OVEREXPRESSION OF RAT LONG CHAIN ACYL-COA SYNTHETASE 1 ALTERS FATTY ACID METABOLISM IN RAT PRIMARY HEPATOCYTES

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Running Title: ACSL1 overexpression alters fatty acid metabolism

ABSTRACT
Long chain acyl-CoA synthetases (ACSL) activate fatty acids (FA) and provide substrates for both anabolic and catabolic pathways. We have hypothesized that each of the five ACSL isoforms partitions FA towards specific downstream pathways. ACSL1 mRNA is increased in cells under both lipogenic and oxidative conditions. To elucidate the role of ACSL1 in hepatic lipid metabolism, we overexpressed an ACSL1 adenovirus construct (Ad-ACSL1) in rat primary hepatocytes. Ad-ACSL1, located on the endoplasmic reticulum but not on mitochondria or plasma membrane, increased ACS specific activity 3.7-fold. With 100 or 750 μM [1-14C]oleate, Ad-ACSL1 increased oleate incorporation into diacylglycerol and phospholipids, particularly phosphatidylethanolamine and phosphatidylinositol, and decreased incorporation into cholesterol esters and secreted triacylglycerol. Ad-ACSL1 did not alter oleate incorporation into triacylglycerol, β-oxidation products, or total amount of FA metabolized. In pulse-chase experiments to examine the effects of Ad-ACSL1 on lipid turnover, more labeled diacylglycerol and phospholipid, but less labeled diacylglycerol remained in Ad-ACSL1 cells, suggesting that ACSL1 increased reacylation of hydrolyzed oleate derived from triacylglycerol and diacylglycerol. In addition, less hydrolyzed oleate was used for cholesterol ester synthesis and β-oxidation. The increase in [1,2,3-3H]glycerol incorporation into diacylglycerol and phospholipid was similar to the increase with [14C]oleate labeling suggesting that ACSL1 increased de novo synthesis. Labeling Ad-ACSL1 cells with [14C]acetate increased triacylglycerol synthesis, but did not channel endogenous FA away from cholesterol ester synthesis. Thus, consistent with the hypothesis that individual ACSLs partition FA, Ad-ACSL1 increased FA reacylation and channeled FA towards diacylglycerol and phospholipid synthesis and away from cholesterol ester synthesis.

INTRODUCTION
Long-chain acyl-CoA synthetases (ACSL) catalyze the first step in FA metabolism by converting long-chain FA into acyl-CoA thioesters. Acyl-CoAs enter both anabolic and catabolic pathways (1), and the disturbance of these pathways is linked to disorders such as hepatic steatosis, hyperlipidemia, and insulin
resistance. Five ACSL isoforms, each the product of a separate gene, have been cloned and characterized in mammals (2,3). Even though individual ACSL isoforms have different substrate preferences, enzyme kinetics, and cellular and subcellular locations, and are regulated uniquely (4-6), the significance of this diversity is unknown. We hypothesized that instead of being redundant, individual ACSL isoforms might channel FA into distinct metabolic pathways.

Evidence for the importance of ACSL in FA channeling comes from studies with triacsin C, an inhibitor of recombinant ACSL1, 3 and 4, but not ACSL5 or 6 (5,6). For example, in hepatocytes, in which ACSL1, 3, 4 and 5 are abundant (7), triacsin C inhibits TAG synthesis 70% but inhibits olate incorporation into phospholipids and β-oxidation only 34% (8). More direct evidence comes from studies that overexpress individual ACSL isoforms. In ACSL1 heart-specific transgenic mice, TAG and PL accumulate in heart muscle in the absence of changes in CE or β-oxidation (9), and when ACSL5 is overexpressed in rat hepatoma McArdle-RH777 cells, it partitions exogenously derived FA towards TAG synthesis and storage, but not towards PL or CE synthesis (10). However, the exact function of other ACSL isoforms in liver remains largely unexplored, in part because we lack inhibitors that can exclusively inhibit one isoform without affecting others.

Although studies suggest that ACSL1 is important for TAG synthesis in adipocytes and fibroblasts (4,11), conflicting data exist concerning the function of ACSL1 in liver. Supporting a role for ACSL1 in TAG synthesis is its location in the ER and MAM, which are sites of TAG synthesis, and its absence from mitochondria, the major site of FA β-oxidation (12). Further, ACSL1 mRNA is induced when previously fasted rats are refed with a high fat or high sucrose diet that favors lipogenesis (13,14), and hepatic ACS specific activity and ACSL1 mRNA is enhanced in obese and hypertriglyceridemic rats that have fatty livers (15,16). On the other hand, in support of a role for ACSL1 in providing FA for β-oxidation are data showing that PPARα agonists, which upregulate genes for both FA β-oxidation and de novo FA synthesis (17), increase total ACS activity in rat liver (18) and ACSL1 mRNA expression in rat liver (18) and in the rat liver-derived cell line AML-12 (19). This upregulated transcription is mediated by a PPAR-responsive element in the promoter of the Acsl1 gene (20).

To elucidate the specific role of ACSL1 in hepatic FA metabolism, we overexpressed rat ACSL1 in rat primary hepatocytes. We hypothesized that ACSL1 would channel FA towards some specific lipid metabolic pathways and away from others.

EXPERIMENTAL PROCEDURES

Materials—DNA restriction endonucleases and ligase for recombinant adenovirus construction were from New England Biolabs. HEK-293 and CHO cells were from the American Type Culture Collection. MEM, nonessential amino acids, FBS and tissue culture dishes were from GIBCO-BRL Life Technologies. Rat-tail collagen I was from Collaborative Biomedical Products. Silica gel G plates were from Whatman (cat. 4865-821). [1-14C]acetate, [1,2,3-3H]glycerol and [1-14C]oleate were obtained from PerkinElmer Life Sciences and [14C]palmitate was from New England Nuclear. Lipid standards were from Sigma and Avanti Polar Lipids. Polyacrylamide stock was from National Diagnostics. Lab-Tek™ II Chamber Slides™ were from NUNC. RNeasy kit was from Qiagen. Chemicals were from Sigma-Aldrich unless otherwise indicated.

Construction of pACCMV-ACSL1FLAG adenovirus—A full-length rat ACSL1 cDNA with a C-terminal FLAG epitope
(DYKDDDDK) was subcloned from a previously constructed pFLAG-CTC plasmid (5) into a shuttle vector, pACCMVpLpA at the Bam HI and Sal I sites. Expression of the inserted ACSL1FLAG cDNA is driven by the cytomegalovirus promoter. The inserted ACSL1FLAG in the pACCMV-ACSL1FLAG construct was verified by restriction enzyme analysis and confirmed by DNA sequencing at the UNC DNA sequencing facility. Expression and activity of the pACCMV-ACSL1FLAG construct were confirmed by transient transfection into CHO cells for 24 h, followed by ACS activity assay and anti-FLAG Western blot. The pACCMV-ACSL1FLAG construct was cotransfected with an adenoviral DNA, pJM17, into HEK293 cells for homologous recombination to form recombinant adenovirus carrying ACSL1FLAG cDNA (Ad-ACSL1) (21). After plaque purification, virions were further purified and amplified by the UNC Vector Core Facility. A virus containing a GFP gene under control of the cytomegalovirus promoter (Ad-GFP) was used for control infections (10).

**Hepatocyte isolation and adenovirus infection**

Animal protocols were approved by the UNC Institutional Animal Care and Use Committee. Male Wistar rats (250-300 g) were housed in a 12:12-h light-dark cycle and were allowed free access to food (Prolab® Rat/Mouse/Hamster 3000 diet, Labdiet) before hepatocyte isolation. Primary hepatocytes were isolated by collagenase perfusion by the UNC Cellular Metabolism and Transport Core. Cell viability, determined by trypan blue exclusion, exceeded 90%. Hepatocytes were seeded at a density of 1.5 x 10⁶ cells per 60 mm or 4.5 x 10⁶ cells per 100 mm collagen-coated dish in MEM supplemented with 10% FBS (v/v), 50 U/ml penicillin, and 50 mg/ml streptomycin (22). After cells attached (4-5 h), recombinant adenoviruses (Ad-GFP or Ad-ACSL1) were added for 2 h at 37°C in serum-free MEM. Infection medium was removed and replaced by MEM containing 10% FBS, 10 nM dexamethasone, and 0.1 mM nonessential amino acid (MEM-DA).

For dose-dependent expression, hepatocytes were infected with Ad-ACSL1 at MOIs of 5, 10, 20 or 50 for 2 h. Uninfected cells or Ad-GFP infected cells (20 MOI) served as controls. After 18 h, cells were washed with cold PBS and homogenates were collected as described below. For the time-course, hepatocytes were infected with Ad-GFP or Ad-ACSL1 at an MOI of 20. Homogenates were collected after 12, 18, 24, 26, or 36 h of incubation for Ad-ACSL1 infected cells and after 18 h of incubation for Ad-GFP infected cells.

**Cell labeling and lipid extraction and analysis**

Twenty-one h after adenoviral infection (Ad-GFP or Ad-ACSL1) at 20 MOI, hepatocytes (1.5 x 10⁶ cells in 60 mm dishes) were labeled with 3 ml MEM containing 1.0 μCi of [1-14C]oleate bound to BSA (essentially FA free) in a 3:1 molar ratio for 3 h (10). Although ACS activity with oleate was ~ 20% lower than that with palmitate in both GFP and ACSL1 adenovirus infected cells (data not shown), oleate was used for labeling in order to avoid lipotoxicity (23,24). The radiolabeling medium, which included 1 mM carnitine, contained a final concentration of 100 μM or 750 μM oleate (22). In some experiments cells were incubated with 250 μM [1,2,3-3H]glycerol (1.1 μCi) or 2.5 mM [1-14C]acetate (1.0 μCi). The medium was collected for ASM measurement or extracted to measure radiolabel incorporation into secreted lipids (10,22). Hepatocytes were washed twice with 1% BSA in PBS at 37°C and cellular lipids were extracted (25). For pulse-chase experiments, hepatocytes were infected with adenovirus for 21 h (as above) and incubated with 750 μM [1-14C]oleate in the presence of 250 μM unlabeled glycerol, or with 250 μM [1,2,3-3H]glycerol in the presence of 750 μM unlabeled oleate. After a 3 h incubation, the cells were either collected...
Aliquots of the lipid extracts from the cells and media were separated by TLC on 0.25-mm silica gel G plates in either hexane:ethyl ether:acetic acid (80:20:1; v/v) for neutral lipids (10) or in chloroform: methanol:acetic acid:water (50:37.5:3.5:2; v/v) for PL (26), together with authentic lipid standards in parallel. The 14C- or 3H-labeled lipids were detected and quantified with a Bioscan 200 Image System.

**Cell homogenate preparations for ACS activity and ACSL protein assays**—Hepatocytes infected with Ad-ACSL1 or Ad-GFP were washed twice with cold PBS and collected in cold Medium A (10 mM Tris pH 7.4, 250 mM sucrose, 1 mM EDTA, 1 mM dithiothreitol and Protease Inhibitor Cocktail [Sigma]) and homogenized on ice with 10 up-and-down strokes with a Teflon-glass motor-driven homogenizer. Homogenate aliquots were stored at –80°C until use. Protein concentrations were determined by the BCA method (Pierce). ACS specific activity was determined by measuring the production of [14C]acyl-CoA in the presence of 175 mM Tris-HCl, pH 7.4, 8 mM MgCl2, 5 mM dithiothreitol, 10 mM ATP, 0.25 mM CoA, and 50 μM [14C]palmitic acid in 0.5 mM Triton X-100, 0.01 mM EDTA. The assays were performed in a total volume of 200 μl at 37 °C for 5 min. The reaction was started by adding 0.5 to 1.5 μg of homogenate protein, terminated with 1 ml Dole’s reagent (isopropanol, heptane, 1M H2SO4; 80:20:2; v/v), and extracted (27). Enzyme assays measured initial rates.

**Western Blot analysis**— Homogenates from the dose-dependent infection and time-course incubations (10 μg) were separated by electrophoresis on a 10% polyacrylamide gel with 0.1% SDS, and transferred to a polyvinylidene fluoride membrane (Bio-Rad). Immunoreactive bands were detected by incubating the membranes with anti-FLAG M2 monoclonal antibody (Sigma), horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G, and SuperSignal West Pico Chemiluminescent Reagent (Pierce) (28).

**Quantitative Real-time-PCR**— Hepatocytes were plated at 4.5 x 10^6 cells per 100 mm dish and infected with Ad-GFP or Ad-ACSL1 at an MOI of 20 for 2 h as described above. Thirty h after infection, RNA was isolated (RNeasy, Qiagen) and stored at -80°C until use. Samples were analyzed on an ABI Prism 7700 sequence detection system (Applied Biosystems). Primers and corresponding FAM probes are listed in Table 1 except for CPT1 (Applied Biosystems, Rn00580702_m1). Data were analyzed using the relative standard curve method (10).

**Immunocytochemistry**— Primary hepatocytes were plated on 8-well chamber slides (100,000 cells/well) and infected with Ad-ACSL1 for 18 h as described above. After washing three times with PBS at room temperature (RT), cells were fixed at RT with 3.7% formaldehyde in PBS for 10 min and permeabilized with 0.2% Triton-X100 in PBS for 10 min. Next, cells were washed three times with PBS and blocked in 10% FBS in PBS (blocking buffer) for 30 min at RT. Cells were then doubly labeled with anti-FLAG M2 monoclonal antibody (1:400) and individual organelle markers (FITC-ConcanavalinA [1:500, Miles-Yeda Ltd.] and anti-calnexin polyclonal antibody [1:100, Abcam] for ER, anti-VDAC polyclonal antibody [1:100, ABR] for mitochondria, or rhodamine phalloidin [1:100, Molecular Probes] for actin fiber) in blocking buffer for 1 h at RT. Cells were washed three times and then incubated in blocking buffer containing...
Alexa Fluor® 488 or Alexa Fluor® 568 goat anti-mouse IgG-specific antibodies (1:1000, Molecular Probes) and Alexa Fluor® 568 goat anti-rabbit IgG-specific antibodies (1:1000, Molecular Probes) or cellular stains for 1 h at RT. After washing three times for 5 min in PBS, mounting media (Biomedia) was added to the chambers on the detached glass slide and fitted with a No.1 cover glass before being sealed and imaged. Confocal microscopy was performed using a Zeiss 510 Meta Inverted Laser Scanning Confocal Microscope, and LSM 510 META software (Zeiss) for image acquisition.

**Cellular free cholesterol measurement**--Hepatocytes (4.5 x 10⁶ cells per 100 mm dish) infected for 21 h with Ad-GFP or Ad-ACSL1 at MOI 20 were incubated with oleate at 0 µM, 100 µM or 750 µM for 3 h, followed by lipid extraction. Free cholesterol was determined using an enzymatic colorimetric assay (Free Cholesterol E, COD-DAOS, Wako).

**Statistical analysis**-- Data from each group were expressed as means ± SE. Data were analyzed by Student’s t-test, and significance was declared at \( p < 0.05 \).

**RESULTS**

**Adenoviral overexpression of ACSL1 increased ACS activity in rat primary hepatocytes**--We infected rat primary hepatocytes with Ad-GFP or an adenovirus containing rat ACSL1 with a FLAG epitope at the C terminus (Ad-ACSL1) and measured the ACS specific activity with palmitate at different virus doses (Figure 1A) and incubation times (Figure 1B). After an 18 h infection with Ad-GFP, ACS specific activity did not change (167.8 ± 4.9 vs. 155.0 ± 2.6 nmol/min/mg protein in uninfected cells). In contrast, Ad-ACSL1 (18 h) increased ACS specific activity 86% to 236% at 5 to 50 MOI, with a linear increase through 20 MOI. Ad-ACSL1 at 20 MOI increased ACS specific activity linearly for 36 h. Western blotting with anti-FLAG primary antibody showed a band of about 75 kDa, confirming that the increase in ACS specific activity was due to the overexpression of ACSL1. Consistent with the increase in activity, the density of the immunoreactive band increased with the time of incubation and the adenovirus dose. For labeling experiments, we chose 20 MOI and 24 h when ACS specific activity was increased 3.7-fold. ACSL4 or ACSL5 are two other major ACSLs expressed in rat liver. Overexpressed ACSL1 increased ACSL5 mRNA expression 2-fold but ACSL4 mRNA expression was not altered (data not shown).

**Overexpressed ACSL1 colocalized with ER but not mitochondria or plasma membrane**--Previous studies used subcellular fractionation to show that endogenous ACSL1 in rat liver is present in ER fractions and MAM fraction but not in purified mitochondrial fractions (8,12). We used confocal microscopy to characterize the intracellular localization of overexpressed ACSL1. FLAG antibody detection of ACSL1-FLAG strongly colocalized with the ER markers Concanavalin A (Figure 2A) and calnexin (Figure 2B), but not with the mitochondrial membrane marker VDAC (Figure 2C). Further, staining of actin fibers which revealed cellular morphology, implied the absence of ACSL1 on the plasma membrane (Figure 2D). We were unable to use an anti-phosphatidylethanolamine N-methyltransferase antibody to identify MAM in hepatocytes.

The ER marker Concanavalin A labels lumen-oriented mannose sugars on proteins in ER and Golgi compartments. The polyclonal calnexin antibody labels epitopes on the luminal side of this ER resident protein. Calnexin co-distributed best with ACSL1-FLAG, possibly because both are integral ER proteins. Because ACSL1-FLAG did not co-distribute with equal intensity on ER membranes labeled with
Concanavalin A, we stained cells for other ER proteins. The chaperone-like luminal ER proteins Protein Disulfide Isomerase (PDI) and BiP also colocalized with ACSL1-FLAG, but reflected staining patterns similar to Concanavalin A (data not shown). The anti-rabbit Alexa-568 antibody (Figure 2B, 2C, 2D, red) was preferred for the detection of endogenous proteins due to lower background levels in secondary antibody controls (data not shown). These data provided evidence for exogenous ACSL1 localization to ER membranes. Thus, the subcellular location of overexpressed ACSL1 in primary hepatocytes was consistent with the endogenous ACSL1 detected in liver fractionation studies (12).

**Ad-ACSL1 increased [1-14C]oleate incorporation into DAG but not TAG, and decreased [1-14C]oleate incorporation into CE**—To determine the effects of overexpressed ACSL1 on lipid metabolism, we incubated the adenovirus infected hepatocytes with 100 µM or 750 µM [1-14C]oleate, representing physiological concentrations of exogenous FA under fed or fasting conditions, respectively. At 100 µM oleate, incorporation of [1-14C]oleate into [14C]DAG doubled with Ad-ACSL1 (Figure 3A). Increasing exogenous oleate to 750 µM resulted in a 1.7-fold increase of [14C]DAG in cells infected with Ad-GFP, and this was doubled again by the presence of Ad-ACSL1. Surprisingly however, despite the Ad-ACSL1-mediated increase in [14C]oleate incorporation into DAG, the incorporation of oleate into TAG remained unchanged at both oleate concentrations (Figure 3B). The addition of 750 µM oleate increased [14C]TAG 5-fold in both Ad-GFP and Ad-ACSL1 infected cells. Cellular TAG mass also remained unchanged by the presence of excess ACSL1 (data not shown). Decreasing the adenovirus dose to 10 MOI did not change the incorporation pattern (data not shown), thereby excluding the possibility that cell toxicity related to excess Ad-ACSL1 had inhibited TAG synthesis. Further, the mRNA level of DGAT2, the major enzyme that converts DAG to TAG in liver (29), was unchanged in Ad-ACSL1 infected cells (data not shown).

Acyl-CoAs are also substrates for cholesterol esterification. Compared to Ad-GFP control hepatocytes, the incorporation of [1-14C]oleate into CE was 70% lower in Ad-ACSL1 cells at both 100 µM and 750 µM (Figure 3C). To determine whether the amount of cholesterol might be limiting in cells that overexpress ACSL1, we measured cellular free cholesterol after cells were incubated with 0 µM, 100 µM and 750 µM of oleate. Overexpression of ACSL1 did not change free cholesterol content, nor did higher concentration of oleate deplete cholesterol, suggesting that ACSL1 channels acyl-CoAs away from cholesterol esterification (Figure 3D).

**ACSL1 overexpression increased [1-14C]oleate incorporation into specific PL**—In addition to its effects on neutral lipids, Ad-ACSL1 increased [1-14C]oleate incorporation into total cellular PL 70% and 21% with 100 µM and 750 µM oleate, respectively (Figure 4A). At 100 µM oleate, Ad-ACSL1 increased oleate incorporation into PE 164%, PI 104% and PC 54% (Figure 4B-D). At 750 µM oleate, Ad-ACSL1 overexpression increased labeled PE 99% and labeled PI 56%, with no change in labeled PC. Thus, it appears that overexpression of ACSL1 enhanced oleate incorporation predominantly into PE and PI, phospholipids that originate from DAG and phosphatidic acid, respectively.

**ACSL1 overexpression did not affect the use of [1-14C]oleate for β-oxidation**—To determine whether overexpressed ACSL1 provided acyl-CoAs for FA oxidation, we measured labeled ASM in the medium as an indicator of FA β-oxidation (22). ASM is considered a more accurate measure of β-oxidation than is CO2 production (30). The incorporation of [1-
Ad-ACSL1 decreased $[^{14}C]$TAG secreted into the medium-- Despite similar incorporation of oleate into cellular TAG in Ad-ACSL1 and Ad-GFP infected hepatocytes, Ad-ACSL1 overexpression decreased the amount of labeled TAG secreted by about 53% (Figure 5B). In contrast, the incorporation of $[^{14}C]$oleate into secreted DAG and PL remained unchanged (data not shown). These data suggested that overexpression of ACSL1 might alter the assembly or/and secretion of lipoproteins.

Overexpression of ACSL1 did not increase the total amount of FA metabolized-- When ACSL5 is overexpressed in McArdle-RH7777 rat hepatoma cells (10), or when ACSL1 is overexpressed in fibroblasts, the uptake of exogenous FA is enhanced (31), suggesting that FA metabolism facilitates the ingress of FA. To determine whether ACSL1 similarly enhances FA uptake by hepatocytes, we calculated the total amount of $[1-{^{14}C}]$ incorporated into cell and medium lipids and into medium ASM. Ad-ACSL1 did not significantly increase the total amount of FA metabolized at either 100 µM oleate ($p=0.06$) or 750 µM oleate ($p=0.09$) (Figure 5C). Thus, in hepatocytes, unlike other cells, exogenous FA uptake was not enhanced by excess ACSL1 activity, perhaps because ACSL1 had a different subcellular location or because there were tissue-specific differences in interacting proteins (31,32).

ACSL1 increased oleate recycling to TAG and PL during the chase-- Cellular complex lipids undergo dynamic changes via hydrolysis, remodeling, and re-esterification (33,34). Thus, we wondered whether the absence of enhanced $[^{14}C]$oleate incorporation into TAG despite a doubling of $[^{14}C]$DAG was due to increased TAG hydrolysis or to diminished TAG reacylation. To examine these possibilities, we labeled cells with 750 µM [1-14C]oleate for 3 h and followed the fate of labeled complex lipids for 14 h afterwards.

In Ad-GFP infected hepatocytes, 33% of the labeled cellular TAG was lost during the 14 h chase (Figure 6A). Overexpression of ACSL1 attenuated the loss of label from TAG, resulting in only an 18% decrease, and also helped to retain label present in PL. In contrast, the amount of labeled PL decreased 19% in the Ad-GFP infected cells. The diminished changes in the amount of $[^{14}C]$-labeled TAG and lack of change in the amount of $[^{14}C]$-labeled PL suggested that Ad-ACSL1 enhanced FA reesterification of TAG and PL. Since the amount of labeled DAG decreased 27% in Ad-GFP controls during the chase, whereas it decreased 59% in the cells that overexpressed ACSL1 (Figure 6A), it appeared that ACSL1 maintained label in TAG and PL partly by reesterifying FA that had been released from DAG.

ACSL1 decreased the recycling of oleate into CE and β-oxidation metabolites-- In both the Ad-GFP and Ad-ACSL1 cells during the 14 h chase, the amount of $[^{14}C]$-label in CE increased markedly compared to the label present at 3 h (Figure 6B). However, 64% less label was recycled into CE in Ad-ACSL1 cells. This decrease in $[^{14}C]$CE was similar to the reduced labeling (70%) observed during the initial 3 h incubation, and was consistent with the interpretation that ACSL1 directs acyl-CoAs away from CE synthesis. In contrast, however, to the lack of effect of Ad-ACSL1 on $[^{14}C]$ASM observed during the 3 h pulse (Figure 5A), Ad-ACSL1 infected cells incorporated 42% less label into ASM during
the chase. These data suggested that the $^{14}$C-label in TAG and PL was retained at the expense of FA oxidation (Figure 6C).

**Overexpressed ACSL1 increased $[^{14}$C$]$oleate incorporation into DAG and PL via both de novo and reacylation pathways**—To determine whether overexpressed ACSL1 affected de novo glycerolipid synthesis, we incubated hepatocytes with 250 μM [1,2,3-$^{3}$H]glycerol in the presence of 750 μM unlabeled oleate for either 3 h or for 3 h followed by a 14 h chase in the absence of labeled glycerol. Similar to the pattern of oleate incorporation during a 3 h incubation, overexpressed ACSL1 increased [3H]glycerol incorporation into DAG and PL 79% and 14%, respectively (Figure 7A), suggesting that ACSL1 increased de novo synthesis of DAG and PL. Since ACSL1 overexpression increased incorporation into DAG and PL from oleate more than from glycerol, it is likely that reacylation contributed to the increase in labeled DAG and PL. Similar to the $[^{14}$C$]$oleate study, [3H]TAG remained unchanged by Ad-ACSL1, again suggesting that ACSL1 overexpression did not increase de novo TAG synthesis despite the increase in [3H]DAG. During the chase, again similar to the effects of ACSL1 on $[^{14}$C$]$oleate recycling, more [3H]TAG and [3H]PL and less [3H]DAG remained in the Ad-ACSL1 infected cells (Figure 7B), confirming that ACSL1 altered lipid recycling in the hepatocytes by retaining labeled TAG and PL and by metabolizing DAG.

**ACSL1 overexpression increased the incorporation of FA derived from de novo synthesis into TAG, DAG and PL**—Liver metabolizes both FA imported from the blood and FA synthesized de novo. It has been suggested that FA derived from different sources might have distinct fates (35). We reported that ACSL5 exclusively activates exogenous FA but not FA synthesized endogenously (10). To examine the selectivity of ACSL1, we labeled the adenovirus infected hepatocytes with 2.5 mM [1-$^{14}$C]acetate. ACSL1 increased [1-$^{14}$C]acetate incorporation into DAG and PL 83%, and 61%, respectively (Figure 7C), similar to the effect observed with $[^{14}$C$]$oleate incorporation (Figure 3). In contrast, however, to the $[^{14}$C$]$oleate study, overexpressed ACSL1 enhanced [1-$^{14}$C]acetate incorporation into TAG 13%, and did not significantly decrease $[^{14}$C$]$acetate incorporation into CE. Lack of effect on [14C]CE may reflect the fact that most of the $[^{14}$C$]$-label in CE was probably derived from de novo synthesized cholesterol rather than from FA synthesis. Thus, it appeared that ACSL1 can activate FA derived from both exogenous and de novo sources and commit them to the synthesis of DAG and PL.

**DISCUSSION**
The major findings of this study are that overexpression of ACSL1 altered FA incorporation into specific pathways in rat primary hepatocytes, and that these pathways differed from those previously observed after overexpression of ACSL1 in other types of cells. Previous studies in NIH3T3 fibroblasts and PC12 neuronal cells strongly suggest that overexpressed ACSL1 provides acyl-CoAs that are incorporated primarily into TAG (11,36), and in vivo studies of ACSL1 heart-specific transgenic mice show a marked increase in heart TAG mass (9). In addition, during the differentiation of 3T3-L1 preadipocytes into adipocytes, ACSL1 mRNA increases 160-fold whereas other ACSL isoforms remain unchanged, concomitant with a 100-fold increase in microsomal ACS specific activity and TAG accumulation (36,37). ACSL1 mRNA is upregulated in liver by PPARα (18,20) and in adipose tissue by PPARγ (38), suggesting that the function of ACSL1 might differ in these tissues.
In contrast to other overexpression studies of ACSL1 (11) and ACSL5 (10), overexpressed ACSL1 in hepatocytes did not increase TAG mass or $[^{14}C]$oleate incorporation into TAG despite a doubling in $[^{14}C]$oleate incorporation into DAG. Because less $[^{14}C]$TAG was hydrolyzed in the Ad-ACSL1 infected cells during the 14 h chase, it appeared that ACSL1 overexpression either diminished the rate of lipid hydrolysis or increased recycling of hydrolyzed $[^{14}C]$oleate back to TAG. We favor the latter explanation because more $[^{14}C]$DAG was lost from the Ad-ACSL1 infected cells. Thus, an increase in glycerolipid turnover cannot explain the inconsistency between the increased $[^{14}C]$DAG without a concomitant increase in $[^{14}C]$TAG.

Incubating cells with $[^{3}H]$glycerol confirmed the pattern seen with $[^{14}C]$oleate incorporation. In addition, consistent with the pattern of oleate recycling during the chase, the $[^{3}H]$-label increased in TAG and PL, and decreased in DAG, again supporting the hypothesis that ACSL1 increases FA reacylation.

In liver, both ER and MAM are enriched with enzymes of TAG and phospholipid synthesis including PS, PE and PC (39,40). Previous studies identified endogenous ACSL1 in both ER and MAM fractions from rat liver. The present studies in hepatocytes showed colocalization of overexpressed ACSL1 with ER, but not mitochondria or plasma membrane. Although colocalization of ACSL1-FLAG and MAM could not be assessed in current study, the increase in phospholipid synthesis by Ad-ACSL1 is consistent with a location in MAM as well as in ER. Thus, it appears that, instead of being channeled towards TAG synthesis, acyl-CoAs were used primarily to synthesize PL. This is consistent with the previous finding that choline- and ethanolamine-glycerophospholipid masses increased 50% and 15%, respectively in heart-specific ACSL1 transgenic mice (9). Because less $[^{14}C]$PL and more $[^{14}C]$DAG was hydrolyzed in Ad-ACSL1 cells during the chase, ACSL1 may have increased FA incorporation into PL by enhancing FA recycling or by increasing the use of labeled DAG. These data are strikingly different from a study of overexpressed ACSL5 in McArdle-RH7777 rat hepatoma cells in which no increase was observed in either oleate or glycerol incorporation into cellular PL despite an increase in incorporation into DAG (10), again suggesting that ACSL1 and ACSL5 commit FA to different metabolic fates.

The incorporation of $[^{14}C]$oleate into CE was markedly lower in cells that overexpressed ACSL1, despite unchanged ACAT2 mRNA abundance or cellular content of free cholesterol. Increasing the exogenous oleate concentration to 750 μM did not change this result, suggesting that neither acyl-CoA nor free cholesterol was limiting. Further, even though both Ad-GFP and Ad-ACSL1 cells were able to use $[^{14}C]$oleate hydrolyzed from labeled glycerolipids to esterify cholesterol during the chase, the Ad-ACSL1 cells continued to incorporate less oleate into CE (Figure 6B). Thus, we conclude that overexpressed ACSL1 diverted oleate away from cholesterol esterification. This diversion by ACSL1 contrasts with studies of ACSL5 overexpression in rat hepatoma cells which did not decrease oleate incorporation into CE (10).

Overexpressed ACSL1 did not alter the amount of FA oxidized during the 3 h oleate incubation, but during the 14 h chase 42% less $[^{14}C]$oleate was released from complex lipids for oxidation. Thus, even though hepatic ACSL1 is a target of PPARα (18-20), our data did not indicate that ACSL1 channels FA into the pathway of β-oxidation. Although PPARα agonists upregulate genes involved in FA oxidation like CPT1 (41), they also upregulate DGAT activity (42). Treatment of mice with a PPARα agonist increased the expression of acetyl-CoA carboxylase and stearoyl-CoA desaturase-1 which are involved in de novo FA and TAG synthesis; this increase was attributed...
to an increase in the amount of nuclear sterol regulatory element binding protein-1c (17). Thus, PPARα regulation of ACSL1 may enhance lipogenesis as well as increase β-oxidation.

Despite unchanged [14C]oleate incorporation into cellular TAG, Ad-ACSL1 cells secreted less [14C]TAG into the medium, perhaps due to insufficient CE for VLDL synthesis. Although the role of CE in the assembly and secretion of VLDL is controversial (43,44), CE availability appears to be important because ACAT inhibitors reduce apoB100 secretion in primary rat hepatocytes and HepG2 cells (45) and overexpression of ACAT1 and ACAT2 stimulates apoB-containing lipoproteins in McArdle-RH7777 cells (44). Additionally, because in rat hepatocytes cytosolic TAG is not incorporated en bloc into the ER for VLDL biogenesis and secretion (46,47), the amount of cell TAG might not correlate directly with TAG secreted in VLDL. Finally, secretion might be affected by ACSL1-mediated changes in the cellular content of FA and acyl-CoA which are ligands for nuclear transcription factors like the PPARs (48) and HNF4α (49,50).

Overexpression of several ACSL isoforms increase the uptake of exogenous FA (10,31,36). Uptake probably occurs because vectorial acylation and enhanced FA metabolism diminish the rate of efflux of unesterified FA from the cell (51). Most studies have measured the initial rate of FA import within 1-2 min, a time frame that may not represent the physiological uptake of FA that is driven by transporters and metabolic demand (51). We measured the total FA metabolized in cell and medium lipids and in β-oxidation products during a period of time that takes metabolic demands into account (10,51). In our study, ACSL1 did not significantly increase the total amount of FA metabolized by hepatocytes. ACSL1 has been reported to interact with FATP1 on the plasma membrane of 3T3-L1 adipocytes (32). Since FATP1 is not present in hepatocytes (52), and since we showed that hepatocyte ACSL1 is not present on the plasma membrane, lack of enhanced FA uptake and metabolism in hepatocytes may reflect the different intracellular location of ACSL1, its interaction with a different FATP isoform, or its association with different downstream enzymes that use acyl-CoAs.

To determine the selectivity of ACSL1 for endogenous versus exogenous FA, we incubated hepatocytes with [14C]acetate, which is used for de novo FA synthesis. In contrast to incubations with [14C]oleate, Ad-ACSL1 increased label incorporation into TAG, as well as DAG and PL, showing enhanced use of de novo synthesized FA for TAG synthesis. It has been suggested that TAG and CE synthesis in hepatocytes requires some FA derived from de novo synthesis (42,53). Our data suggest that overexpression of ACSL1 channeled exogenous FA into DAG and PL, but not into TAG, in part due to insufficient endogenous FA. However, during the 14 h chase, the required pool of endogenous FA may no longer have been limiting because of the hydrolysis of TAG and PL, so that overexpressed ACSL1 could increase [14C]oleate incorporation into TAG. Differing from the decrease in exogenous oleate used for CE synthesis, ACSL1 overexpression did not diminish [14C]acetate incorporation into CE, suggesting that the [14C]-label in CE was derived primarily from cholesterol rather than FA.

In summary, consistent with our hypothesis that ACSL1 channels FA towards specific pathways, adenovirus-mediated overexpression of rat ACSL1 in rat primary hepatocytes channeled [14C]oleate towards DAG, PE, PI and PC synthesis and away from cholesterol esterification. Overexpressed ACSL1 also increased the reacylation of hydrolyzed oleate to TAG and PL, but diminished the amount of hydrolyzed oleate used for β-oxidation. In
contrast to its role in adipocytes (54), fibroblasts (31) and heart muscle (9), overexpression of ACSL1 in hepatocytes did not increase incorporation of [\(^{14}\)C]oleate into TAG or increase the total amount of FA metabolized. It seems likely that ACSL1 channels FA differently in different tissues, perhaps depending on the subcellular location of ACSL1 or the presence of interacting proteins specific to each cell type. In addition, overexpressed ACSL1 activated both exogenous FA and FA derived from de novo synthesis, but channeled the resulting acyl-CoA products into different pathways. Our study suggests that ACSL1 in hepatocytes plays an important role in directing FA into pathways of phospholipid synthesis, and away from cholesterol esterification and β-oxidation.

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Abbreviations. ACS, acyl-CoA synthetase; ACSL, long chain acyl-CoA synthetase; ACAT2, acyl-CoA:cholesterol acyltransferase 2; Ad, adenovirus; ASM, acid-soluble metabolites; BSA, bovine serum albumin; CE, cholesterol ester; CPT1, carnitine palmitoyltransferase 1; DAG, diacylglycerol; DGAT2, acyl-CoA:diacylglycerol acyltransferase 2; DPM, disintegrations per minute; ER, endoplasmic reticulum; FA, fatty acid; FBS, fetal bovine serum; GFP, green fluorescent protein; MAM, mitochondria-associated membrane; MEM, Minimal essential medium; MEM-DA, MEM plus dexamethasone and nonessential amino acids; MOI, multiplicities of infection; PBS, phosphate-buffered saline; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PL, phospholipid; PPAR, peroxisome proliferator-activated receptor; TAG, triacylglycerol; TLC, thin layer chromatography; VLDL, very low-density lipoprotein

REFERENCES
Table 1. Primer and probe sequences used for gene expression by qRT-PCR for rat primary hepatocytes.

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<tr>
<th>Isoform</th>
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<th>Reverse Primer</th>
<th>Probe</th>
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<td>CACTATCGCAATGACGTTGCG</td>
</tr>
</tbody>
</table>

14
FIGURE LEGENDS

Figure 1. Acyl-CoA synthetase (ACS) activity increased in rat hepatocytes overexpressing ACSL1. Rat primary hepatocytes (1.5 x 10^6 cells/60 mm dish) were uninfected (control) or infected with adenoviruses carrying either GFP (Ad-GFP) or rat ACSL1 (Ad-ACSL1). A) Cells were infected with Ad-ACSL1 at different MOI as indicated. After an 18 h incubation, cells were scraped and homogenized; B) Cells were infected with Ad-GFP or Ad-ACSL1 (MOI = 20) and homogenates were collected at the indicated times. Total ACS activity was measured and Western blot with anti-FLAG monoclonal antibody was performed as described under “Experimental procedures”. Data are reported as means ± SE from triplicate dishes. All Ad-ACSL1 versus Ad-GFP, p < 0.001.

Figure 2. Ad-ACSL1 is an ER protein in primary hepatocytes. Primary hepatocytes infected with Ad-ACSL1 for 18 h were fixed and labeled with primary antibodies against the FLAG epitope. Detection of ACSL1-FLAG was attained with either secondary anti-mouse IgG-specific Alexa-568 antibodies (A; red), or secondary anti-mouse IgG-specific Alexa-488 antibodies (B, C, and D; green). A) Ad-ACSL1 infected cells were co-stained with FLAG antibodies (red) and FITC-conjugated ConcanavalinA (green). Arrows in each panel and inset point to regions of intense colocalization on reticulated endomembranes; B) Ad-ACSL1 infected cells co-stained with FLAG antibodies (green) and the ER membrane protein calnexin (red). Arrows point to areas of intense colocalization (upper cell) and areas with less intensity that show colocalization near the cell periphery (central cell); C) Co-staining for FLAG (green) and mitochondria marker VDAC (red) with arrows pointing to VDAC immunopositive structures devoid of Ad-ACSL1; D) Co-staining with FLAG antibodies (green) and rhodamine-phalloidin (red) with an arrow pointing to an area on the cell surface that is devoid of Ad-ACSL1. Cells not stained with FLAG antibody are uninfected cells. Bar: 10 μm. Images are representative of experiments repeated at least three times. Cells were imaged by confocal microscopy as 0.5 μm slices with a 63x oil immersion objective at 2x magnification.

Figure 3. ACSL1 overexpression increased [1-14C]oleate incorporation into cellular DAG but not TAG. Primary hepatocytes (1.5 x 10^6 cells/60 mm dish) were infected with Ad-GFP (open bar) or Ad-ACSL1 (filled bar) at 20 MOI. After a 21 h infection, cells were incubated with 100 μM or 750 μM [1-14C]oleate for 3 h and harvested. Cellular lipids were extracted and [1-14C]oleate incorporation into neutral lipid species was determined by TLC as described under “Experimental procedures”. [1-14C]oleate incorporation into cellular A) DAG; B) TAG; C) CE; and D) cell free cholesterol, Ad-GFP or Ad-ACSL1 infected cells were incubated with 0 μM, 100 μM or 750 μM oleate for 3 h and free cholesterol was determined; E) unesterified fatty acid (FA). Data are reported as means ± SE from a representative experiment performed in triplicate dishes that was repeated four times. Ad-ACSL1 versus Ad-GFP, * p < 0.05, ** p < 0.01.

Figure 4. ACSL1 overexpression increased [1-14C]oleate incorporation into cellular PL. Hepatocytes were infected with either Ad-GFP (open bar) or ACSL1 (filled bar) for 21 h, and incubated with 100 μM or 750 μM [1-14C]oleate for 3 h. Cellular lipids were extracted and [1-14C]oleate incorporation into different PL species was determined by TLC. [1-14C]oleate incorporation into cellular A) PL; B) PE; C) PI; and D) PC. Data are reported as means ± SE from a representative experiment performed in triplicate dishes that was repeated four times. Ad-ACSL1 versus Ad-GFP, **p < 0.01, *** p < 0.001.
Figure 5. ACSL1 overexpression decreased [1-14C]oleate metabolism to medium TAG but not to β-oxidation. Hepatocytes were seeded and infected with Ad-GFP (open bar) or Ad-ACSL1 (filled bar) as described under “Experimental procedures”. Cells were incubated with 100 μM or 750 μM [1-14C]oleate for 3 h and the cells and medium were collected and extracted. Total FA metabolized includes cell and medium lipids and ASM (acid soluble metabolites). [1-14C]oleate incorporation into A) medium ASM; B) medium TAG; C) total [1-14C]oleic acid metabolized. Data are shown as means ± SE from a representative experiment performed in triplicate dishes and that was repeated four times. Ad-ACSL1 versus Ad-GFP, ** p < 0.01, *** p < 0.001.

Figure 6. Overexpression of ACSL1 altered [14C]oleate recycling in hepatocytes. Hepatocytes were plated and infected with Ad-GFP (open bar) or Ad-ACSL1 (filled bar) for 21 h. Cells were labeled with 750 μM [1-14C]oleate for 3 h, and then were either collected for lipid extraction (pulse), or were washed and incubated with new media containing no FA for 14 h (chase), as described under “Experimental procedures”. The remaining [14C]-label was analyzed in cellular lipid extracts or medium ASM in chased cells, and compared with pulsed cells. A) Remaining label in cellular lipids as a percentage of the label present at 3 h pulse, label in TAG decreased from 179,114 ± 4,242 DPM to 120,231 ± 5,106 DPM/1.5 x 10^6 Ad-GFP cells and from 167,514 ± 4,843 DPM to 138,119 ± 2,032 DPM/1.5 x 10^6 Ad-ACSL1 cells, label in DAG decreased from 11,732 ± 588 DPM to 8,577 ± 242 DPM/1.5 x 10^6 Ad-GFP cells and from 21,740 ± 488 DPM to 8,912 ± 24 DPM/1.5 x 10^6 Ad-ACSL1 cells; B) [14C]oleate incorporation into cellular CE during the 3 h pulse and 14 h chase; C) [14C]oleate incorporation into ASM during the 14 h chase. Data are shown as means ± SE from a representative experiment performed in triplicate dishes and that was repeated three times. # Significantly different from pulsed cells, p < 0.01; Ad-ACSL1 versus Ad-GFP, * p < 0.05, ** p < 0.01, *** p < 0.001.

Figure 7. ACSL1 overexpression increased [1,2,3-3H]glycerol incorporation into DAG and PL, and activates FA derived from do novo synthesis. Primary hepatocytes (1.5 x 10^6 cells/60 mm dish) were infected with Ad-GFP (open bar) or Ad-ACSL1 (filled bar) at 20 MOI for 21 h. Cells were labeled with 250 μM [1,2,3-3H]glycerol in the presence of 750 μM unlabeled oleate (A, B) or 2.5 mM [1-14C]acetate (C). Cells were either collected for lipid extraction after 3 h labeling (pulse) (A, C), or were washed and incubated with new media containing no glycerol nor FA for 14 h (chase) (B), as described under “Experimental procedures”. Remaining [14C] label was analyzed in cellular lipid extracts in chased cells, and compared with pulsed cells. Data are shown as means ± SE from 6 dishes. # Significantly different from pulsed cells, p < 0.05; Ad-ACSL1 versus Ad-GFP, * p < 0.05, ** p < 0.01, *** p < 0.001.
Figure 1

**Panel A**

ACS Specific Activity (nmol/min/mg protein)

- Control
- Ad-GFP 20
- Ad-GFP 10
- Ad-GFP 20
- Ad-GFP 50

**Panel B**

ACS Specific Activity (nmol/min/mg protein)

- Ad-GFP 18 h
- Ad-GFP 12 h
- Ad-GFP 24 h
- Ad-GFP 26 h
- Ad-GFP 36 h
- Ad-ACSL1 18 h
- Ad-ACSL1 24 h

**Western Blot**

- 75 kD band
Figure 2

A

FITC-ConA FLAG Merge

B

FLAG Calnexin Merge

C

FLAG VDAC Merge

D

FLAG Actin Merge
Figure 4

A

Cellular PL
(nmol/mg/3 h)

100 μM 750 μM
[1-14C]oleate

B

Cellular PE
(nmol/mg/3 h)

10 μM 750 μM
[1-14C]oleate

C

Cellular PI
(nmol/mg/3 h)

100 μM 750 μM
[1-14C]oleate

D

Cellular PC
(nmol/mg/3 h)

100 μM 750 μM
[1-14C]oleate
Figure 6

(A) [14C] label remaining in cellular lipid (% of pulsed cells DPM/14 h)

- TAG
- DAG
- PL

(B) [14C] oleate incorporation into CE (DPM)

- Pulse
- Chase

(C) [14C] lipid → medium ASM (DPM 14 h)

- Ad-GFP
- Ad-ACSL1

** indicates significant differences compared to control group (Ad-GFP)

*** indicates ** significant differences compared to Ad-ACSL1
Figure 7

**A**

[3H]glycerol incorporated (nmol/mg/3 h)

- **TAG**
- **DAG**
- **PL**

**B**

[3H] label remaining in cellular lipid (% of pulsed cells DPM/14 h)

- **TAG**
- **DAG**
- **PL**

**C**

[14C]acetate incorporated (nmol/mg/3 h)

- **CE**
- **TAG**
- **DAG**
- **PL**

* * * ***

0 5 10 15 20 25

0 20 40 60 80 100 120

0 5 10 15 20 25 30 35

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