter of L-FABP (14 kDa) is only about 36 Å [48;52]. Because small proteins such as the 13 kDa (cytochrome C) diffuse freely through the nuclear pores (90 Å diameter), L-FABP is sufficiently small to passively diffuse through nuclear pores. Double immunofluorescence colocalization, co-immunoprecipitation, and transactivation studies in transfected tumorigenic cells suggest that L-FABP is in close proximity and may directly interact with PPAR α to facilitate bound LCFA transfer in the nucleus (rev. in [10]. Potential importance of such an interaction is underscored by studies with the closely related cellular retinoic acid binding protein-2 (CRABP-2). As shown by stopped flow fluorescence spectroscopy, CRABP-2 has entered nuclei and directly interacted with retinoid X receptor to transfer bound ligand (rev. in [10;53;54].

In summary, earlier studies performed *in vitro* and with transfected transformed cells suggested that L-FABP plays a direct, short-acting, role in facilitating LCFA uptake and delivery of bound ligands to metabolic enzymes/proteins (rev. [10]. The contribution of the present work was to resolve new, longer-acting roles of L-FABP in regulation of the ligand (LCFA) activated nuclear regulatory protein PPAR α . The data presented herein with cultured primary hepatocytes from wild-type L-FABP $^{+/+}$ and L-FABP $^{-/-}$ mice were consistent with the hypothesis that L-FABP and PPAR α coordinately regulate LCFA metabolism by a mechanism whereby L-FABP enhances LCFA uptake, co-transport bound LCFA into the nucleus, and delivers bound LCFA ligand to PPAR α for enhancing transcriptional activity. These findings, in a physiologically relevant system (primary hepatocytes), combined with new *in vitro* and imaging experiments point to a functional interaction of L-FABP with PPAR α in LCFA metabolism.

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Abbreviations

L-FABP

Liver fatty acid binding protein

PPAR α

peroxisome proliferator activated receptor- α

CPT1	carnitine palmitoyl CoA acyltransferase
ACADS	short chain acyl (butyryl) CoA dehydrogenase
ACOX1	acyl CoA oxidase
CRABP	cellular retinoic acid binding protein
RXR	retinoid x receptor
SRC-1	steroid receptor coactivator-1
N-CoR	nuclear receptor corepressor
LCFA	unesterified long chain fatty acid
LCFA-CoA	long chain fatty acyl CoA
LSCM	laser scanning confocal microscopy
BODIPY FL C₁₆	4, 4-difluoro-5, 7-dimethyl-4-bora-3a, 4a-diaza-s-indacene-hexadecanoic acid

References

1. Francis GA, Fayard E, Picard F, Auwerx J. Nuclear receptors and the control of metabolism. *Annu Rev Physiol* 2003;65:261–311. [PubMed: 12518001]
2. Desvergne B, Wahli W. Peroxisome proliferator activated receptors: nuclear control of metabolism. *Endocrine Rev* 1999;20:649–688. [PubMed: 10529898]
3. Kersten S, Desvergne B, Wahli W. Roles of PPARs in health and disease. *Nature* 2000;405:421–424. [PubMed: 10839530]
4. Hostetler HA, Petrescu AD, Kier AB, Schroeder F. Peroxisome proliferator activated receptor alpha (PPARalpha) interacts with high affinity and is conformationally responsive to endogenous ligands. *J Biol Chem* 2005;280:18667–18682. [PubMed: 15774422]
5. Hostetler HA, Kier AB, Schroeder F. Very-long-chain and branched-chain fatty acyl CoAs are high affinity ligands for the peroxisome proliferator-activated receptor alpha (PPARalpha). *Biochemistry* 2006;45:7669–7681. [PubMed: 16768463]
6. Lin Q, Ruuska SE, Shaw NS, Dong D, Noy N. Ligand selectivity of the peroxisome proliferator-activated receptor α . *Biochem* 1999;38:185–190. [PubMed: 9890897]
7. Huang H, Starodub O, McIntosh A, Kier AB, Schroeder F. Liver fatty acid binding protein targets fatty acids to the nucleus: real-time confocal and multiphoton fluorescence imaging in living cells. *J Biol Chem* 2002;277:29139–29151. [PubMed: 12023965]
8. Elholm M, Garras A, Neve S, Tarnehave D, Lund TB, Skorge J, Flatmark T, Kristiansen K, Berge RK. Long chain acyl CoA esters and acyl CoA binding protein are present in the nucleus of rat liver cells. *J Lipid Res* 2000;41:538–545. [PubMed: 10744774]

9. Huang H, Starodub O, McIntosh A, Atshaves BP, Woldegiorgis G, Kier AB, Schroeder F. Liver fatty acid binding protein colocalizes with peroxisome proliferator receptor alpha and enhances ligand distribution to nuclei of living cells. *Biochemistry* 2004;43:2484–2500. [PubMed: 14992586]
10. Schroeder F, Petrescu AD, Huang H, Atshaves BP, McIntosh AL, Martin GG, Hostetler HA, Vespa A, Landrock K, Landrock D, Payne HR, Kier AB. Role of fatty acid binding proteins and long chain fatty acids in modulating nuclear receptors and gene transcription. *Lipids* 2008;43:1–17. [PubMed: 17882463]
11. Lawrence JW, Kroll DJ, Eacho PI. Ligand dependent interaction of hepatic fatty acid binding protein with the nucleus. *J Lipid Res* 2000;41:1390–1401. [PubMed: 10974046]
12. Schroeder F, Atshaves BP, Starodub O, Boedeker AL, Smith R, Roths JB, Foxworth WB, Kier AB. Expression of liver fatty acid binding protein alters growth and differentiation of embryonic stem cells. *Mol Cell Biochem* 2001;219:127–138. [PubMed: 11354243]
13. Atshaves BP, Petrescu A, Starodub O, Roths J, Kier AB, Schroeder F. Expression and Intracellular Processing of the 58 kDa Sterol Carrier Protein 2/3-Oxoacyl-CoA Thiolase in Transfected Mouse L-cell Fibroblasts. *J Lipid Res* 1999;40:610–622. [PubMed: 10191285]
14. Bradford M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Anal Biochem* 1976;72:248–254. [PubMed: 942051]
15. Martin GG, Danneberg H, Kumar LS, Atshaves BP, Erol E, Bader M, Schroeder F, Binas B. Decreased liver fatty acid binding capacity and altered liver lipid distribution in mice lacking the liver fatty acid binding protein (L-FABP) gene. *J Biol Chem* 2003;278:21429–21438. [PubMed: 12670956]
16. Lanford RE, Carey KD, Estlack LE, Smith GC, Hay RV. Analysis of plasma protein and lipoprotein synthesis in long term primary cultures of baboon hepatocytes maintained in serum-free medium. *In Vitro Cell Devel Biol* 1989;25:174–182. [PubMed: 2921235]
17. Atshaves BP, McIntosh AL, Payne HR, Gallegos AM, Landrock K, Maeda N, Kier AB, Schroeder F. Sterol carrier protein-2/sterol carrier protein-x gene ablation alters lipid raft domains in primary cultured mouse hepatocytes. *J Lipid Res* 2007;48:2193–2211. [PubMed: 17609524]
18. Atshaves BP, Storey S, McIntosh AL, Petrescu AD, Lyuksyutova OI, Greenberg AS, Schroeder F. Sterol carrier protein-2 expression modulates protein and lipid composition of lipid droplets. *J Biol Chem* 2001;276:25324–25335. [PubMed: 11333258]
19. Atshaves BP, McIntosh AL, Lyuksyutova OI, Zipfel WR, Webb WW, Schroeder F. Liver fatty acid binding protein gene ablation inhibits branched-chain fatty acid metabolism in cultured primary hepatocytes. *J Biol Chem* 2004;279:30954–30965. [PubMed: 15155724]
20. Atshaves BP, Storey SM, Petrescu AD, Greenberg CC, Lyuksyutova OI, Smith R, Schroeder F. Expression of fatty acid binding proteins inhibits lipid accumulation and alters toxicity in L-cell fibroblasts. *Am J Physiol* 2002;283:C688–C703.
21. Atshaves BP, Storey SM, Schroeder F. Sterol carrier protein-2/sterol carrier protein-x expression differentially alters fatty acid metabolism in L-cell fibroblasts. *J Lipid Res* 2003;44:1751–1762. [PubMed: 12810824]
22. Atshaves BP, Storey S, Huang H, Schroeder F. Liver fatty acid binding protein expression enhances branched-chain fatty acid metabolism. *Mol Cell Biochem* 2004;259:115–129. [PubMed: 15124915]
23. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* 2001;25:402–408. [PubMed: 11846609]
24. Jefferson JR, Slotte JP, Nemezc G, Pastuszyn A, Scallen TJ, Schroeder F. Intracellular sterol distribution in transfected mouse L-cell fibroblasts expressing rat liver fatty acid binding protein. *J Biol Chem* 1991;266:5486–5496. [PubMed: 2005092]
25. Patsouris D, Mandard S, Voshol PJ, Escher P, Tan NS, Havekes LM, Koenig W, Maerz W, Tafuri S, Wahli W, Mueller M, Kersten S. PPARalpha governs glycerol metabolism. *J Clin Invest* 2004;114:94–103. [PubMed: 15232616]
26. Leid M, Kastner P, Lyons R, Nakshatri H, Saunders M, Zacharewski T, Chen JY, Staub A, Garnier JM, Mader S, Chambon P. Purification, cloning, and RXR identity of the hela cell factor with which RAR or TR heterodimerizes to bind target sequences efficiently. *Cell* 1992;58:377–395. [PubMed: 1310259]

27. Kliewer SA, Umesono K, Noon DJ, Heyman RA, Evans RM. Convergence of 9-cis-retinoic acid and peroxisome proliferator signalling pathways through heterodimer formation of their receptors. *Nature* 1992;358:771–774. [PubMed: 1324435]
28. Hostetler HA, Huang H, Kier AB, Schroeder F. Glucose directly links to lipid metabolism through high-affinity interaction with peroxisome proliferator activated receptor- α . *J Biol Chem* 2008;283:2246–2254. [PubMed: 18055466]
29. Spector AA, Hoak JC. An improved method for the addition of long chain free fatty acid to protein solutions. *Anal Biochem* 1969;32:297–302. [PubMed: 5407820]
30. Hara A, Radin NS. Lipid extraction of tissues with a low toxicity solvent. *Anal Biochem* 1978;90:420–426. [PubMed: 727482]
31. Martin GG, Huang H, Atshaves BP, Binas B, Schroeder F. Ablation of the liver fatty acid binding protein gene decreases fatty acyl CoA binding capacity and alters fatty acyl CoA pool distribution in mouse liver. *Biochem* 2003;42:11520–11532. [PubMed: 14516204]
32. Larson TR, Graham IA. A novel technique for the sensitive quantification of fatty acyl CoA esters from plant tissues. *The Plant Journal* 2001;25:115–125. [PubMed: 11169187]
33. Huang H, Atshaves BP, Frolov A, Kier AB, Schroeder F. Acyl-coenzyme A binding protein expression alters liver fatty acyl coenzyme A metabolism. *Biochemistry* 2005;44:10282–10297. [PubMed: 16042405]
34. McIntosh A, Gallegos A, Atshaves BP, Storey S, Kannoju D, Schroeder F. Fluorescence and multiphoton imaging resolve unique structural forms of sterol in membranes of living cells. *J Biol Chem* 2003;278:6384–6403. [PubMed: 12456684]
35. Demandolx D, Davoust J. Multicolour analysis and local image correlation in confocal microscopy. *Journal of Microscopy* 1997;185:21–36.
36. Atshaves BP, Starodub O, McIntosh AL, Roths JB, Kier AB, Schroeder F. Sterol carrier protein-2 alters HDL-mediated cholesterol efflux. *J Biol Chem* 2000;275:36852–36861. [PubMed: 10954705]
37. Schroeder F, Jolly CA, Cho TH, Frolov AA. Fatty acid binding protein isoforms: structure and function. *Chem Phys Lipids* 1998;92:1–25. [PubMed: 9631535]
38. McArthur MJ, Atshaves BP, Frolov A, Foxworth WD, Kier AB, Schroeder F. Cellular uptake and intracellular trafficking of long chain fatty acids. *J Lipid Res* 1999;40:1371–1383. [PubMed: 10428973]
39. Martin GG, Atshaves BP, McIntosh AL, Mackie JT, Kier AB, Schroeder F. Liver fatty acid binding protein (L-FABP) gene ablation alters liver bile acid metabolism in male mice. *Biochem J* 2005;391:549–560. [PubMed: 15984932]
40. White AL. Biogenesis of Lp (a) in transgenic mouse hepatocytes. *Clin Genet* 1997;52:326–337. [PubMed: 9520123]
41. Murphy EJ, Prows DR, Jefferson JR, Schroeder F. Liver fatty acid binding protein expression in transfected fibroblasts stimulates fatty acid uptake and metabolism *Biochim. Biophys Acta* 1996;1301:191–198.
42. Murphy EJ. L-FABP and I-FABP expression increase NBD-stearate uptake and cytoplasmic diffusion in L-cells. *Am J Physiol* 1998;275:G244–G249. [PubMed: 9688651]
43. Prows DR, Murphy EJ, Schroeder F. Intestinal and liver fatty acid binding proteins differentially affect fatty acid uptake and esterification in L-Cells. *Lipids* 1995;30:907–910. [PubMed: 8538377]
44. Wolfrum C, Buhlman C, Rolf B, Borchers T, Spener F. Variation of liver fatty acid binding protein content in the human hepatoma cell line HepG2 by peroxisome proliferators and antisense RNA affects the rate of fatty acid uptake. *Biochim Biophys Acta* 1999;1437:194–201. [PubMed: 10064902]
45. Torra ISP, Jamshidi Y, Flavell DM, Fruchart JC, Staels B. Characterization of the human PPAR α promoter: identification of a functional nuclear receptor response element. *Mol Endocrinology* 2002;16:1013–1028.
46. Fujishiro K, Fukui Y, Sato O, Kawabe K, Seto K, Motojima K. Analysis of tissue-specific and PPAR α dependent induction of FABP gene expression in the mouse liver by and in vivo DNA electroporation method. *Mol Cell Biochem* 2002;239:165–172. [PubMed: 12479582]

47. Murphy EJ, Edmondson RD, Russell DH, Schroeder F. Isolation and characterization of two distinct forms of liver fatty acid binding protein from the rat. *Biochim Biophys Acta* 1999;1436:413–425. [PubMed: 9989272]
48. Frolov A, Cho TH, Murphy EJ, Schroeder F. Isoforms of rat liver fatty acid binding protein differ in structure and affinity for fatty acids and fatty acyl CoAs. *Biochemistry* 1997;36:6545–6555. [PubMed: 9174372]
49. Norris AW, Spector AA. Very long chain n-3 and n-6 polyunsaturated fatty acids bind strongly to liver fatty acid binding protein. *J Lipid Res* 2002;43:646–653. [PubMed: 11907148]
50. McIntosh, A.; Atshaves, BP.; Huang, H.; Gallegos, AM.; Kier, AB.; Schroeder, F.; Xu, H.; Zhang, W.; Liu, S. Multiphoton laser scanning microscopy and spatial analysis of dehydroergosterol distributions on plasma membranes of living cells. McIntosh, T., editor. *Lipid Rafts*, Humana Press Inc; Totowa, N.J: 2007. p. 85-105.
51. Bordewick U, Heese M, Borchers T, Robenek H, Spener F. Compartmentation of hepatic fatty-acid-binding protein in liver cells and its effect on microsomal phosphatidic acid biosynthesis. *Biol Chem Hoppe-Seyler* 1989;370:229–238. [PubMed: 2653363]
52. Thompson J, Winter N, Terwey D, Bratt J, Banaszak L. The crystal structure of the liver fatty acid-binding protein. *J Biol Chem* 1997;272:7140–7150. [PubMed: 9054409]
53. Budhu AS, Noy N. Direct channeling of retinoic acid between cellular retinoic acid binding protein II and retinoic acid receptor sensitizes mammary carcinoma cells to retinoic acid induced growth arrest. *Mol Cell Biol* 2002;22:2632–2641. [PubMed: 11909957]
54. Tan NS, Shaw NS, Vinckenbosch N, Liu P, Yasmin R, Desvergne B, Wahli W, Noy N. Selective cooperation between fatty acid binding proteins and peroxisome proliferator activated receptors in regulating transcription. *Mol Cell Biol* 2002;22:5114–5127. [PubMed: 12077340]

Fig. 1. Primary mouse hepatocytes in culture

Inverted light transmission microscopy on liver hepatocytes isolated from L-FABP null (A) and wild-type (B) mice. Live/dead staining with ethidium bromide homodimer (red) and calcein (green) on hepatocytes isolated from L-FABP wild-type (C) mice. Live cells are indicated by a green cytoplasm with dark nucleus; dead cells by a dark cytoplasm and red nucleus. Western blotting (D–F) was performed by plating hepatocytes on 35 mm dishes (5×10^5 cells/dish, $n = 4$) for culture up to 5 days. The medium was removed each day and replaced with fresh serum-free medium. Removed medium was centrifuged to remove cell debris and proteins from the hepatocyte cell monolayer and aliquots from the medium over the 5 day period were subjected to Western blotting with antibodies to detect L-FABP or albumin. Western blots of incubation medium from hepatocytes isolated from L-FABP null (D) and wild-type (E) mice were probed with anti-albumin: Lane 1, molecular weight standard; Lane 2, albumin standard, Lane 3–7, days 1–5 in culture. Western blot of hepatocytes isolated from L-FABP wild-type (F) mice were probed with anti-LFABP: Lane 1 = molecular weight marker; Lane 2–6 = days 1–5 in culture, Lane 7, L-FABP standard. GAPDH signal was used as a loading control to normalize protein expression.

Fig. 2. Western blot and real-time PCR analysis of effects of L-FABP gene ablation in cultured primary hepatocytes

Western blots of homogenates from cultured primary hepatocytes derived from L-FABP^{+/+} and L-FABP^{-/-} mice were probed with the following affinity-purified antibodies: (A). anti-PPAR α . (B). anti-L-FABP. (C). GAPDH (used as a loading control to normalize protein expression). (D). Hepatic protein expression levels in wild type L-FABP^{+/+} and L-FABP^{-/-} mice were quantitated densitometrically and expressed as the mean integrated density values as described in Methods. (E). Quantitative real-time PCR was used to determine relative mRNA levels of PPAR α in the liver as well as PPAR α , carnitine palmitoyl CoA acyltransferase 1 (CPT1), short chain acyl (butyryl) CoA dehydrogenase (ACADS), and acyl CoA oxidase (ACOX1) in hepatocytes derived from L-FABP^{+/+} and L-FABP^{-/-} mice as described in Methods. Mean $\pm$ standard error, n = 3–5; (*) indicate significance P<0.05 and (#) indicate significance P<0.02 using two way ANOVA.

Fig. 3. Effect of L-FABP gene ablation on [9,10-³H] palmitic acid uptake and oxidation in mouse cultured primary hepatocytes

Total uptake (**A**) and oxidation (**B**) of palmitic acid was determined in cultured primary hepatocytes derived from L-FABP^{+/+} (open bar) and L-FABP^{-/-} (black bar) mice after 6 hours incubation with palmitic acid (50 nM) containing trace levels of [9,10-³H] palmitic acid (1.5 μ Ci/nmol) as described in Methods. Values represent means $\pm$ SEM. (*) indicates significance $P < 0.04$, $n = 4-6$ as compared to hepatocytes derived from L-FABP^{+/+} mice.

Fig. 4. Effect of L-FABP gene ablation on co-immunoprecipitation of PPARα with coactivator and corepressor proteins

Co-immunoprecipitation from liver homogenates was performed with anti-PPARα followed Western blot probing with anti-PPARα, anti-SRC-1, and anti-N-CoR as described in Methods. **(A)** Western blots of co-immunoprecipitated samples for steroid receptor coactivator-1 (SRC-1) and PPARα (as loading and elution control) from liver homogenates of wild type (L-FABP +/+, left columns) and L-FABP gene ablated (L-FABP -/-, right columns) mice in the absence and presence of palmitic acid. Western blot figures for SRC-1 and PPARα align with the bar graph below. **(B)** Fold change in co-immunoprecipitation of SRC-1 with PPARα from liver homogenates of wild type (L-FABP+/+) and L-FABP gene ablated (L-FABP-/-) mice in the absence (filled bars) and presence (open bars) of palmitic acid using a PPARα monoclonal antibody for the Co-IP. Protein values were standardized to protein values obtained for PPARα and normalized for each co-factor such that L-FABP +/+ sample in the absence of palmitic acid was equal to 1. Values represent the mean fold change in protein levels ± SEM, n = 4–5. (*) signify significant differences between wild type (L-FABP+/+) and L-FABP gene ablated (L-FABP-/-) samples, P<0.05. **(C)** Western blots of co-immunoprecipitated samples for the nuclear receptor corepressor (N-COR) and PPARα (as loading and elution control) from liver homogenates of wild type (L-FABP +/+, left columns) and L-FABP gene ablated (L-FABP -/-, right columns) mice in the absence and presence of palmitic acid. Western blot figures for N-COR and PPARα align with the bar graph below. **(D)** Fold change in co-immunoprecipitation of N-COR with PPARα from liver homogenates of wild type (L-FABP +/+) and L-FABP gene ablated (L-FABP -/-) mice in the absence (filled bars) and presence (open bars) of palmitic acid using a PPARα monoclonal antibody for the Co-IP. Protein values were standardized to protein values obtained for PPARα and normalized for each co-factor such that L-FABP +/+ sample in the absence of palmitic acid was equal to 1. Values represent the mean fold change in protein levels ± SEM, n = 4–5. (*) signify significant differences between wild type (L-FABP+/+) and L-FABP gene ablated (L-FABP-/-) samples, P<0.05.

Fig. 5. Effect of L-FABP overexpression on PPARα transactivation of acyl-CoA oxidase Control and L-FABP overexpressing COS-7 cells transfected with (A) PPARα, (B) PPARα and RXRα were analyzed for transactivation of an acyl-CoA oxidase reporter construct in the absence (open bars) and presence (filled bars) of palmitic acid. Transactivation values are presented as percent firefly luciferase activity normalized to *Renilla* luciferase (internal control) where PPARα activity in control cells in the absence of palmitic acid was arbitrarily set to 100%. Bar graph values represent the mean value ± the standard error (n = 4). (*) indicate significant differences between the control and L-FABP overexpressing cells, P < 0.05. (#) indicate significant differences due to the addition of palmitic acid, P < 0.05.

Fig. 6. Binding of fluorescent, poorly-metabolizable BODIPY- C_{16} fatty acid to PPAR α
 Direct binding of BODIPY FL C_{16} based on quenching of PPAR α aromatic amino acid fluorescence emission. The data are presented as the change in fluorescence intensity ($F - F_0$) plotted as a function of ligand concentration. PPAR α (0.1 μ M) was titrated with 0–1000 nM of BODIPY FL C_{16} . Analysis of binding curve as described in methods yielded $K_d = 8.3 \pm 1.2$ nM BODIPY FL C_{16} . Values are the mean $\pm$ standard error, $n = 4$. *Inset*, linear plot of the binding curve from each panel.

Fig. 7. Fatty acid targeting to the nucleus in cultured primary hepatocytes from L-FABP^{+/+} (A–C) and L-FABP^{-/-} (D–F) mice imaged by laser scanning confocal microscopy (LSCM) Hepatocytes were labeled with BODIPY FL C₁₆ fatty acid and SYTO 61 as described in Materials and Methods and imaged by LSCM. **(A and D)** Images of BODIPY FL C₁₆ fatty acid fluorescence emission in hepatocytes from L-FABP^{+/+} and L-FABP^{-/-} mice, respectively. **(B and E)** Images of fluorescence SYTO 61 labeling of hepatocytes from L-FABP^{+/+} and L-FABP^{-/-} mice, respectively. **(C and F)** Merged images showing the fluorescence emission of BODIPY FL C₁₆ fatty acid (green) and SYTO 61 (red) from only the nuclei of L-FABP^{+/+} and L-FABP^{-/-} mice, respectively. Segmentation of the nuclei using SYTO 61 was described in Materials and Methods.

Fig. 8. Effect of L-FABP gene ablation on BODIPY FL C₁₆ fatty acid distribution in cultured primary hepatocytes

Quantitative image analysis was performed on the images acquired by LSCM to yield the nuclear (A) and cytoplasmic (B) distribution of the fluorescent fatty acid analog BODIPY FL C₁₆ in hepatocytes derived from wild type L-FABP^{+/+} and L-FABP^{-/-} mice. (C) Ratio of BODIPY FL C₁₆ intensities in nucleus/cytoplasm. Data are presented as the mean $\pm$ standard error fluorescent intensity, n = 21–46. (*) indicates significance $P < 0.05$.

Fig. 9. BODIPY FL C₁₆ distribution across the nucleus of cultured primary hepatocytes from wild-type L-FABP^{+/+} mice

The fluorescently labeled nuclei of hepatocytes cultured from livers of wild type L-FABP^{+/+} mice were segmented as described in Materials and Methods. (A). An intensity profile of BODIPY FL C₁₆ fatty acid across a typical nucleus of a wild-type L-FABP^{+/+} mice hepatocyte at 30 minute incubation. (B). Fluorogram of the merged image in Fig. 7C. The population of pixels was divided into Regions I with lower green intensities and Region II with higher green intensities: (C). Image of nuclei showing the colocalized pixels in Region I (pseudo-colored magenta) and Region II (pseudo-colored yellow). (D). The inverted image of nuclei showing only the selected intensity population from Region II. These pixels show the higher concentration of BODIPY FL C₁₆ (green) labeling when compared to SYTO 61 emission (red), indicative of fatty acid in the nuclear membrane region. (E). The inverted image of nuclei showing only the selected intensity population Region I. These pixels have a lower

concentration of BODIPY FL C₁₆ (green) labeling when compared to SYTO 61 emission (red), indicative of fatty acid in the nucleoplasm.

Fig. 10. Non-cytoplasmic BODIPY FL C₁₆ distribution in cultured primary hepatocytes from wild-type L-FABP^{+/+} mice

Nile red (pseudo-colored red) was used to label neutral lipids within the cytoplasmic regions of primary hepatocytes cultured from livers of wild type L-FABP^{+/+} mice that were incubated with BODIPY FL C₁₆ fatty acid. (A). Hepatocyte with BODIPY FL C₁₆ (pseudo-colored green) and Nile red (pseudo-colored red). (B). Image of (A) showing only the pixels of BODIPY FL C₁₆ fatty acid that did not colocalize with Nile red. A multi-colored lookup table (LUT) was chosen with intensity ranges indicated by color bar (right side) for rapid visualization. The nucleus and plasma membrane (including microvilli) were distinctive as a result of removing the cytoplasmic distribution of the fatty acid pixels from the image.

Fig. 11. BODIPY FL C₁₆ distribution across the nucleus of cultured primary hepatocytes from L-FABP^{-/-} mice

The fluorescently labeled nuclei of hepatocytes cultured from livers of wild type L-FABP^{-/-} mice acquired using LSCM were segmented as described in Materials and Methods. (A). An intensity profile of BODIPY FL C₁₆ fatty acid across a typical nucleus of a wild-type L-FABP^{-/-} mice hepatocyte at 30 minute incubation. (B). Fluorogram of the merged image in Fig. 7F. The population of pixels was divided into Regions I with lower green intensities and Region II with higher green intensities: (C). Image of nuclei showing the colocalized pixels in Region I (pseudo-colored magenta) and Region II (pseudo-colored yellow). (D). The image of nuclei showing only the selected intensity population from Region II. These pixels show significant

amounts of BODIPY FL C₁₆ (green) labeling when compared to SYTO 61 emission (red), indicative of the fatty acid in the nuclear membrane region.. (**E**). The image of nuclei showing only the selected intensity population Region I. These pixels have a lower amount of BODIPY FL C₁₆ (green) labeling when compared to SYTO 61 emission (red), indicative fatty acid in the nucleoplasm.

Table 1

Effect of L-FABP gene ablation on total mass of LCFA and LCFA-CoA in cultured mouse primary hepatocytes.

Ligand	Mass (nmol/mg cell protein)	
	L-FABP +/+	L-FABP -/-
LCFA	108 ± 10	70 ± 4 *
LCFA-CoA	0.097 ± 0.012	0.205 ± 0.038 *

Values reflect the mean ± SE.

* indicates significance ($P \leq 0.05$, $n = 4-8$) as compared to cultured primary hepatocytes derived from livers of wild-type L-FABP+/+ mice.

Table 2

Effect of L-FABP gene ablation on acyl chain distribution of unesterified long chain fatty acids (LCFAs) in cultured primary hepatocytes.

Fatty Acyl Chain Distribution				
Fatty Acid	nmol/mg cell protein		% Composition	
	L-FABP (+/+)	L-FABP (-/-)	L-FABP (+/+)	L-FABP (-/-)
12:1 n-8	1.18 ± 0.08	1.3 ± 0.3	1.0 ± 0.07	1.8 ± 0.4
14:0	1.0 ± 0.1	1.0 ± 0.2	0.91 ± 0.09	1.4 ± 0.3
14:1 n-5	0.7 ± 0.1	0.9 ± 0.1	0.7 ± 0.1	1.3 ± 0.2
16:0	20 ± 4	16 ± 3	18 ± 4	22 ± 4
16:1 n-7	1.4 ± 0.2	1.1 ± 0.2	1.3 ± 0.2	1.6 ± 0.3
18:0	26 ± 4	11 ± 2 [*]	24 ± 4	16 ± 3
18:1 n-9	18 ± 1	11 ± 2 [*]	16.5 ± 0.9	16 ± 3
18:2 n-6	13 ± 2	7 ± 1 [*]	12 ± 2	10 ± 1
18:3 n-3	1.1 ± 0.1	0.6 ± 0.1 [*]	1.0 ± 0.1	0.9 ± 0.1
18:3 n-6	0.5 ± 0.1	0.7 ± 0.1	0.6 ± 0.1	0.9 ± 0.1
20:0	0.66 ± 0.02	0.8 ± 0.1	0.60 ± 0.02	1.1 ± 0.2
20:1 n-9	1.1 ± 0.1	1.2 ± 0.2	1.0 ± 0.1	1.7 ± 0.3
20:2 n-6	0.8 ± 0.2	0.6 ± 0.1	0.7 ± 0.2	0.9 ± 0.1
20:3 n-6	1.4 ± 0.2	1.2 ± 0.2	1.3 ± 0.2	1.7 ± 0.3
20:4 n-6	6 ± 1	2.6 ± 0.6 [*]	5.5 ± 0.9	3.7 ± 0.8
20:5 n-3	1.7 ± 0.7	0.7 ± 0.2	1.6 ± 0.6	1.0 ± 0.3
22:0	0.7 ± 0.1	0.8 ± 0.1	0.6 ± 0.1	1.1 ± 0.2
22:1 n-9	0.6 ± 0.1	0.6 ± 0.2	0.6 ± 0.1	0.9 ± 0.3
22:2 n-6	1.3 ± 0.3	0.9 ± 0.3	1.2 ± 0.2	1.3 ± 0.4
22:3 n-3	1.0 ± 0.1	1.2 ± 0.3	0.9 ± 0.1	1.7 ± 0.4
22:4 n-6	2.4 ± 0.5	2.0 ± 0.5	2.2 ± 0.5	2.9 ± 0.7
22:5 n-6	0.89 ± 0.09	1.3 ± 0.5	0.80 ± 0.08	1.9 ± 0.7
22:6 n-3	4.9 ± 0.6	2.8 ± 0.6 [*]	4.5 ± 0.5	4.0 ± 0.9
24:0	0.93 ± 0.06	0.9 ± 0.2	0.85 ± 0.06	1.3 ± 0.3
24:1 n-9	0.7 ± 0.1	0.7 ± 0.1	0.6 ± 0.1	1.0 ± 0.1
Sat/protein	49 ± 6	30 ± 3 [*]	45 ± 6	43 ± 4
Unsat/protein	59 ± 8	40 ± 3	54 ± 7	57 ± 4
MUFA/protein	24 ± 1	17 ± 2 [*]	22.0 ± 0.9	24 ± 3
PUFA/protein	35 ± 3	22 ± 2 [*]	32 ± 3	31 ± 3
Sat/Unsat	0.8 ± 0.1	0.8 ± 0.1		
PUFA/MUFA	1.5 ± 0.1	1.3 ± 0.2		

Values reflect the mean ± SE.

^{*} indicates significance ($P \leq 0.05$, $n = 4$) as compared to cultured primary hepatocytes derived from livers of wild-type L-FABP+/+ mice.