Loss of intracellular lipid binding proteins differentially impacts saturated fatty acid uptake and nuclear targeting in mouse hepatocytes

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The impact of L-FABP and SCP-2 on LCFA uptake has been examined in transformed cell lines and in L-FABP gene-targeted mice. Overexpression of L-FABP in L-cell fibroblasts, cells with a very low level of endogenous FABP or SCP-2, enhanced cellular LCFA uptake (7, 49). While L-FABP anti-sense treatment of HepG2 hepatoma cells decreased cellular LCFA uptake, such hepatoma cells normally express 3- to 10-fold less L-FABP and SCP-2 than the liver (34, 83). This issue was resolved with L-FABP null mice, which exhibit decreased hepatic LCFA uptake in vivo (40, 51). SCP-2 overexpression in L-cell fibroblasts also enhanced LCFA uptake (8, 48). Conversely, nothing is known about the effect of SCP-2 gene ablation on hepatic LCFA uptake (40, 51). Furthermore, interpretation of hepatic LCFA uptake in mouse models is complicated by the high prevalence of SCP-2 and L-FABP, as well as concomitant upregulation of L-FABP expression, in SCP-2 null liver (21, 68, 72). Thus the relative contributions of L-FABP and SCP-2 to LCFA uptake in liver hepatocytes remain to be resolved.

Although L-FABP facilitates intracellular targeting of LCFA to nuclei of transformed cell lines, its role in LCFA targeting into hepatocyte nuclei is not completely resolved, and nothing is known regarding the role of SCP-2 in LCFA nuclear targeting. In vitro studies showed that L-FABP stimulated cotransport of LCFA into purified nuclei (35). Real-time confocal and multiphoton imaging demonstrated that L-FABP overexpression in L-cell fibroblasts preferentially increased the targeting of fluorescent analogs of saturated and polyunsaturated LCFA into nuclei (30, 31, 45). While L-FABP gene ablation reduced the distribution of nuclear LCFA uptake, such hepatoma cells normally express 3- to 10-fold less L-FABP and SCP-2 than the liver (34, 83). This issue was resolved with L-FABP null mice, which exhibit decreased hepatic LCFA uptake in vivo (40, 51). SCP-2 overexpression in L-cell fibroblasts also enhanced LCFA uptake (8, 48). Conversely, nothing is known about the effect of SCP-2 gene ablation on hepatic LCFA uptake (40, 51). Furthermore, interpretation of hepatic LCFA uptake in mouse models is complicated by the high prevalence of SCP-2 and L-FABP, as well as concomitant upregulation of L-FABP expression, in SCP-2 null liver (21, 68, 72). Thus the relative contributions of L-FABP and SCP-2 to LCFA uptake in liver hepatocytes remain to be resolved.

Additionally, studies with primary hepatocytes may be complicated by glucose concentrations that are much higher (11–28 mM) than physiological (6 mM) in the culture medium (32, 46, 53). Glucose concentration is much higher in liver and liver-derived cells than in most other tissues, and intracellular glucose levels rapidly rise in response to high (20–30 mM) extracellular glucose (17, 25, 27, 81). High glucose alters L-FABP interaction with peroxisome proliferator-activated receptor-α (PPARα), a key nuclear receptor in transcription of LCFA β-oxidative enzymes (28, 29, 71). However, the impact of high glucose on L-FABP-mediated LCFA uptake and dis-
distribution to the nucleus of cultured primary hepatocytes is not known.

The work presented here begins to address these questions by real-time confocal imaging of 12-N-methyl-(7-nitrobenz-2-oxa-1,3-diazo)-aminostearic acid (NBD-stearic acid) uptake in cultured primary hepatocytes. Hepatocytes were obtained from livers of wild-type (WT), L-FABP null, SCP-2/SCP-x null, and L-FABP/SCP-2/SCP-x null mice. Studies were performed not only with radiolabeled stearic acid, but also with NBD-stearic acid, a poorly metabolized fluorescent LCFA that reflects direct uptake, rather than secondary uptake driven by intracellular LCFA metabolism (30, 31).

MATERIALS AND METHODS

Materials. Antisera against FATP2, FATP4, glucokinase (GCK), and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antisera against FATP5, insulin receptor, glucose transport protein (GLUT)-1, GLUT2, and cytochrome c oxidase subunit IV were obtained from Abcam (Cambridge, MA). Rabbit anti-glutamic oxaloacetic transaminase (GOT) was produced as described elsewhere (6). Rabbit polyclonal antibodies against rat L-FABP and human SCP-2 were produced as previously described (40, 41). NBD-stearic acid was obtained from Avanti Polar Lipids (Alabaster, AL). Alkaline phosphatase-conjugated rabbit anti-goat IgG and goat anti-rabbit IgG were purchased from Sigma-Aldrich (St. Louis, MO). Donkey anti-rabbit IgG conjugated to 15-nm gold, as well as LR White resin, were obtained from Electron Microscopy Sciences (Hatfield, PA). d(+)-glucose and d(+)-maltose were obtained from Sigma (St. Louis, MO). Rat liver recombinant L-FABP was isolated as described elsewhere (37). D-[1-3H]glucose (8.00 Ci/mmol) was purchased from Amersham/GE Healthcare (Piscataway, NJ). [9,10-3H]stearic acid (1 nmol/63.5 μCi) was obtained from Avanti Polar Lipids (Alabaster, AL). Alkaline phosphatase-conjugated rabbit anti-goat IgG and goat anti-rabbit IgG were purchased from Sigma-Aldrich (St. Louis, MO). Donkey anti-rabbit IgG conjugated to 15-nm gold and donkey anti-goat IgG conjugated to 6-nm gold, as well as LR White resin, were obtained from Electron Microscopy Sciences (Hatfield, PA). d(+)-glucose and d(+)-maltose were obtained from Sigma (St. Louis, MO). Rat liver recombinant L-FABP was isolated as described elsewhere (37). D-[1-3H]glucose (8.00 Ci/mmol) was purchased from Amersham/GE Healthcare (Piscataway, NJ). [9,10-3H]stearic acid (1 nmol/63.5 μCi) was obtained from Moravek Biochemicals (Brea, CA). All reagents and solvents were of the highest grade available and were cell culture tested as necessary.

Animals. All animal protocols were approved by the Institutional Animal Care and Use Committee at Texas A & M University. Male WT C57BL/6NCr mice (8–10 wk old; 20–25 g body wt) were purchased from the National Cancer Institute (Frederick Cancer Research and Developmental Center, Frederick, MD). L-FABP null mice were produced as described earlier by our laboratory and backcrossed to C57BL/6NCr mice to ≥10 generations (40). SCP-2/SCP-x null mice were generated by this laboratory by targeted disruption of the SCP-2 gene through homologous recombination and backcrossed to C57BL/6NCr mice to ≥10 generations (3). L-FABP/SCP-2/SCP-x null mice were generated by this laboratory by targeted disruption of the SCP-2 gene through homologous recombination and backcrossed to C57BL/6NCr mice to ≥10 generations (40). SCP-2/SCP-x null mice were generated by this laboratory by targeted disruption of the SCP-2 gene through homologous recombination and backcrossed to C57BL/6NCr mice to ≥10 generations (3). L-FABP/SCP-2/SCP-x null mice were generated by this laboratory by targeted disruption of the SCP-2 gene through homologous recombination and backcrossed to C57BL/6NCr mice to ≥10 generations by this laboratory, as described elsewhere (72). Mice were kept under a 12:12-h light-dark cycle in a temperature-controlled facility (25°C) with access to food (standard rodent chow mix, 4% fat calories) and water ad libitum. All mice in the facility were free of all known rodent pathogens, as monitored quarterly.

Hepatocyte isolation and culture. Cultured primary hepatocytes were isolated from the livers of 10- to 12-wk-old male WT or null mice, as described elsewhere (72). Briefly, mice were euthanized by CO2 asphyxiation, and the liver was removed and perfused with buffer A [10 mM HEPES, pH 7.4, in calcium/magnesium-free HBSS, gentamicin sulfate (1 mg/ml medium), and 0.5 mM EGTA]. Hepatocytes were released by perfusion in buffer B [buffer A without EGTA, supplemented with 5 mM CaCl2 and 0.2 mg/ml collagenase] with gentle palpation of the liver capsule during perfusion. Hepatocytes were washed twice in cold DMEM-F12 medium with 5% FBS and plated on collagen-coated dishes in the DMEM-F12 medium with 5% FBS. DMEM-F12 medium contained 17.5 mM d-glucose, while the only insulin available in the culture medium was in the FBS.

NBD-stearic acid uptake by cultured primary hepatocytes: laser scanning microscopy. Hepatocytes isolated from male WT and L-FABP null, SCP-2/SCP-x null, and SCP-2/SCP-x/L-FABP null mice were plated at a density of 2 × 10^5 cells/well on collagen-coated two-chamber cover glasses (Nalge Nunc Lab-Tek). After the cells were cultured overnight in DMEM-F12 medium with 5% FBS, individual wells were processed for a time-course analysis of NBD-stearic acid uptake. Cells were washed twice with warm (37°C) PBS, incubated for 15 min in PBS at 37°C under 5% CO2, and transferred to a 37°C heated microscope stage on a laser scanning confocal microscopy system (MRC-1024MP, Bio-Rad Laboratories, Hercules, CA) equipped with an inverted microscope (Zeiss Axiovert, Carl Zeiss Microimaging, Thornwood, NY), a band-pass filter (no. HQ530/40, Chroma Technology, Bellow Falls, VT), and a ×40 oil objective (Zeiss Apochromat). The 488-nm excitation line of the argon-krypton ion laser source was set to 1% power, and LaserSharp 3.0 software (Bio-Rad) was used to capture images of the cells at 1-min intervals after addition of 0.01–0.10 μM NBD-stearic acid.

For examination of the effects of high or low glucose, hepatocytes were washed as described above and then incubated with 0, 6, or 20 mM glucose or maltose before (15 min) and during uptake of NBD-stearic acid. Hepatocytes were only used for 2 days in culture after isolation (Fig. 1).

Quantitation of NBD-stearic acid uptake by cultured primary hepatocytes: laser scanning microscopy. Metamorph 4.0 ( Molecular Devices, Sunnyvale, CA) was used to quantify the fluorescence intensities of individual cells and nuclei throughout each time course. Excel (Microsoft, Redmond, WA) and SigmaPlot (Systat Software, San Jose, CA) were used for statistical analyses. All data are means ± SE; n = 6–8. *P < 0.05 vs. day 0.

Fig. 1. Expression of proteins involved in transport and metabolism of fatty acids and glucose across membranes in cultured primary hepatocytes from days 0–4 postisolation. Western blotting of wild-type (WT) hepatocyte lysates taken at days 0–4 after cell isolation was performed using GAPDH or other housekeeper internal control. A: fatty acid transport protein (FATP)-5. B: FATP2. C: glutamic oxaloacetic transaminase (GOT). D: FATP4. E: insulin receptor. F: glucose transporter (GLUT)-1. G: GLUT2. H: glucokinase (GCK). Values are means ± SE; n = 6–8. *P < 0.05 vs. day 0.
The freshly isolated mouse hepatocytes (71) are primary hepatocytes described elsewhere (72, 73). The cells were washed three times with TBST and incubated for 2 h at room temperature with the appropriate alkaline phosphatase-conjugated secondary antibodies diluted in 1% gelatin in TBST. After a final wash with TBST, the blots were incubated in AP buffer (100 mM Tris-base, pH 9, 100 mM NaCl, and 10 mM MgCl₂) for 10 min and then in 5-bromo-4-chloro-3-indolyl phosphate-nitro blue tetrazolium according to the manufacturer’s protocol (Sigma Aldrich) until bands developed. Images of blots were acquired using a dual-lens scanning blot scanner and produce the final multipanel images.

Quantitation of NBD-stearic acid uptake and metabolism by cultured primary hepatocytes. Isolated WT hepatocytes were plated on collagen-coated six-well plates at a density of 1 × 10⁶ cells/well and incubated overnight in presence of 0, 1, 2, or 6 h. The cells were washed twice with PBS and incubated for 2 h in PBS supplemented with 0.5 μM cold stearic acid and [9,10⁻³H]stearic acid (0.01 nmol/well) with 6 or 20 mM glucose. Medium was removed and saved, the cells were washed twice with PBS, and the washes were pooled with the appropriated medium samples. The labeled cells were scraped in a hexane-isopropanol (3:2, vol/vol) mixture and centrifuged at 1,500 rpm for 15 min, and the lipid extracts were transferred to acid-washed glass tubes. The remaining protein samples were dried overnight at room temperature and dissolved overnight in 0.2 M KOH (1 ml/sample) for Bradford protein assay. Cellular lipids were dried under nitrogen and resolved by TCL using silica gel G plates (Analtech, Newark, DE) and a petroleum ether-diethyl ether-methanol-acetic acid (80:14:4:1, vol/vol/vol/vol) solvent system. Individual lipid bands were identified with lipid standards (TLC 18.5A, Nu-Chek Prep, Elysian, MN), which were then removed from the plate for disintegration-per-minute (DPM) quantification by scintillation counting. The DPM of the medium/wash samples was quantified, and a chloroform-methanol (2:1, vol/vol) solution in combination with a 30-min 1,500-rpm centrifugation was used to remove the cold + labeled stearic acid from the medium/wash samples to quantify DPM of aqueous cellular oxidation products. Samples extracted from cell-free wells and unlabeled feedings were used to control for loss/preservation of the labeled probe and for label specificity/background, respectively.

Quantitation of [¹¹C]glucose uptake and fatty acid metabolism by cultured primary hepatocytes. Isolated hepatocytes were plated on collagen-coated six-well culture plates at a density of 1 × 10⁶ cells/well, cultured overnight in DMEM-F12 medium (57, 58, 72), rinsed twice with PBS, and cultured in glucose-free DMEM (1 ml/well) supplemented with 6 or 20 mM glucose for 0, 1, 2, or 6 h (n = 3 wells per medium type per time point). For radiolabeled experiments, 1 μL of [¹¹C]glucose was added to 13 ml of the 6 mM medium (5,171 cpm/μmol glucose), and 1 μL of [¹¹C]glucose was added to 13 ml of the 20 mM medium (1,551 cpm/μmol glucose). At each time point, the medium was removed, and the cells were rinsed twice with 500 μL of PBS; the 1-mL medium and the two 500-μL PBS washes were pooled to give the 2-ml medium for each sample. The cells were frozen/floated in liquid nitrogen, and each well was scraped twice in 500 μL of PBS (1-ml cell sample for each time point).

Hepatocyte cell homogenates were prepared as described previously (2). Cell homogenate and medium total protein and glucose were quantified by the Bradford protein assay (Bio-Rad, Richmond, CA) and the Wako Autokit Glucose (Wako Chemicals, Richmond, VA), respectively, utilizing a microplate reader (Spectra 2, Biotek Instruments, Winooski, VT). Concentration was determined using hepatocyte volume (42). Cell homogenate and
medium lipid masses were determined as described previously (2).

For nonradioactive samples, the lipid classes were identified by comparison with known standards utilizing TLC according to the method of Marzo et al. (43). Radioactive samples were prepared as described above. The lipids were separated by TLC and visualized by iodine, and each lipid spot was scraped into a scintillation vial. After addition of ScintiSafe gel (Fisher Scientific, Pittsburgh, PA) (5 ml), the amount of radioactivity was quantified utilizing a liquid scintillation counter (model 1600 TR, Packard, Meriden, CT). No detectable [3H]lipid signal above background was measured in any of the medium samples (data not shown).

Statistical analysis. Values are means ± SE based on the replication number for each experiment. Statistical variance between mean values within groups of three or more was determined using one-way ANOVA and Dunnett’s post tests; analysis of groups of two was performed using unpaired t-tests. P < 0.05 indicates statistical significance. All statistical tests were performed within GraphPad Prism (GraphPad Software, San Diego, CA).

RESULTS

Cultured primary mouse hepatocytes maintain expression of key proteins involved in LCFA and glucose uptake and metabolism. Primary hepatocytes may lose expression of proteins with increasing time in culture (33, 47). Thus it was important to first determine whether mouse hepatocytes in culture stably express the transporters involved in LCFA and glucose uptake. Western blotting was performed on primary mouse hepatocytes immediately after isolation (day 0) or at 1, 2, 3, and 4 days in culture.

Several integral membrane proteins involved in hepatic LCFA uptake are localized in the plasma membrane and peroxisomal membranes. Hepatocytes expressed both key plasma membrane LCFA transporters, FATP5 (Fig. 1A) and GOT (Fig. 1C) at levels similar to those in the liver (day 0) for up to 2 days in culture. Expression of the peroxisomal membrane LCFA transporters FATP2 (Fig. 1B) and FATP4 (Fig. 1D) was similar to that in the liver (day 0) at 2 and 4 days in culture, respectively.

Quantitative Western blotting demonstrated that WT cultured primary mouse hepatocytes expressed nearly fourfold more L-FABP than SCP-2, while L-FABP gene ablation did not alter SCP-2 expression (72). Conversely, ablating SCP-2 elicited 1.7-fold upregulation of L-FABP, as seen previously (72). In contrast, hepatic expression of L-FABP (2) and SCP-2 (not shown) was maintained at the same level as in the liver and remained constant for 3 and 4 days in culture, respectively. Hepatocytes also express several key proteins involved in glucose uptake, plasma membrane integral proteins and soluble enzymes. Expression of the insulin receptor was similar to that in the liver (day 0) and remained constant or slightly increased for at least 4 days in culture (Fig. 1E). Expression of the plasma membrane glucose transporter found in all hepatocytes, GLUT2, was constant for 4 days in culture (Fig. 1G). Similarly, expression of plasma membrane GLUT1 (Fig. 1F), present in only a small subset (1%) of hepatocytes localized near the central vein (9, 74), was also constant for 4 days in culture. The level of the rate-limiting enzyme in glucose
metabolism, GCK, was also similar to that in the liver (day 0) and was maintained for 4 days in culture (Fig. 1H).

Thus expression of all hepatocyte soluble and membrane LCFA transporters was similar to that in the liver and was maintained at constant levels for 3–4 days and 2 days, respectively, in culture. Concentrations of key proteins involved in glucose uptake and metabolism were maintained at levels similar to those in the liver and were stable for at least 4 days. On the basis of these findings, mouse primary hepatocytes were cultured for ≤2 days for all subsequent studies.

Real-time confocal imaging of fluorescent LCFA uptake in cultured primary mouse hepatocytes. To examine the impact of LCFA binding protein gene ablation on LCFA uptake in living hepatocytes, NBD-stearic acid concentration and incubation time were optimized. Representative confocal images showed that, with increasing time, the primary hepatocytes took up NBD-stearic acid into the cytoplasm and less intensely into nuclei (Fig. 2A). Uptake exhibited saturation by 20–60 min at all concentrations (Fig. 2B). The maximum fatty acid uptake or cellular capacity saturated quickly at >0.5 μM NBD-stearic acid (Fig. 2C). The maximal rate of uptake (Fig. 2D) saturated at around four- to fivefold higher concentration. Therefore, all subsequent studies were undertaken with a nonsaturating NBD-stearic acid concentration of 0.1 μM, a level in the linear range of both curves.

NBD-stearic acid was poorly metabolized in cultured primary mouse hepatocytes. To determine if the NBD-stearic acid uptake and distribution as seen by confocal laser scanning microscopy represented free LCFA and not a metabolite, cultured primary mouse hepatocytes isolated from the liver of WT mice were incubated with NBD-stearic acid in PBS for 1 or 2 h. Lipids were extracted, separated by TLC, and quantitated as described in MATERIALS AND METHODS. First, when Silica Gel G plates were developed with a petroleum ether-diethyl ether-glacial acetic acid (70:30:1, vol/vol) solvent system, NBD-stearic acid remained at the origin, with no other fluorescent bands observed above the origin (data not shown). When the same TLC plate was developed with petroleum ether-diethyl ether-glacial acetic acid (180:14:41, vol/vol) solvent system, NBD-stearic acid remained at the origin, with no other fluorescent bands observed above the origin (data not shown). The majority of the fluorescence above the origin was NBD-stearic acid (Fig. 3A), as shown by the NBD-stearic acid loaded on the plate for standard curve quantitation. A faint fluorescent band between the origin and the brighter NBD-stearic acid band (Fig. 3A) accounted for ~2% of the fluorescence as well as the mass (Fig. 3, B and C). No NBD-stearic acid phospholipids or NBD-stearic acid-CoA was detected after 1 or 2 h of incubation (data not shown).

LCFA binding protein gene ablation reduced cellular LCFA uptake. L-FABP gene ablation decreased the cellular uptake of NBD-stearic acid at all time points (Fig. 4A), primarily due to a 60% decrease in maximal uptake (Fig. 4B), rather than a maximal rate of uptake (Fig. 4C). In contrast, SCP-2 gene ablation did not reduce LCFA uptake (Fig. 4A) but, instead, significantly increased maximal uptake (Fig. 4B), likely due to concomitant upregulation of L-FABP (72, 73). Ablation of L-FABP and SCP-2 did not further reduce cellular LCFA uptake into hepatocytes, as did L-FABP ablation alone (Fig. 4, A–C).

Thus ablation of L-FABP, but not SCP-2, reduced cellular LCFA uptake in cultured primary hepatocytes. The residual ability of hepatocytes to take up LCFA in low quantity in L-FABP null and L-FABP/SCP-2/SCP-x null hepatocytes indicated potential contributions of membrane binding, compartmentalization, or as yet unresolved additional cellular factors (2).

LCFA binding protein gene ablation differentially reduced LCFA uptake into nuclei. L-FABP gene ablation (L-FABP null and L-FABP/SCP-2/SCP-x null) nearly abolished NBD-stearic acid uptake into the nucleus of the cultured hepatocytes at all time points (Fig. 5, A–C). Consequently, the ratio of nuclear to cytoplasmic LCFA uptake was reduced >90% relative to that in WT hepatocytes. SCP-2 gene ablation did not impact LCFA uptake into the nucleus (Fig. 5, A–C). This was likely due to the concomitant upregulation of L-FABP in SCP-2-ablated hepatocytes (72, 73). Conversely, SCP-2 was not upregulated in L-FABP null hepatocytes (72).
Double-immunogold labeling of FATP5 and L-FABP at the plasma membrane of primary cultured hepatocytes from WT control mice. It has been suggested that FATP5, which localizes to the basolateral plasma membrane of liver hepatocytes, might interact with L-FABP (15, 16, 70). This possibility was explored by double-immunogold-labeling electron microscopy focusing on the plasma membrane regions of fixed hepatocytes isolated from the liver of WT mice. Representative images of FATP5 and L-FABP in close proximity as determined by immunogold (6 and 15 nm, respectively) are shown in Fig. 6A. A histogram of the edge-to-edge separation distance of FATP5 and L-FABP pairs was prepared from multiple images obtained at or near the plasma membranes of mouse hepatocytes (Fig. 6B). The diameter of each IgG is ~28 nm (60). On the basis of an interaction distance of 10 nm for the two proteins, FATP5 and L-FABP, the gold particles would distribute at edge-to-edge distances of up to ~60 nm. The first bin of the histogram (Fig. 6B) was colored black to denote the distribution of immunogold-labeled FATP5 and L-FABP close enough to form protein-protein interactions.

LCFA binding protein gene ablation did not reduce cellular LCFA uptake by downregulating membrane LCFA transport proteins. The possibility that the reduced LCFA uptake was due at least in part to net reduction of the membrane LCFA transporters was examined. Ablation of L-FABP, SCP-2, or both did not decrease expression of the two major integral plasma membrane proteins involved in LCFA uptake into the hepatocyte. Expression of FATP5 was unaltered (Fig. 7A), while expression of GOT was increased (Fig. 7C). With regard to peroxisomal membrane LCFA transport proteins, ablation of L-FABP, SCP-2, or both decreased expression of FATP2 (Fig. 7B) and increased expression of FATP4 (Fig. 7D). Taken together, these findings indicate that ablation of L-FABP, SCP-2, or both did not result in net downregulation of integral membrane proteins involved in LCFA uptake localized in the plasma membrane or peroxisomal membranes.

LCFA binding protein gene ablation did not reduce cellular LCFA uptake by downregulating key hepatocyte proteins involved in glucose uptake and metabolism. The possibility that LCFA binding protein gene ablation adversely impacted key

![Fig. 4. Alterations in uptake of cellular NBD-stearic acid uptake due to loss of SCP-2 and/or L-FABP. A: time-course analysis of relative cellular fluorescence in WT, L-FABP null, SCP-2/SCP-x null, and SCP-2/SCP-x/L-FABP null hepatocytes after addition of 0.01 μM NBD-stearic acid. Uptake curves were fit using the Wiebull equation. B and C: maximal cellular fluorescence intensity and maximal uptake rate. Values are means ± SE; n = 10–15 cells. *P < 0.05 vs. WT.](image-url)

![Fig. 5. Alterations in NBD-stearic acid nuclear transport due to loss of SCP-2 and/or L-FABP. A: time-course analysis of relative nuclear fluorescence in WT, L-FABP null, SCP-2/SCP-x null, and SCP-2/SCP-x/L-FABP null hepatocytes after addition of 0.01 μM NBD-stearic acid. Nuclear uptake curves from each genotype were fit using the Wiebull equation. B and C: maximal cellular fluorescence intensity and maximal uptake rate. Values are means ± SE; n = 10–15 cells. *P < 0.05 vs. WT.](image-url)
proteins in glucose uptake and metabolism was examined. Loss of L-FABP reduced the expression of insulin receptors in hepatocytes from L-FABP null and L-FABP/SCP-2/SCP-x null mice (Fig. 7E). Expression of the major glucose transporter GLUT2, which is present in all hepatocytes (Fig. 7G), as well as expression of the minor glucose transporter GLUT1, which is present in a small fraction of hepatocytes (Fig. 7F), was not altered by L-FABP gene ablation, but GLUT2 was reduced in hepatocytes from SCP-2/SCP-x null and L-FABP/SCP-2/SCP-x null mice (Fig. 7G). The rate-limiting enzyme in glucose metabolism, GCK, was upregulated in hepatocytes from L-FABP null and SCP-2/SCP-x null mice, but not from L-FABP/SCP-2/SCP-x null mice (Fig. 7H). Taken together, these findings suggest that reduced cellular LCFA uptake in these null hepatocytes did not correlate with the patterns of expression of key plasma membrane proteins involved in glucose transport or intracellular metabolism.

High glucose reduces cellular LCFA uptake and LCFA transport into the nuclei. Most prior studies of LCFA metabolism in primary hepatocytes have been performed with culture media containing glucose levels much higher than physiological (32, 46, 53). Therefore, the impact of different extracellular glucose concentrations on NBD-stearic acid uptake and nuclear targeting was examined.

Increasing glucose resulted in decreased cellular LCFA uptake in cultured primary hepatocytes from WT mice. Increasing glucose from 0 to 6 mM (normal physiological level) decreased LCFA uptake at all time points (Fig. 8). Further increasing glucose from 6 to 20 mM reduced cellular (Fig. 8) and maximal (Fig. 9A) LCFA uptake even more extensively. The inhibitory effects of high glucose on cellular LCFA uptake in hepatocytes did not correlate directly with L-FABP or SCP-2 expression, since cellular LCFA uptake was decreased 65–70% in all cases (Fig. 9, A–D). An osmotic control, maltose, did not significantly impact LCFA uptake into hepatocytes (not shown).

However, the impact of high glucose on LCFA uptake into nuclei in general correlated with L-FABP expression. High glucose decreased LCFA distribution to the nuclei of L-FABP null (Fig. 9F) and L-FABP/SCP-2/SCP-x null (Fig. 9H) hepatocytes to near the limit of detection. In contrast, glucose decreased nuclear LCFA uptake less in WT (Fig. 9E) and
SCP-2/SCP-x null hepatocytes (upregulate L-FABP) (Fig. 9H) by 80% and 49%, respectively. An osmotic control, maltose, did not significantly impact LCFA nuclear targeting in hepatocytes (not shown).

To determine if the decrease in LCFA cellular uptake and/or nuclear targeting was related to glucose directly affecting the binding affinity of NBD-stearic acid to L-FABP or potentially affecting its environment in either ligand binding pocket and, thus, its quantum yield, ligand binding experiments were performed in the presence of 0, 6, and 20 mM glucose. The resulting curves (Fig. 10) overlapped, with no differences in binding capacity, and yielded the same $K_d$ for NBD-stearic acid binding. Therefore, glucose did not enhance or inhibit the binding of NBD-stearic acid to recombinant L-FABP. Thus high glucose-induced decrease in NBD-stearic acid uptake was not due to altered L-FABP binding affinity for NBD-stearic acid.

Since insulin receptor expression significantly decreased as a result of the L-FABP gene ablation (Fig. 7E), the glucose-dependent effects on NBD-stearic acid uptake were examined in the presence of insulin. Insulin (58 nM) did not have an effect on NBD-stearic acid uptake in the absence or presence of 6 mM glucose (Fig. 10B). Insulin did not further decrease the maximal uptake of NBD-stearic acid (Fig. 10B), as observed in the presence of 20 mM glucose (Fig. 8).

[3H]glucose uptake and subsequent incorporation of the label into lipids in primary cultured hepatocytes. The possibility that de novo synthesis of LCFA from high glucose might result in inhibition of LCFA uptake was examined. d-[1-3H]glucose was used as a tracer for intracellular measurements of the incorporation of the glucose carbons and radiolabel into fatty acid biosynthesis. Glucose was taken up rapidly into WT hepatocytes incubated with 6 and 20 mM extracellular glucose (Table 1). For 60 min, total intracellular lipid content was relatively unchanged. Even with the addition of 20 mM glucose, there were no dramatic increases in the incorporation of the glucose radiolabel into the intracellular lipid. These results indicate that there is no significant rise in the intracellular glucose-derived LCFA content of the hepatocytes as a result of increases in extracellular glucose concentrations.

Effects of extracellular glucose concentration on [3H]stearic acid uptake and metabolism in cultured primary mouse hepatocytes. WT primary hepatocytes were plated in medium with 6 or 20 mM glucose supplemented with 0.5 μM stearic acid/ [3H]stearic acid (Fig. 11), and total uptake of the [3H]stearic acid (esterified + oxidized), as well as incorporation into cellular free fatty acids, esterified lipids, and oxidation products, was determined. High glucose decreased the total uptake of [3H]stearic acid (Fig. 11A), primarily through incorporation into esterified lipids (Fig. 11B), such as triglycerides (Fig. 11C).

DISCUSSION

Hepatic LCFA’s and glucose are metabolically linked. Once taken up by the liver, exogenous LCFA’s are stored/secreted (triglycerides/VLDL), partially oxidized to two-carbon units for gluconeogenesis (for storage as glycogen or maintenance of...
blood glucose levels), or fully oxidized to produce energy. L-FABP impacts hepatic LCFA and glucose metabolism in the short term by enhancing LCFA uptake and in the longer term by enhancing LCFA targeting to nuclei for induction of PPARα transcription of LCFA β-oxidative enzymes (reviewed in Ref. 1). These actions of L-FABP are attributed in part to L-FABP’s high affinity for LCFA and their CoA thioesters (reviewed in Refs. 19, 20, 52, 59, 82). Although SCP-2 also binds LCFA and LCFA-CoA with high affinity and enhances LCFA uptake, the impact of SCP-2 on LCFA nuclear targeting is not known (19, 23). Use of L-FABP null and SCP-2/SCP-x null mice alone to resolve the physiological roles of SCP-2 and L-FABP has been complicated by the continued presence of SCP-2 or upregulation of L-FABP in these models, respectively (21, 40, 41, 68). The data presented here, especially with L-FABP/SCP-2/SCP-x null hepatocytes, further our understanding of the respective roles of L-FABP and SCP-2 in cellular LCFA uptake and nuclear targeting.

Ablation of L-FABP or SCP-2/SCP-x differentially regulated expression of the respective nontargeted gene. While concomitant upregulation of SCP-2 was observed in hepatocytes from early-backcross-generation L-FABP null mice (2), this was not the case in cultured primary hepatocytes from a ≥10-backcross generation. Similarly, upregulation of SCP-2 was observed in livers of early-backcross generations of L-FABP null mice but not later-backcross generations (39, 40). These findings suggest that any effects of L-FABP gene ablation on LCFA uptake and nuclear targeting in hepatocytes are not likely to be complicated by concomitant upregulation of SCP-2. In contrast, L-FABP was upregulated two- to fourfold in ≥10-backcross-generation hepatocytes from SCP-2 null hepatocytes, as well as in the liver (21, 68, 72). Thus upregulation of L-FABP may confound interpretation of SCP-2’s effects on LCFA uptake and nuclear targeting in hepatocytes.

Ablation of L-FABP decreased cellular LCFA uptake, as demonstrated using the fluorescent probe NBD-stearic acid not significantly metabolized by hepatocytes and [3H]stearic acid. Roles for L-FABP and SCP-2 in hepatocyte LCFA uptake were also supported by the finding that L-FABP overexpression in L-cell fibroblasts (control L-cell fibroblasts do not express L-FABP and express very low levels of SCP-2) enhanced LCFA uptake (7, 49). Similarly, SCP-2 overexpression in these fibroblasts also increased LCFA uptake (8, 48). The fact that the effects of L-FABP and SCP-2 ablation on LCFA uptake in cultured primary hepatocytes are not additive suggests that the two proteins may have overlapping functions in LCFA uptake in hepatocytes. An analogous potential overlap in function relating to HDL-mediated cholesterol uptake was observed in relation to the distribution of proteins in cholesterol-poor and -rich microdomains (44). Hepatocyte plasma membrane-associated SCP-2 and L-FABP are enriched in cholesterol-rich and -poor microdomains, respectively, in WT control mice (44). Loss of L-FABP resulted in an increase of SCP-2 in the cholesterol-poor microdomains (44). This remodeling of the SCP-2 distribution in hepatocyte plasma membranes in L-FABP gene-ablated mice may give rise to the differences in the maximal LCFA uptake rates compared with those in hepatocytes from SCP-2/SCP-x/L-FABP null mice. Additionally, even when both L-FABP and SCP-2 were ablated, the hepatocytes maintained a residual LCFA uptake into the hepatocyte. The latter may be attributed to a diffusional component and/or integral membrane fatty acid transport proteins known to enhance LCFA uptake through their localization at the plasma membrane (FATP5 and GOT) or at the peroxisomal membrane (FATP2 and FATP4) (78). Since ab-

![Fig. 9. Effect of glucose on maximal NBD-stearic acid uptake into hepatocytes and hepatocyte nuclei. Primary mouse hepatocytes were isolated from WT, L-FABP null, SCP-2/SCP-x null, and SCP-2/SCP-x/L-FABP null mice and incubated with 0 (control) or 20 mM glucose and 0.01 μM NBD-stearic acid. Maximal NBD-stearic acid uptake into hepatocytes (A–D) and nuclei (E–H) was determined as maximal fluorescence intensity from a 0- to 60-min time curve. Values are means ± SE; n = 10–15 cells. *P < 0.05 vs. WT with the same treatment. $P < 0.05 vs. control with the same genotype.](http://ajpgi.physiology.org/doi/10.1152/ajpgi.00489.2011)
nuclear targeting. However, differential uptake of bound ligand (i.e., retinoic acid) has been observed with other FABP family members (i.e., cellular retinoic acid binding proteins I and II) (12, 75). The latter finding suggests that entry of the binding protein into nuclei is essential for ligand-mediated activation of nuclear receptor target (12, 75). Thus L-FABP’s role in LCFA nuclear targeting shares more functional similarity to these other LCFA binding proteins than with SCP-2.

High glucose reduced cellular LCFA uptake in all mouse models, but the impact of high glucose on LCFA uptake into nuclei in general correlated with L-FABP expression. High glucose especially reduced nuclear uptake of LCFA in the SCP-2/SCP-x null hepatocytes. This was due not only to concomitant upregulation of L-FABP in SCP-2/SCP-x null hepatocytes but also to glucose interacting with L-FABP and increasing L-FABP’s affinity for PPARα (28, 71). Glucose also enhanced LCFA transport into nuclei of L-FABP-overexpressing L cells (28). Taken together, these findings, including the observations from the nonmetabolizable NBD-stearic acid, as well as from [3H]stearic acid and [3H]glucose, suggest that L-FABP and glucose together significantly impact cellular LCFA uptake and nuclear targeting, but not by utilization of glucose through fatty acid biosynthesis or major changes in fatty acid metabolism.

Dietary improvements, exercise, and statins significantly decrease incidence and severity of CVD. However, CVD risk remains higher among individuals with diabetes mellitus, even with optimum statin therapy. Dyslipidemia, particularly hypertriglyceridemia, is the major basis for this high residual CVD risk in statin-treated subjects, especially hypertriglyceridemic diabetic patients. Therapeutic interest in L-FABP has increasingly focused on L-FABP’s protection against hepatocellular oxidative stress, as in diabetes (56, 76, 77, 85), and on developing fibrate analogs better bound and targeted by L-FABP for activating PPARα (13, 62, 82). Part of this interest is based on the discovery of the L-FABP T94A variant, which is the most frequently occurring polymorphism in the entire FABP protein family, with a 26–38% minor allele frequency (8.3 ± 1.9% frequency of homozygous variant) in multiple populations tested worldwide [mutation annotation format for 1,000 genomes in National Center for Biotechnology Information single-nucleotide polymorphism database; ALFRED database] (11, 18, 38, 54, 61, 80, 84). The human L-FABP T94A variant is

Table 1. [3H]glucose uptake and lipid incorporation in cultured wild-type primary hepatocytes

<table>
<thead>
<tr>
<th>Time, min</th>
<th>6 mM Glucose</th>
<th>20 mM Glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>0.94 ± 0.02</td>
<td>3.75 ± 0.09</td>
</tr>
<tr>
<td>60</td>
<td>2.28 ± 0.01</td>
<td>10.47 ± 0.03</td>
</tr>
</tbody>
</table>

| 2         | 6.0 ± 0.4    | 9.4 ± 0.3     |
| 60        | 7.0 ± 0.3    | 6.6 ± 0.8     |

| 2         | 390 ± 20     | 390 ± 20      |
| 60        | 450 ± 30     | 370 ± 30      |

Values are means ± SE (n = 3).
associated with high plasma triglyceride (10, 18) and high LDL cholesterol (18, 54), features associated with increased risk of CVD and type 2 diabetes (10, 18). It has been suggested that the L-FABP null mouse has a phenotype similar to that of humans with the L-FABP T94A polymorphism (10, 18). Consistent with this possibility, overexpression of mouse L-FABP or human L-FABP increases LCFA uptake in transfected L-cell fibroblasts, human HepG2 cells, and human Chang liver cells (24, 49, 55, 83). Conversely, L-FABP gene ablation or expression of the human L-FABP T94A variant decreases LCFA uptake (24, 49, 55, 83). Furthermore, the elevated serum triglycerides in L-FABP null mice share a phenotype similar to that of humans with the L-FABP T94A polymorphism (10, 18). Finally, clofibrate- and/or phytol-treated L-FABP null mice with elevated serum triglycerides share a phenotype similar to that of fenofibrate-treated humans expressing the L-FABP T94A variant (10). Fibrates were first developed as less toxic analogs of dietary phytol and its metabolite PPARα agonists. Low levels of clofibrate and/or dietary phytol decreased serum and hepatic triglycerides in WT, but not L-FABP null, mice (4, 5).

In summary, resolving fatty acid and glucose signaling pathways is of great importance to our understanding of human metabolic disorders such as diabetes mellitus and its complications, which is the sixth-leading cause of death in the United States (22, 63, 79, 86). Serum glucose and LCFA are elevated in diabetes (69, 87). The uptake and metabolism of LCFA and glucose respond to increased levels of both in primary rat hepatocytes and podocytes (26, 36). Our findings suggest that the elevated serum LCFA in diabetes may be due not only to increased hepatic de novo synthesis of LCFA from glucose, but also at least in part to glucose inhibiting LCFA uptake. Our results from cultured primary hepatocytes from L-FABP/SCP-2/SCP-x null mice help clarify the interwoven roles of L-FABP and SCP-2 in hepatic fatty acid uptake. L-FABP and SCP-2 are highly expressed in the liver, and L-FABP is concomitantly upregulated in SCP-2/SCP-x null hepatocytes and the liver (21, 68). In a glucose concentration-dependent manner, our findings demonstrate that L-FABP, much more than SCP-2, impacted hepatoocyte cellular LCFA uptake and LCFA targeting to nuclei. Ablation of SCP-2, resulting in the increased expression of L-FABP, enhanced LCFA distribution to the nucleus, consistent with the entry of L-FABP, but not SCP-2, into the nuclei (23, 67). Within the nuclei, L-FABP interaction with and activation of PPARα transcription of LCFA β-oxidative enzymes may be greatly affected by high glucose concentrations (28, 29, 46, 67).

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

REFERENCES


54. Stewart JM, Dewling VF, Wright TG. Fatty acid binding to rat liver fatty acid-binding protein is modulated by early glycolytic intermediates. Biochim Biophys Acta 1391: 1–6, 1998.


