FATP1 channels exogenous FA into 1,2,3-triacyl-*sn*-glycerol and down-regulates sphingomyelin and cholesterol metabolism in growing 293 cells

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Abstract Biosynthesis of lipids was investigated in growing 293 cells stably expressing fatty acid (FA) transport protein 1 (FATP1), a bifunctional polypeptide with FA transport as well as fatty acyl-CoA synthetase activity. In short-term (30 s) incubations, FA uptake was increased in FATP1 expressing cells (C8 cells) compared with the vector (as determined by BODIPY 3823 staining and radioactive FA uptake). In long-term (4 h) incubations, incorporation of [^{14}C]acetate, [3H]oleic acid, or [^{14}C]lignoceric acid into 1,2,3-triacyl-*sn*-glycerol (TG) was elevated in C8 cells compared with vector, whereas incorporation of radiolabel into glycerophospholipids was unaltered. The increase in TG biosynthesis correlated with an increase in 1,2-diacyl-*sn*-glycerol acyltransferase activity in C8 cells compared with vector. In contrast, incorporation of [^{14}C]acetate into sphingomyelin (SM) and cholesterol, and [3H]oleic acid or [^{14}C]lignoceric acid into SM was reduced due to a reduction in de novo biosynthesis of these lipids in C8 cells compared with vector. The results indicate that exogenously supplied FAs, and their subsequently produced acyl-CoAs, are preferentially channeled by an FATP1 linked mechanism into the TG biosynthetic pathway and that such internalized lipids down-regulate de novo SM and cholesterol metabolism in actively growing 293 cells.—Hatch, G. M., A. J. Smith, F. Y. Xu, A. M. Hall, and D. A. Bernlohr. **Fatty acid transport protein 1 channels exogenous fatty acid into 1,2,3-triacyl-*sn*-glycerol and down-regulates sphingomyelin and cholesterol metabolism in growing 293 cells. *J. Lipid Res.* 2002.** 43: **1380–1389.**

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Fatty acids (FAs) are key components of membrane glycerolipids, are involved in signal transduction mechanisms, and serve as an important source of metabolic fuel though β-oxidation (1). Circulating FAs are taken up into cells either by diffusion due to their solubility in the membrane or by one or more proteins that facilitate their transport into the cell (2, 3). To date, at least three separate families of proteins have been described: FA translocase/CD 36 (FAT/CD 36), plasma membrane (PM) FA binding protein (FABPpm), and FA transport protein (FATP). Five members of the FATP family have been described in murine cells and a sixth identified from analysis of the human genome (labeled fatty acid transport protein 1 (FATP1)-6) (4). The N-terminal 51 amino acids of FATP are variable and may represent specific targeting of the protein to different subcellular compartments. One of the members of this family, FATP1, is expressed on the PM as well as intracellular membranes (5, 6).

FAs enter into glycerolipids by de novo biosynthesis by successive acyl-CoA dependent acylation of *sn*-glycerol-3-phosphate to form lysophosphatidic acid followed by phosphatidic acid (PA) (7). PA is then converted to 1,2-diacyl-*sn*-glycerol (DG) by PA phosphohydrolase (8). DG is then condensed with another molecule of acyl-CoA to form 1,2,3-triacyl-*sn*-glycerol (TG) and free CoA in a reaction catalyzed by DG:acyl-CoA acyltransferase. In addition to TG, DG produced from PA phosphohydrolase may be utilized for de novo phosphatidylcholine (PC) and phosphatidylethanolamine (PE) biosynthesis by their respective cytidine diphosphate (CDP)-choline and CDP-ethanolamine pathways (7). PA may also serve as a precursor for phosphatidylinositol (PI) synthesis via CDP-DG production catalyzed by PA:CTP cytidylyltransferase. In addition to glycerolipid de novo biosynthesis, fatty acyl-CoAs may enter into glycerophospholipids via deacylation followed by reacylation pathways (9). Fatty acyl-CoA enters into sphingolipids via two routes: condensation of serine with palmitoyl-CoA catalyzed by serine palmitoyltransferase

Abbreviations: ACS, acyl-CoA synthetase; DG, 1,2-diacyl-*sn*-glycerol; DGAT, 1,2-diacyl-*sn*-glycerol acyltransferase; ER, endoplasmic reticulum; FA, fatty acid; FATP1, fatty acid transport protein 1; GPAT, *sn*-glycerol-3-phosphate acyltransferase; MHC, myosin heavy chain; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PM, plasma membrane; PS, phosphatidylserine; SM, sphingomyelin; TG, 1,2,3-triacyl-*sn*-glycerol.

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and *N*-acylation of sphinganine (sphingosine) catalyzed by sphinganine (sphingosine) *N*-acyltransferase (10). Numerous experimental models and human metabolic disorders have shown that sphingomyelin (SM) biosynthesis is regulated in concert with cholesterol biosynthesis (11).

Acyl-CoA synthetases (ACSs) are a family of proteins that ligate FA to CoA with concomitant pyrophosphoryl-mediated hydrolysis of ATP (12). Recent studies have indicated that there may be selectivity of acyl-CoA pool for various glycerolipid biosynthetic pathways (13, 14). For example, incubation of hepatocytes, prepared from fasted or fed rats, with triacsin C inhibited TG biosynthesis but not FA oxidation (13). FATP1 exhibits FA ligase activity toward long chain and very long chain FAs suggesting that FA transport may be linked to CoA esterification (15). The above studies prompted us to examine whether or not the CoA synthetase component of FATP1 activity mechanistically linked FA uptake to selection of acyl-CoA pools for various lipid biosynthetic pathways. Our surprising results indicate that FATP1 upregulates de novo TG biosynthesis while down regulating de novo SM and cholesterol metabolism in growing 293 cells.

MATERIALS AND METHODS

[^{3}H]oleic acid, L-[^{3}H]serine, [*methyl*-^{3}H]choline, [1-^{14}C]acetate, [^{3}H]ethanolamine, and [*myo*-^{3}H]inositol were obtained from Amersham. [1-^{14}C]lignoceric acid was obtained from American Radiochemicals Company. Thin layer plates (silica gel G 0.25 mm) were obtained from Fisher Scientific. Lipid standards were obtained from Avanti Polar Lipids Inc. Triacsin C was obtained from BIOMOL. Cell culture reagents were obtained from GIBCO. Oleic acid and lignoceric acid were obtained from Nu-Chek Prep., Inc., Elysian, MN. Phloretin was obtained from ICN Biomedicals, Inc. Fluorescein-conjugated secondary antibody was obtained from Organon Teknika. Polyclonal anti-caveolin-1 (C13630) antibody was obtained from Transducin Laboratories. All other reagents were of analytical grade and obtained from Sigma Chemical Co., St. Louis, MO. The murine FATP1 cDNA was a generous gift of Dr. Jean Schaffer, St. Louis, MO.

Generation of stable cells expressing FATP1

FATP1 subcloned into pcDNA3.0 (InVitrogen) or vector control was linearized and introduced into 293 cells by electroporation (BTX Corp., San Diego, CA) according to the manufacturers instructions. 293 cells are an excellent model system to evaluate FATP1 function in lipid metabolism for they are a well defined cell system, grow easily in culture, and have been shown to tolerate FATP expression (5). Dilutions of the electroporated cells were plated in DMEM containing 10% FBS and incubated at 37°C, 5% CO_2, and after 48 h selection was initiated with the addition of 400 μg/ml geneticin (GIBCO/BRL Life Technologies). After 8 days colonies were selected using cloning rings and individual lines developed. Once established, several lines were analyzed for FATP1 expression using a commercially generated (bioWORLD, Dublin OH) affinity-purified polyclonal anti-peptide antibody made in rabbits and directed toward murine FATP1 amino acid sequences 192–215. A cell line stably expressing a high level of mFATP1 (C8) was selected for further study along with a vector-transformed line (vector). C8 and vector cells were maintained on 400 μg/ml geneticin.

Localization of FATP1 in C8 cells

Vector and C8 cells were plated onto 13 mm coverslips and fixed with 3% formaldehyde and 0.1% glutaraldehyde in the presence or absence of 0.01% Triton X-100 at 37°C. After fixation, cells were incubated for 2 h at 37°C with anti-FATP1, washed with PBS, and incubated with fluorescein-conjugated secondary antibody for 1 h at 25°C. Cells were viewed using a Nikon Eclipse E800 photomicroscope equipped with brightfield, DIC, phase and fluorescence optics including a 100 W mercury lamp epifluoresence illumination with standard fluorescein/GFP (excitation filter 470–490 nm, barrier 520–580 nm) filter sets. Digital images were collected using a CoolCam liquid-cooled, three-chip color CCD camera (Cool Camera Co., Decatur, GA) and captured to a personal computer using Image Pro Plus version 4.1. software (Media Cybernetics, Silver Springs, MD).

FA uptake experiments

C8 or vector cells (200,000 per well) were plated on 13 mm coverslips in 12-well plates pre-treated with polylysine for enhanced cell adherence and incubated for 48 h in DMEM containing 10% FBS. Uptake experiments were performed on cells at 50–90% confluence. Cells were incubated for 2 h with serum-free DMEM, washed twice with PBS, and then incubated for 10–20 min at 37°C in Krebs-Ringer Hepes containing 2 mM glucose, pH 7.4, before initiating uptake studies. Stock solutions (5 mM) of non-radioactive oleic acid or lignoceric acid were prepared as described (16), and radioactive lipids were added to achieve the indicated specific radioactivity. To initiate uptake, cells were incubated at 37°C with KRH, 2 mM glucose, pH 7.4, containing 50 μM [^{3}H]oleic acid (8 mC_i/mmol) or 50 μM [^{14}C]lignoceric (3 mC_i/mmol) acid bound to FA free BSA (1:1 molar ratio). After 30 s, the cells were rapidly washed three times in ice cold PBS containing 200 μM phloretin, incubated at room temperature in 0.5% SDS for 30 min, and the radioactivity determined by liquid scintillation counting. A duplicate set of coverslips treated similarly were utilized for phosphorus determinations. FA uptake was expressed as nmol FA internalized/30 s/nmol lipid phosphorus. The concentration of phosphorus did not differ between vector and C8 cells. For experiments using BODIPY 3823, cells were plated onto coverslips at 250,000 cells/well and after 48 h cells were incubated with 20 μM BODIPY 3823 in BSA (1:1 molar ratio) and uptake determined as described (5) using a Nikon E800 Fluorescence microscope.

Radiolabeling of phospholipid and triacylglycerol pools

Cells were plated on 10 cm plates and incubated in DMEM containing 10% FBS. Subconfluent cells were incubated for 4 h with serum-free DMEM containing 0.1 mM [^{3}H]oleic acid (2 μCi / dish) or 0.1 mM [^{14}C]lignoceric acid (2 μCi /dish) bound to albumin (1:1 molar ratio). For other labeling, cells were incubated for 4 h with either 0.1 μM [^{14}C]acetate (2 μCi/dish), 0.5 mM L-[^{3}H]serine (10 μCi /dish), 28 μM [*methyl*-^{3}H]choline (2 μCi / dish), 28 μM [^{3}H]ethanolamine (2 μCi /dish), or 0.4 mM [*myo*-^{3}H]inositol (2 μCi /dish). For all labeling, the medium was removed and the cells washed twice with ice-cold PBS and then harvested from the dish with 2 ml of methanol-water (1:1, v/v) using a rubber policeman. A 25 μl aliquot was taken for the determination of total radioactivity associated with the cells and another for protein determination. To facilitate phase separation, chloroform and water were added until a final ratio of chloroform-methanol-water of 4:2:3 (v/v/v) was achieved. The mixture was centrifuged and the aqueous phase removed. The organic phase was washed once with theoretical aqueous phase (chloroform-methanol-water, 3:48:47, v/v/v). The aqueous phase was removed and combined with the aqueous phase from the first centrifugation and a 0.5 ml aliquot of the combined aqueous fraction was taken for the deter-

mination of radioactivity. The organic phase was dried under nitrogen and resuspended in 125 μl of chloroform-methanol (2:1, v/v) and 50 μl aliquots were placed on thin-layer plates for the separation of neutral lipids and phospholipids. Phospholipids were separated by ascending TLC in a solvent system containing chloroform-methanol-acetic acid-water (70:30:8:3, v/v/v/v). The TG, DG, and FA fractions were separated by TLC in a solvent system containing benzene-diethylether-absolute ethanol-acetic acid (50:45:2:0.2, v/v/v/v). Cholesterol was separated by TLC in a solvent system containing hexanes-diethylether-acetic acid (70:30:2, v/v/v). Silica gel corresponding to the lipids of interest was removed and radioactivity in these determined by liquid scintillation counting as described (17).

Enzymatic assays and other determinations

Phospholipid and cholesterol pool sizes were determined as described (18, 19). FA and TG pool sizes were measured by gas chromatography using C19 as internal standard as described (20). For Oil Red O staining a 0.5% solution was prepared in isopropanol then diluted with 1.5 ml water, filtered, and used immediately as described (21). Preparation of caveolin-1 containing membrane fractions and separation using Optiprep™ were exactly as described (22). Microsomal fractions were prepared from vector and C8 cells and 1,2-diacyl-*sn*-glycerol acyltransferase (DGAT) activity determined in these fractions as described (23). Microsomal and mitochondrial fractions were prepared from vector and C8 cells and *sn*-glycerol-3-phosphate acyltransferase (GPAT) activity determined in these fractions as described (24). FATP1 dependent FA CoA ligase activity was measured as described by Coe et al., (15). Protein was measured by the method of Lowry (25). Student's *t*-test or Dunnet's *t*-test for multiple comparisons with a single mean were used for determination of statistical significance. The level of significance was defined as $P < 0.05$.

RESULTS

FATP1 is localized to non-caveolin-1 containing PMs and internal membranes in C8 cells

The subcellular localization of FATP1 was investigated in vector and in C8 cells over-expressing FATP1. As shown in **Fig. 1**, the level of FATP1 was markedly elevated in C8 cells compared with vector. In addition, FATP1 was clearly localized to the PM in vector and C8 cells. Consistent with the results of Schaffer and colleagues (5, 6), additional fluorescence was detected on internal membranes and suggested that some FATP1 may reside within the ER. In agreement with the immunofluorescence studies, semi quantitative analysis of Western blots of FATP1 expression suggested approximately 20× more FATP1 in C8 cells as compared with vector cells (Fig. 1, inset). Although FATP1 is expressed to high levels, the abundance of the protein in 293 cells is less than in cultured 3T3-L1 adipocytes (results not shown). As such, while overexpressed, the amount of FATP1 is not in excess of that normally found. Fractionation of membranes by gradient density centrifugation in the presence of Optiprep™ (22) and subsequent Western blotting indicated that FATP1 did not co-localize with caveolin-1 (**Fig. 2**). Hence, heterologously expressed FATP1 is localized in 293 cells to non-caveolin-1 containing regions of the plasma and internal membranes.

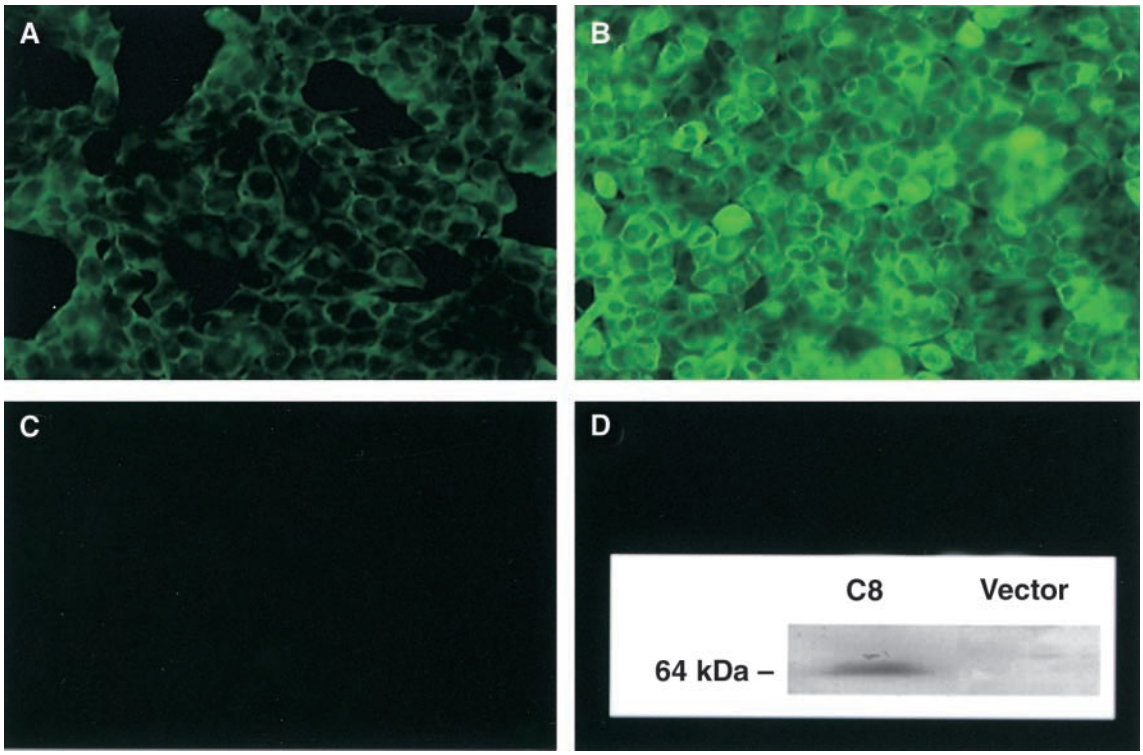

Fig. 1. Localization of fatty acid transport protein 1 (FATP1) protein in vector and C8 cells. Vector and C8 cells were glutaraldehyde/formaldehyde fixed and incubated with anti-FATP1 antibody followed by fluorescein-tagged goat anti-rabbit secondary antibody and analyzed by immunofluorescence microscopy. A and C: Represent vector cells. B and D: C8 cells. Fluorescence of monolayers treated identically but lacking primary antibody is depicted in C and D. The inset in D represents Western blotting of total cell membrane from C8 and vector cells with anti-FATP1 antibody.

FA-CoA ligase activity and the uptake of FAs are elevated in 293 cells over-expressing FATP1

The FA-CoA ligase activity of cellular extracts expressing FATP1 was compared with that from vector cells using both oleic acid and lignoceric acid. Consistent with previous reports for FATP1 (15) and FATP4 (26), FATP1 containing fractions had elevated esterification activity using both long chain (18:1) and very long chain (24:0) FAs. The amount of esterification activity attributable to FATP1 was similar for long chain and very long chain FA (**Fig. 3**). This indicated that FATP1 is a broad substrate range enzyme as opposed to more familiar ACS family of CoA ligases that exhibit much more specificity for long chain over very long chain FAs (27). Since the ACS family of CoA ligases are sensitive to inhibition by triacsin C (28, 29), we evaluated if FATP1 was similarly inhibited. As shown in Fig. 3, extracts from both vector and FATP1 expressing cells were sensitive to increasing triacsin C levels using oleic acid as a substrate but were markedly resistant to triacsin C inhibition using lignoceric acid as a substrate. Moreover, the fraction of activity inhibited by triacsin C in vector extracts was greater than from C8 cells. These results suggest that FATP1 is a broad range CoA ligase that is relatively insensitive to triacsin C inhibition and that triacsin C sensitivity can be used as a tool to evaluate lipid trafficking through either the ACS or FATP family of enzymes.

To examine if lipid uptake was elevated in C8 cells over-expressing FATP1, cells were incubated for 30 s with FA analog BODIPY 3823 and the incorporation evaluated by fluorescence microscopy. C8 cells exhibited elevated BODIPY 3823 staining compared with vector suggesting that FA uptake was elevated in cells over-expressing FATP1 (**Fig. 4**). This was anticipated since FATP1 was isolated by Schaffer and Lodish (5) as having BODIPY 3823 uptake activity. To examine if long chain and very long chain FA uptake were elevated in cells over-expressing FATP1, 293 vector and C8 cells were incubated for 30 s at 37°C with KRH, 2 mM glucose, pH 7.4, containing 50 μM [^{3}H]oleic acid or 50 μM [^{14}C]lignoceric acid bound to BSA (1:1 molar ratio) and radioactivity incorporated into cells determined. [^{3}H]oleic acid incorporation into vector was 7.5 ± 2.7 × 10^2 nmol/30 s/nmol Pi and increased 1.8-fold to 13.5 ± 3.9 × 10^2 nmol/30 s/nmol P_i in C8 cells. [^{14}C]lignoceric acid incorporation into vector was 9.3 ± 0.1 × 10^2 nmol/30 s/nmol Pi and increased 2.6-fold to 24.3 ± 0.7 × 10^2 nmol/30 s/nmol Pi in C8 cells (**Fig. 5**). These data indicate that C8 cells over-expressing FATP1 exhibit a functional elevation in FA uptake compared with vector and that uptake activity is, generally, proportional to FATP1 enzymatic activity. It should be noted that the radiolabeling methodology used in this study simply measures cell-associated radioactivity and does not mechanistically distinguish between bilayer transit (transport) vs. intercalation and lateral diffusion in the PM. As such, the term uptake has been used to signify such.

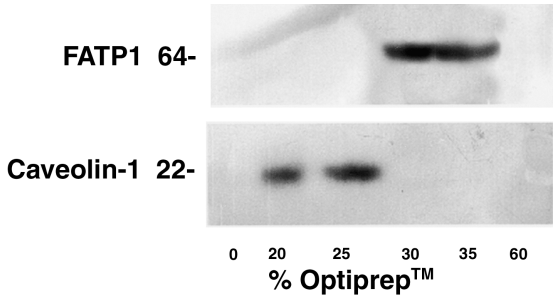

Fig. 2. FATP1 does not co-localize with caveolin-1 in C8 cells. Detergent-solubilized C8 cell extract was analyzed exactly as described by Oliferenko et al. (22) using a floatation gradient system in the presence of varying concentrations of Optiprep™. Following centrifugation, fractions were recovered and proteins analyzed by SDS-PAGE. The presence of FATP1 and caveolin-1 in each fraction was assessed using immunoblotting and detected using enhanced chemiluminescence. Numbers represent molecular mass in kDa.

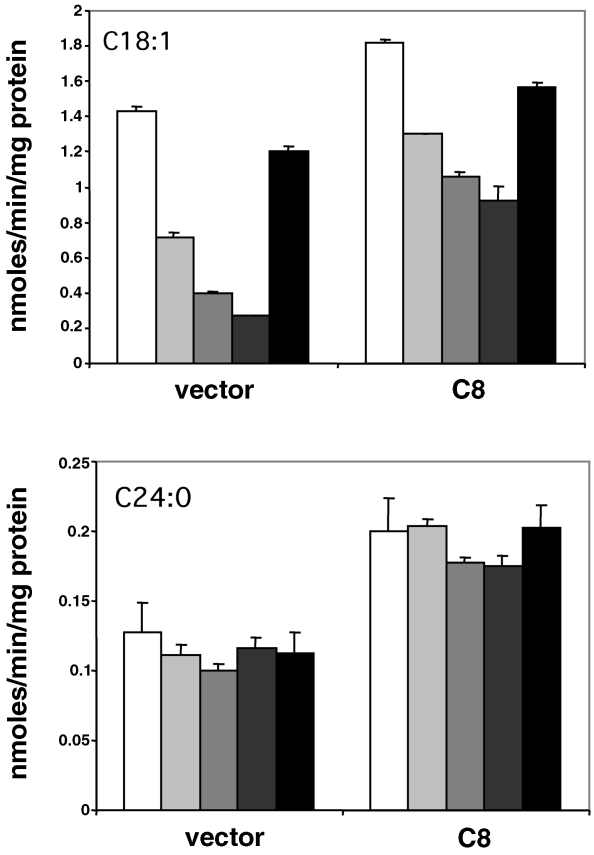

Fig. 3. Fatty acid (FA) CoA ligase activity of FATP1 and its sensitivity to triacsin C. Vector or C8 cells were lysed by repeated freeze-thaw cycles and total protein assayed for FA-CoA ligase activity using either oleic acid (18:1) or lignoceric acid (24:0) in the presence or absence of the indicated concentration of triacsin C. Control activity (white bar), 1μM triacsin C (light gray bar), 10 μM triacsin C (gray bar), 100 μM triacsin C (dark gray bar), DMSO control (black bar).

TG synthesis is increased and SM synthesis decreased in 293 C8 cells over-expressing FATP1

Since some of FATP1 was localized to internal membranes in 293 cells, and the endoplasmic reticulum (ER) is the principal site of lipid synthesis in mammalian cells, we investigated if over-expression of FATP1 affected de novo biosynthesis of lipids from long chain FA. To evaluate

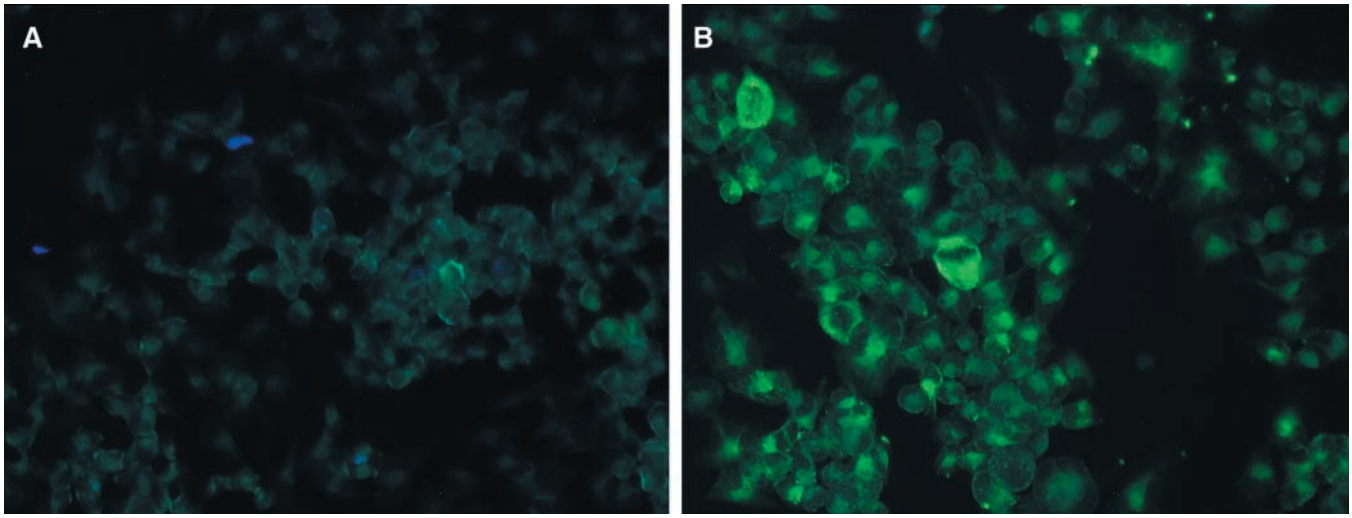

Fig. 4. FA uptake in vector and C8 cells as assessed by BODIPY 3823 incorporation. Vector and C8 cells were incubated for 30 s with BODIPY 3823 and analyzed by fluorescence microscopy.

this possibility, 293 vector and C8 cells were cultured for 7 days, stained with Oil Red O (21), and analyzed by light microscopy. Oil Red O staining of C8 cells was enhanced compared with vector indicating increased accumulation of neutral lipid in 293 cells over-expressing FATP1 (**Fig. 6**) and suggestive of effects on de novo biosynthesis of lipids. A similar accumulation of TG has been reported by Souza et al., (30) who co-expressed ACS1 and FATP1.

To evaluate de novo lipid biosynthesis in FATP1 expressing cells, 293 vector and C8 cells were incubated for 4 h with 0.1 mM [^{3}H]oleic acid bound to albumin (1:1 molar ratio) and radioactivity incorporated into lipids determined. Oleic acid in the quantity of 0.1 mM was chosen to approximate the physiological level of plasma FA (31). Total radioactivity incorporated into 293 C8 cells was elevated 30% ($P < 0.05$) compared with vector (**Table 1**). Radioactivity incorporated into TG in 293 C8 cells was increased 72% ($P < 0.05$) compared with vector, indicating an apparent elevation in TG synthesis from oleic acid. Radioactivity observed as free FA accounted for less than 1% of the total radioactivity associated with the cells and was unaltered in 293 C8 cells compared with vector. In the phospholipid fraction, radioactivity incorporated into SM was reduced 34% ($P < 0.05$) in 293 C8 cells compared with vector indicating an apparent reduction in SM biosynthesis. In contrast, radioactivity incorporated into DG and the glycerophospholipids PC, PE, phosphatidylserine (PS), and PI/PA were unaltered in 293 C8 cells compared with vector. Thus, in 293 C8 cells over-expressing FATP1, de novo TG biosynthesis from oleic acid was elevated and SM biosynthesis from oleic acid reduced.

Long chain sphingoid bases are synthesized de novo from serine and palmitate (32). To examine if the reduction in oleic acid incorporation into SM in 293 cells was truly due to a reduction in de novo SM biosynthesis, 293 vector and C8 cells were incubated for 4 h with 0.5 mM L-[^{3}H]serine and radioactivity incorporated into SM determined. Total radioactivity incorporated into 293 C8 cells was unaltered compared with vector (**Table 2**). In contrast, radioactivity incorporated into SM was reduced 26% ($P < 0.05$) in 293 C8 cells compared with vector. Radioactivity incorporated into PS and PE was unaltered compared with the vector, indicating that PS biosynthesis and its subsequent decarboxylation to PE was unaffected in 293 C8 cells. Thus, in 293 C8 cells over-expressing FATP1, de novo biosynthesis of SM was reduced.

Since PC is the major phospholipid found in mammalian cells (7), we examined if elevation in expression of FATP1 altered de novo biosynthesis of PC or of other phospholipids. C8 cells were incubated with [*methyl*-^{3}H]choline for 4 h and radioactivity incorporated into PC, SM, and LPC determined. Total radioactivity incorporated into 293 C8 cells was unaltered compared with vector (**Table 3**). In addition, radioactivity incorporated into PC and LPC was unaltered in C8 cells compared with vector. In contrast, radioactivity incorporated into SM was reduced 55% ($P < 0.05$) in C8 cells compared with controls confirming the

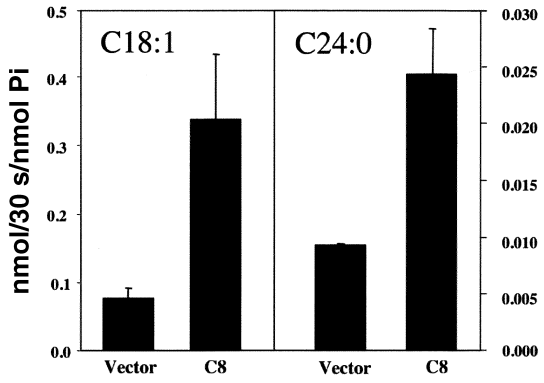

Fig. 5. FA uptake in vector and C8 cells. FA uptake using 50 μM total lipid in a 1:1 molar complex with BSA for 30 s. was performed as described in Materials and Methods. A: Represents oleic acid uptake while B represents lignoceric acid. Error bars are SD. $P < 0.05$.

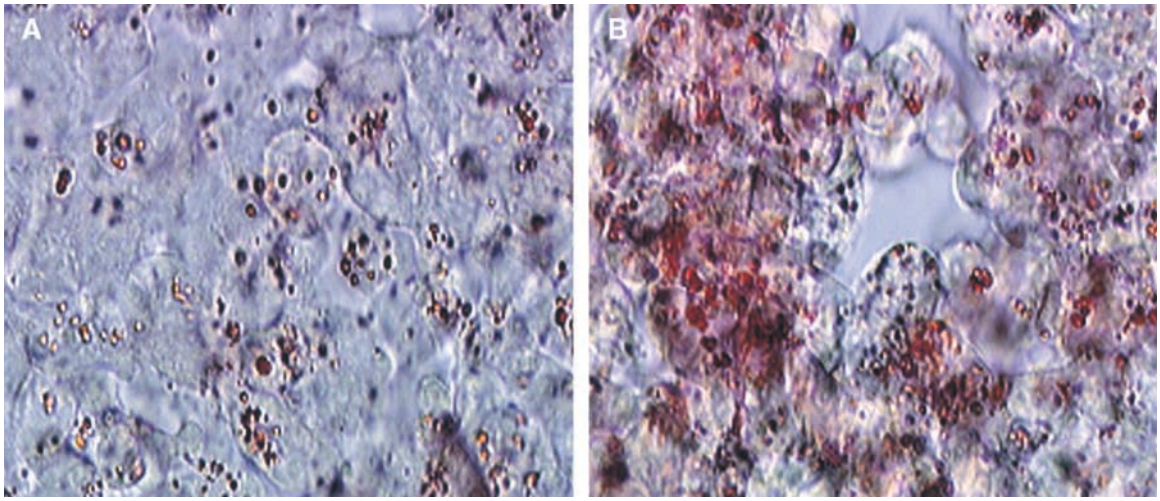

Fig. 6. Oil Red O staining for neutral lipids in vector and C8 cells. Vector and C8 cells were formaldehyde fixed and incubated with Oil Red O and visualized by light microscopy.

decrease in de novo SM synthesis. In addition, FATP1 overexpression in C8 cells did not affect [*myo*-^{3}H]inositol uptake or incorporation into PI nor [^{3}H]ethanolamine incorporation into PE (data not shown). Thus, the reduction in de novo SM biosynthesis from [^{3}H]serine, [^{3}H]oleic acid, and [*methyl*-^{3}H]choline in C8 cells was specific for this phospholipid. PC and PE were the major phospholipids in 293 cells and accounted for 56% and 24%, respectively, of the total phospholipid mass. Over-expression of FATP1 in 293 C8 cells did not affect the PC and PE pool sizes compared with the vector (data not shown).

Elevated TG biosynthesis is correlated with decreased cholesterol and SM biosynthesis in 293 cells over-expressing FATP1

A previous study had indicated a negative correlation between TG and SM-cholesterol levels in the surfactant-accommodated epidermis (33). To explore if this relationship was observed in 293 cells over-expressing FATP1, we simultaneously examined TG, SM, and cholesterol biosynthesis in 293 C8 and vector cells. 293 vector and C8 cells were incubated for 4 h with [^{14}C]acetate and radioactivity incorporated into TG, SM, and cholesterol determined. Total incorporation of radioactivity into 293 C8 cells was unaltered compared with the vector (**Table 4**). Radioactivity incorporated into TG was increased 69% ($P < 0.05$) in 293 C8 cells compared with the vector. Since total uptake of [^{14}C]acetate into the 293 C8 cells was unaltered but incorporation into TG elevated compared with vector, de novo TG biosynthesis was truly stimulated in C8 cells. In contrast, radioactivity incorporated into SM and cholesterol was reduced 33% ($P < 0.05$) and 57% ($P < 0.05$), respectively, in 293 C8 cells compared with vector. Radioactivity incorporated into all other phospholipids was unaffected (data not shown). These data indicate that a negative correlation between de novo TG and SM-cholesterol biosynthesis exists in 293 cells over-expressing FATP1. We examined the pool sizes of FA, TG, SM, and cholesterol in these cells. As seen in **Table 5**, the pool sizes of FA and TG were elevated 60% ($P < 0.05$) and 2.2-fold ($P < 0.05$), respectively, in 293 C8 cells compared with vector. In contrast, SM and cholesterol pool sizes were unaltered. Since the cholesterol and SM pool sizes were unaltered but cholesterol and SM de novo biosynthesis were decreased in 293 C8 cells, we interpret this data to suggest that SM and cholesterol metabolism in C8 cells was lower than that of the vector. The mechanism for the elevation

TABLE 1. Incorporation of [^{3}H]oleic acid into lipids of 293 vector and C8 cells

	Vector	C8	
Total incorporation	2.0 ± 0.1	2.6 ± 0.3[a]	(dpm × 10^6/mg protein)
Cellular lipid class			
TG	3.9 ± 0.3	6.7 ± 0.6[a]	(dpm × 10^5/mg protein)
DG	3.2 ± 0.4	3.3 ± 0.8	(dpm × 10^4/mg protein)
PC	2.1 ± 0.1	2.0 ± 0.2	(dpm × 10^5/mg protein)
PE	6.9 ± 0.5	6.8 ± 0.4	(dpm × 10^4/mg protein)
PI/PA	1.6 ± 0.4	2.0 ± 0.4	(dpm × 10^4/mg protein)
FA	9.0 ± 1.9	9.4 ± 2.4	(dpm × 10^3/mg protein)
PS	4.5 ± 0.6	5.1 ± 0.7	(dpm × 10^3/mg protein)
SM	3.2 ± 0.1	2.1 ± 0.1[a]	(dpm × 10^3/mg protein)

293 vector and C8 cells were incubated with [^{3}H]oleic acid bound to albumin for 4 h and the radioactivity incorporated into cells and lipids determined. Data represent the mean ± SD of three determinations.

[a] $P < 0.05$ for comparison of C8 with vector.

TABLE 2. Incorporation of L-[^{3}H]serine into SM, PE, and PS of 293 vector and C8 cells

	Vector	C8	
Total incorporation	2.2 ± 0.1	2.1 ± 0.1	(dpm × 10^5/mg protein)
Cellular lipid class			
PE	7.5 ± 0.7	6.9 ± 0.3	(dpm × 10^3/mg protein)
PS	4.1 ± 0.3	3.6 ± 0.5	(dpm × 10^3/mg protein)
SM	2.7 ± 0.3	2.0 ± 0.2[a]	(dpm × 10^3/mg protein)

293 vector and C8 cells were incubated with L-[^{3}H]serine for 4 h and the radioactivity incorporated into cells, SM, PE, and PS determined. Data represent the mean ± SD of three determinations.

[a] $P < 0.05$ for comparison of C8 with vector.

TABLE 3. Incorporation of [*methyl*-^{3}H]choline into SM, PC, and LPC of vector and C8 cells

	Vector	C8	
Total incorporation	1.9 ± 0.3	1.7 ± 0.2	(dpm × 10^5/mg protein)
Cellular lipid class			
PC	6.1 ± 0.4	5.9 ± 0.2	(dpm × 10^4/mg protein)
LPC	1.2 ± 0.2	1.0 ± 0.2	(dpm × 10^2/mg protein)
SM	6.0 ± 0.6	2.7 ± 0.2[a]	(dpm × 10^2/mg protein)

293 vector and C8 cells were incubated with [*methyl*-^{3}H]choline for 4 h and the radioactivity incorporated into cells, SM, PC, and LPC determined. Data represent the mean ± SD of three determinations.

[a] $P < 0.05$ for comparison of C8 with vector.

TABLE 5. Pool size of FA, cholesterol, TG, and SM of 293 vector and C8 cells

	Vector	C8	
Cellular lipid class			
TG	11 ± 5	24 ± 3[a]	(nmol/mg protein)
FA	5 ± 1	8 ± 1[a]	(nmol/mg protein)
SM	0.28 ± 0.02	0.24 ± 0.02	(nmol P_i/mg protein)
Cholesterol	1.48 ± 0.23	1.55 ± 0.05	(μg/mg protein)

Lipids were extracted from 293 vector and C8 cells and the pool size of FA, cholesterol, TG, and SM determined. Data represent the mean ± SD of three determinations.

[a] $P < 0.05$ for comparison of C8 with vector.

in TG pool size was examined. Mitochondrial and microsomal GPAT activities were unaltered between vector and C8 cells (**Table 6**). In contrast, DGAT activity was increased 40% ($P < 0.05$) in C8 cells compared with vector. Thus, the increase in TG formation in C8 cells was due to an increase in DGAT activity.

We next examined whether or not the inverse correlation between de novo TG and SM biosynthesis in 293 cells over-expressing FATP1 held true for very long chain FAs. 293 vector and C8 cells were incubated for 4 h with 0.1 mM [^{14}C]lignoceric acid bound to albumin (1:1 molar ratio) and radioactivity incorporated into lipids determined. Incorporation of [^{14}C]lignoceric acid into TG was increased 35% ($P < 0.05$) from 4.3 ± 0.4 dpm × 10^2/mg protein in vector to 5.8 ± 0.5 dpm × 10^2/mg protein in C8 cells (average of three separate dishes). In contrast, incorporation of [^{14}C]lignoceric acid into SM was reduced 53% ($P < 0.05$) from 3.8 ± 0.7 dpm × 10^2/mg protein in vector to 1.8 ± 0.3 dpm × 10^2/mg protein in C8 cells (average of three separate dishes). Incorporation of [^{14}C]lignoceric acid into other phospholipids was unaffected (data not shown). Thus, in 293 C8 cells over-expressing FATP1 de novo TG biosynthesis from very long chain FA was elevated and SM biosynthesis from very long chain FA reduced.

FATP1-mdediated uptake of oleic acid is insensitive to triacsin C

Since FATP1 CoA synthetase activity was found to be insensitive to triacsin C inhibition whereas ACS forms 1 and 4 are sensitive to the inhibitor [28, 29], we examined how the presence of triacsin C would affect FA incorporation and its subsequent conversion into TG in C8 cells over-expressing FATP1. Cells were incubated for 4 h with [^{3}H]oleic acid bound to albumin (1:1 molar ratio) and total radioactivity incorporated into cells and TG determined. Incorporation of [^{3}H]oleic acid into 293 C8 cells was elevated 41% ($P < 0.05$) compared with vector (**Table 7**). The presence of triacsin C (10 μM) resulted in a 47% reduction ($P < 0.05$) in incorporation of [^{3}H]oleic acid into 293 vector cells compared with untreated vector cells. In addition, the presence of triacsin C resulted in a 33% reduction ($P < 0.05$) in incorporation of [^{3}H]oleic acid into C8 cells over-expressing FATP1 compared with untreated C8 cells. Incorporation of [^{3}H]oleic acid into untreated 293 vector and triacsin C-treated C8 cells was similar. Incorporation of [^{3}H]oleic acid into TG in 293 C8 cells was elevated 83% ($P < 0.05$) compared with vector. The presence of triacsin C resulted in a 86% reduction ($P < 0.05$) in incorporation of [^{3}H]oleic acid into TG in 293 vector cells compared with untreated vector cells. In addition, the presence of triacsin C resulted in a 41% reduction ($P < 0.05$) in incorporation of [^{3}H]oleic acid into TG in C8 cells compared with untreated C8 cells. Incorporation of [^{3}H]oleic acid into TG of untreated 293 vector and triacsin C-treated C8 cells was similar. Consistent with triacsin C not affecting FATP1 enzymatic activity, triacsin C did not affect the FATP1 mediated elevation in [^{3}H]oleic acid incorporation into C8 cells and its subsequent conversion into TG.

DISCUSSION

The objective of this study was to examine the regulation of lipid metabolism from long chain and very long chain FAs and to explore metabolic channelling of metabolites derived from FATP1 action. Although some FATP1

TABLE 4. Incorporation of [^{14}C]acetate into cholesterol, TG, and SM of 293 vector and C8 cells

	Vector	C8	
Total incorporation	1.8 ± 0.1	1.7 ± 0.1	(dpm × 10^5/mg protein)
Cellular lipid class			
TG	5.1 ± 1.1	8.6 ± 1.0[a]	(dpm × 10^3/mg protein)
SM	2.4 ± 0.1	1.6 ± 0.2[a]	(dpm × 10^3/mg protein)
Cholesterol	7.4 ± 1.6	3.2 ± 0.4[a]	(dpm × 10^2/mg protein)

293 vector and C8 cells were incubated with [^{14}C]acetate for 4 h and the radioactivity incorporated into cells, cholesterol, TG, and SM determined. Data represent the mean ± SD of three determinations.

[a] $P < 0.05$ for comparison of C8 with vector.

TABLE 6. GPAT and DGAT activities of 293 vector and C8 cells

Enzyme activity	Vector	C8
	nmol/min·mg protein	
GPAT microsomal	1.44 ± 0.08	1.50 ± 0.11
Mitochondrial	1.27 ± 0.07	1.28 ± 0.08
DGAT	0.15 ± 0.01	0.21 ± 0.02[a]

Mitochondrial and microsomal fractions were prepared from 293 vector and C8 cells and mitochondrial and microsomal GPAT and microsomal DGAT activities determined. Data represent the mean ± SD of three determinations.

[a] $P < 0.05$ comparing C8 cells to vector cells.

TABLE 7. Effect of triacsin C on incorporation of [^{3}H]oleic acid into 293 vector and C8 cells

	Vector	C8	Vector	C8
	(−Triacsin C)		(+10 μM Triacsin C)	
Total incorporation (dpm × 10^6/mg protein)	1.7 ± 0.1	2.4 ± 0.1[a]	0.9 ± 0.1[b]	1.6 ± 0.2[c]
Cellular lipid class (dpm × 10^5/mg protein)				
TG	3.5 ± 0.4	6.4 ± 0.5[a]	0.5 ± 0.1[b]	3.8 ± 0.5[c]

293 vector and C8 cells were incubated with [^{3}H]oleic acid for 4 h and the radioactivity incorporated into cells and TG determined. Data represent the mean ± SD of three determinations.
[a] $P < 0.05$ vector versus C8, [b] $P < 0.05$ vector versus vector + triacsin C, [c] $P < 0.05$ C8 versus C8 + triacsin C.

is found in internal membranes, the protein is largely localized to the PM in 293 cells (5). Our findings confirm this observation and are consistent with a recent report demonstrating the presence of FATP1 on internal membranes in NIH-3T3 cells (6). The results presented herein clearly indicate the surprising finding that FATP1 channels exogenous FA toward TG biosynthesis while down regulating SM and cholesterol metabolism in growing 293 cells.

The elevation in DGAT activity and TG biosynthesis from oleic acid or lignoceric acid in growing 293 C8 cells over-expressing FATP1 and lack of altered GPAT activity and incorporation of these FAs into DG and glycerophospholipids indicate that FATP1 may play a role in TG synthesis at the ER. These observations support the hypothesis of functionally independent acyl-CoA pools within mammalian cells which may be channelled toward specific fates rather than being freely available for all possible enzymatic reactions (27, 28, 29). The question might be asked does PM FATP1 regulate TG metabolism? PM FATP4 has been shown to regulate FA absorption in intestinal mucosal cells (34). In these cells, the PM is in close association with the ER. As reviewed by Stremmel et al. (3), it is possible that FATP4 may play a role in activation of FAs for TG synthesis at the ER in these cells. The same mechanism of activation of FAs might hold true for FATP1 in 293 cells since the immunofluorescence micrographs indicated some areas of PM FATP1 association with ER.

The ACSs referred to as the ACS family are a family of enzymes that catalyze the condensation of long chain FA with CoA to produce acyl-CoA (12). The acyl-CoAs formed are utilized for glycerolipid synthesis, esterification of cholesterol, and β-oxidation, as well as signal transduction (35). At least five different isoforms of ACS exist in mammalian tissue. Both FATP1 and FATP4 have been shown to exhibit broad range FA CoA synthetase activity (4, 15, 26) and are members of a related, five-member gene family in mice (six members in the human FATP family). However, the specific activity of the ACS family appears to be at least an order of magnitude greater than that of the FATP enzymes (**Fig. 3**). Triacsin C is a non-specific competitive inhibitor of the ACS class of enzymes and was shown to be potently effective against ACS1 and ACS4 isoforms (28, 29). Recently the existence of a triacsin C-insensitive ACS (ACS 5) localized to the mitochondrial membrane was demonstrated (28). In the current study, treatment of C8 293 cells with triacsin C did not affect the enzymatic activity of FATP1 or the FATP1-mediated contribution of [^{3}H]oleic acid incorporation into cells nor [^{3}H]oleic acid incorporation into TG. These data in sum suggest that FATP1 is a triacsin C-insensitive CoA synthetase in growing 293 cells.

The results presented herein should be contrasted with the findings of Chui et al., (36) who developed a transgenic mouse line overexpressing ACS1 specifically in the cardiomyocyte under control of myosin heavy chain (MHC) promoter. Such MHC-ACS1 transgenic mice developed cardiac hypertrophy and accumulated 12-fold more TG than did wild-type animals. In contrast to our findings, MHC-ACS1 cardiomyocytes also exhibited a 50% increased accumulation of choline glycerophospholipids and a 15% increase in ethanolamine glycerophospholipids, but no increase in free FA. 293 cells expressing FATP1 similarly accumulated TG had an expanded free FA pool, but did not exhibit any increased accumulation of glycerophospholipids nor alterations in mitochondrial and microsomal GPAT activity. Since overexpression of mitochondrial GPAT activity was associated with increased TG biosynthesis and a reduction in phospholipid biosynthesis in CHO and HEK293 cells (37), it would be of interest to determine in MHC-ACS1 cardiomyocytes if there is an upregulation of DGAT and downregulation of GPAT to account for the bifurcated lipid trafficking. It is not clear if the difference in lipid metabolism reflects differences in cell types, or in specific lipid trafficking events linked to either ACS1 or FATP1. Clearly, NIH-3T3 cells overexpressing both ACS1 and FATP1 exhibit elevated FA uptake and TG accumulation (30, 38). However, the effect of overexpression of both ACS1 and FATP1 on phospholipid metabolism in these cells is unknown. Since acyl-CoAs have been implicated as potential natural ligands for nuclear transcription factors, one attractive hypothesis is that cells overexpressing FATP1 and/or ACS1 affect acyl-CoA pools sufficiently to alter expression of genes whose protein products are linked to complex lipid synthesis.

Many studies have indicated a coordinated link between SM and cholesterol biosynthesis (11). In addition, PUFA regulation of SM and cholesterol biosynthesis is well documented. For example, addition of exogenous oleic acid or other PUFAs to hepatocytes resulted in reduced de novo SM synthesis via inhibition of serine palmitoyltransferase

activity (39). Moreover, addition of exogenous oleic acid and other PUFAs to various cell lines reduced the nuclear content of sterol responsive element binding protein-1 and transcription of various sterol responsive element regulated genes including hydroxymethylglutaryl-CoA reductase, which would result in a reduction in cholesterol biosynthesis (40, 41). In C8 cells over-expressing FATP1, the pool size of FA was elevated and incorporation of [^{3}H] serine or [*methyl*-^{3}H]choline into SM and [^{14}C]acetate into both SM and cholesterol were reduced compared with vector cells. This indicates a concerted regulation of these lipids at the biosynthetic level in growing 293 cells. The current study is the first to show coordination between cholesterol and SM biosynthesis in growing 293 cells. Since the pool sizes of cholesterol and SM were not altered in 293 C8 cells over-expressing FATP1, but de novo biosynthesis from various metabolic precursors was reduced, cholesterol and SM metabolism in these cells must be lower. Hence, FATP1 may play a role in the regulation of cholesterol and SM metabolism through provision of exogenous FAs to down-regulate the biosynthesis of these lipids. In summary, the data present in this paper clearly indicate that in these actively growing 293 cells overexpressing FATP1, there is a mediated metabolic channelling of exogenous FA toward TG formation and down-regulation of cholesterol and SM metabolism.

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