Rat Long Chain Acyl-CoA Synthetase 5 Increases Fatty Acid Uptake and Partitioning to Cellular Triacylglycerol in McArdle-RH7777 Cells*

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Long chain acyl-CoA synthetase (ACSL) catalyzes the initial step in long chain fatty acid metabolism. Of the five mammalian ACSL isoforms cloned and characterized, ACSL5 is the only isoform found to be located, in part, on mitochondria and thus was hypothesized to be involved in fatty acid oxidation. To elucidate the specific roles of ACSL5 in fatty acid metabolism, we used adenoviral-mediated overexpression of ACSL5 (Ad-ACSL5) in rat hepatoma McArdle-RH7777 cells. Confocal microscopy revealed that Ad-ACSL5 colocalized to both mitochondria and endoplasmic reticulum. When compared with cells infected with Ad-GFP, Ad-ACSL5-infected cells at 24 h after infection had 2-fold higher acyl-CoA synthetase activities and 30% higher rates of fatty acid uptake when incubated with 500 μM [1-^{14}C]oleic acid. Metabolism of [1-^{14}C]oleic acid to cellular triacylglycerol (TAG) increased 42% in Ad-ACSL5-infected cells, but when compared with control cells, metabolism to acid-soluble metabolites, phospholipids, and medium TAG did not differ substantially. The incorporation of [1-^{14}C]oleate and [1,2,3-^{3}H]glycerol into TAG was similar in Ad-ACSL5-infected cells, thus indicating that Ad-ACSL5 increased TAG synthesis through both *de novo* and reacylation pathways. However, [1-^{14}C]acetic acid incorporation into cellular lipids showed that, when compared with control cells, Ad-ACSL5-infected cells did not increase the metabolism of fatty acids that were derived from *de novo* synthesis. These results suggest that uptake of fatty acids into cells is regulated by metabolism and that overexpressed ACSL5 partitions exogenously derived fatty acids toward TAG synthesis and storage.

Acyl-CoA synthetase catalyzes the initial step in mammalian fatty acid metabolism. In this reaction, fatty acid, CoA, and ATP are used to form acyl-CoA and AMP. Acyl-CoAs have diverse metabolic fates within the cell and can be used to acylate proteins or be metabolized through catabolic pathways such as β-oxidation or anabolic pathways such as *de novo* synthesis and reacylation of triacylglycerol (TAG),[2] phospholipids, and cholesterol esters.

To date, five isoforms of long chain acyl-CoA synthetase have been cloned and shown to possess activity toward long chain fatty acids. The unique pattern of tissue expression, subcellular localization, and differences in substrate preference among the isoforms suggests that individual isoforms have distinct functions. ACSL1, ACSL4, and ACSL5 appear to be the predominant isoforms in rat liver (1–5). ACSL1 and ACSL4 are located in liver endoplasmic reticulum and mitochondrial-associated membrane (6), an endoplasmic reticulum fraction possibly involved in lipoprotein synthesis (7). In addition, ACSL4 is also present on peroxisomes and has a marked preference for C20:4 and C20:5 (2). ACSL5 is the only isoform found in both mitochondrial membranes and endoplasmic reticulum (6) and, similar to ACSL1, has its highest preference for saturated and unsaturated fatty acids of 16–20 carbons (1).

Because ACSL5 is the only ACSL isoform known to be located on mitochondria, it is logical to speculate that it has a role in the β-oxidation of fatty acids. In support of this hypothesis, rat liver mitochondrial protein content of ACSL5 increases following a 48-h fast but declines when rats are refed a high sucrose diet for 24 h (6). To date, the most direct studies that focus on ACSL used triacsin C, an inhibitor of ACSL1, ACSL3, and ACSL4 but not ACSL5 or ACSL6 (8, 9). In hepatocytes obtained from fed rats, triacsin C decreases [1-^{14}C]oleic acid incorporation into TAG by 70% (10). In contrast, triacsin C decreases the metabolism of [1-^{14}C]oleic acid to phospholipids by 34% and to ASM by 33%. Therefore, inhibiting ACSL1, ACSL3, and ACSL4 decreases fatty acid incorporation into TAG more than into phospholipids or into pathways of fatty acid oxidation. Because hepatocytes express little ACSL6 (4), ACSL5 could account for the triacsin C-resistant activity in hepatocytes and might activate fatty acids destined for β-oxidation.

Despite its putative role in fatty acid oxidation, several lines of evidence do not support a catabolic role for ACSL5. Tissue expression of ACSL5 mRNA is highest in intestinal mucosa (1) and brown adipose tissue (11). Although brown adipose tissue is characterized by high levels of fatty acid oxidation and thermogenesis, both intestinal mucosa and brown adipose tissue have a high capacity for TAG synthesis (12, 13). Additionally, regulation of ACSL5 mRNA does not support a role in fatty acid β-oxidation. Hepatic ACSL5 message abundance does not change in response to peroxisomal proliferator-activated receptor α agonists (14) but increases nearly 4-fold in SREBP-1c transgenic mice and is reduced 40% in SREBP cleavage-activating protein knock-out mice (15). Administration of leptin to C57BL/6J obese *ob/ob* mice causes a 1.5-fold decrease in ACSL5 mRNA expression together with down-regulation of lipogenic genes and increased expression of numerous oxidative genes (16). In addition, hepatic ACSL5 message level increases 1.6-fold in zinc-deficient mice with fatty livers (17) and decreases 1.5-fold in estrogen-related receptor knock-out mice, which exhibit a reduction in lipogenic gene expression and reduced total body fat (18). Taken together, there are many discrepancies in the literature between regulation of ACSL5 mRNA and protein abundance and the

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[2] The abbreviations used are: TAG, triacylglycerol; DAG, diacylglycerol; ACSL, long chain acyl-CoA synthetase; ACSVL, very long chain acyl-CoA synthetase; ASM, acid-soluble metabolites; GFP, green fluorescent protein; Grp78, 78-kDa glucose regulated protein; SREBP-1c, sterol regulatory element binding protein; VDAC, voltage-dependent anion channel; PBS, phosphate-buffered saline; PIPES, 1,4-piperazinediethanesulfonic acid; TRITC, tetramethylrhodamine isothiocyanate .

effects of triacsin C on fatty acid channeling. Therefore, our goal was to use a direct approach to elucidate the function of ACSL5. We ectopically overexpressed ACSL5 in rat hepatoma McArdle-RH7777 cells to determine its role in fatty acid activation and partitioning.

EXPERIMENTAL PROCEDURES

Materials—Silica gel G plates were from Whatman. [1-^{14}C]Oleic acid, [1,2,3-^{3}H]glycerol, and [1-^{14}C]acetic acid were from PerkinElmer Life Sciences. Tissue culture dishes were from Corning, and media were obtained from Invitrogen. All chemicals were from Sigma unless otherwise indicated.

Construction of Recombinant GFP and ACSL5-FLAG Adenovirus—ACSL5 cDNA was cloned from rat liver total RNA and amplified using primers designed to include the entire open reading frame (8). The amplification product was ligated into the pET-FLAG vector containing a T7 promoter and a C-terminal FLAG epitope sequence (DYKD-DDDK) to form pET-ACSL5F. A 2091-bp construct was amplified from the pET-ACSL5F vector, including the initiator AUG codon, the entire ACSL5 coding region, a 24-bp FLAG epitope sequence at the 3′ end, and the TGA stop codon. The PCR product was digested with XhoI and XbaI and ligated into the 9.2-kb pAdTrack-CMV shuttle vector between the cytomegalovirus promoter and the polyadenylation site. This vector contains a *GFP* gene driven by a second cytomegalovirus promoter, which allows direct observation of the efficiency of infection by fluorescence microscopy. The pAdTrack/ACSL5F construct was sequenced at the University of North Carolina (UNC) DNA Sequencing Facility. The UNC Vector Core Laboratory produced recombinant adenovirus (Ad-ACSL5) by homologous recombination of the shuttle vector and the pAdEasy-1 adenoviral plasmid (19). An identical pAdTrack-CMV shuttle vector without the ACSL5 insert was used to produce a recombinant adenovirus (Ad-GFP) for control infections.

McArdle-RH7777 Cell Culture and Adenoviral Infection—McArdle-RH7777 cells were maintained in Dulbecco's modified Eagle's medium containing 25 mM glucose, 10% fetal bovine serum, 10% horse serum, plus 100 units/ml penicillin and 100 μg/ml streptomycin on collagen-coated dishes. Cells were trypsinized the day before adenoviral infection, and 1.2×10^6 cells were plated per 6-cm dish. Cells at 60–80% confluence were infected with 10 multiplicity of infection of either Ad-GFP or Ad-ACSL5. After 2 h, the media were changed.

Cell Radiolabeling, Lipid Extraction, and Measurement of Radioactive Lipid Products and Fatty Acid Uptake Assays—Radiolabeling media contained 1.0 μCi of [1-^{14}C]oleic acid bound to bovine serum albumin in a 3:1 molar ratio. The concentration of oleic acid in media was 20, 100, or 500 μM. In some experiments, 250 μM [1,2,3-^{3}H]glycerol or 2.5 mM [1-^{14}C]acetic acid was used. Additionally, 10 μM triacsin C in Me$_2$SO was added to the radiolabeling media in some experiments to inhibit ACSL1, ACSL3, and ACSL4. Untreated cells were incubated with the same volume of Me$_2$SO (0.2% of the medium). Radiolabeled media were added 24 h after adenoviral infection. After 3 h, aliquots of the radiolabeling media were removed and used to measure ASM or extracted (20) to measure radiolabel incorporation into media lipids. Residual radiolabel was removed by washing the cells once with PBS containing 1% bovine serum albumin at 37 °C. Cellular lipids were extracted (20) and separated on silica gel G plates in hexane:ethyl ether:acetic acid (80:20:1; v/v). Known standards (Avanti) were used to identify lipids that were then visualized by iodine vapor. The Bioscan 200 system was used to quantify radiolabeled lipids. Initial rates of fatty acid uptake were measured as described (21) except that 35-mm culture dishes were used and the radiolabeling medium contained 3.0 μCi of [1-^{14}C]oleic acid.

Cell Homogenate Preparations for ACSL Activity and Protein Assays—McArdle-RH7777 cells were washed with PBS, scraped in cold Medium I (250 mM sucrose, 10 mM Tris, pH 7.4, 1 mM EDTA, 1 mM dithiothreitol), and homogenized on ice with 10 up-and-down strokes in a Teflon glass motor-driven homogenizing vessel, and aliquots were stored at −80 °C. Acyl-CoA synthetase activity was measured in the presence of 175 mM Tris, pH 7.4, 8 mM MgCl$_2$, 5 mM dithiothreitol, 10 mM ATP, 250 μM CoA, and 50 μM [1-^{14}C]palmitic acid (22). Only initial rates were measured. Protein was measured by the BCA method (Pierce).

Immunocytochemistry—McArdle-RH7777 cells were plated on 8-well chamber slides (Nunc) and infected with Ad-ACSL5 as described above. After 24 h, cells were washed in PBS and fixed with 3% paraformaldehyde for 20 min at room temperature. Following several PBS washes at room temperature, cells were permeabilized with buffer A (10 μg/μl digitonin, 300 mM sucrose, 2.5 mM MgCl$_2$, 100 mM KCl, 1 mM EDTA, 10 mM PIPES, pH 6.8) for 30 min on ice. Cells were then incubated with 1.5% goat serum containing a 1/500 dilution of mouse anti-FLAG M2 monoclonal antibody (Sigma), 1/125 dilution of rabbit anti-VDAC, 1/500 dilution of rabbit anti-Grp78, or a combination of anti-FLAG with the organelle markers for 1 h at room temperature. Following several PBS washes, cells were incubated in 1.5% goat serum anti-mouse Alexa Fluor 633 or anti-rabbit Alexa Fluor 546 (Molecular Probes) for 1 h at room temperature in the dark. After washing with PBS, Gel/Mount (Biomedia) was added to the cells, and coverslips were applied. Confocal microscopy was performed on an Olympus FV500 laser scanning microscope. TRITC and CY5 filters were used to visualize the Alexa Fluor 546 and 633, respectively. Image J software (National Institutes of Health) was used for image analysis.

Quantitative Real-time-PCR—Livers from Sprague-Dawley rats (~130 g) were homogenized with a rotor stator homogenizer, and RNA was isolated with the RNeasy kit (Qiagen). Forward and reverse primers for rat ACSL5 were 5′-GGCCAAACAGAATGCACAG-3′ and 5′-GGAGTCCCAACATGACCTG-3′. The FAM probe corresponding to the ACSL5 amplicon was 5′-TGTCCAGTCCCCAGGTGATG-TAAT-3′. The rat β-actin forward primer was 5′-TGCCTGACGGT-CAGGTCA-3′, and the reverse primer was 5′-CAGGAAGGAAG-GCTGGAAG-3′. The probe corresponding to the β-actin amplicon was 5′-CACTATCGGCAATGAGCGGTTCCG-3′. Samples were analyzed on an ABI Prism 7700 sequence detection system (Applied Biosystems). Data were analyzed using the relative standard curve method as described in User Bulletin Number 2 in the product manual.

Statistics—Data were analyzed by Student's *t* test, and significance was declared at $p < 0.05$.

RESULTS

Ad-ACSL5 Increases Total and Triacsin C-resistant Activity—To determine the function of ACSL5, we infected McArdle-RH7777 cells, a rat hepatoma cell line, with an adenoviral construct containing FLAG-tagged ACSL5 and GFP. Infection efficiency monitored by fluorescent microscopy was close to 100% for most infections and was always greater than 90%. Ad-ACSL5 infection of McArdle-RH7777 cells increased total ACSL activity 2-fold when compared with Ad-GFP-infected cells (Fig. 1*A*). Since ACSL5 is only one of five known ACSL isoforms involved in long chain fatty acid activation, we used triacsin C, an inhibitor of ACSL1, ACSL3, and ACSL4, to better gauge the increase in activity due to ACSL5 overexpression. In the presence of 20 μM triacsin C, ACSL activity in Ad-ACSL5-infected cells was 17-fold higher than the Ad-GFP-infected controls. Previous quantitative real-time-PCR analysis revealed that ACSL6, the only other known ACSL not
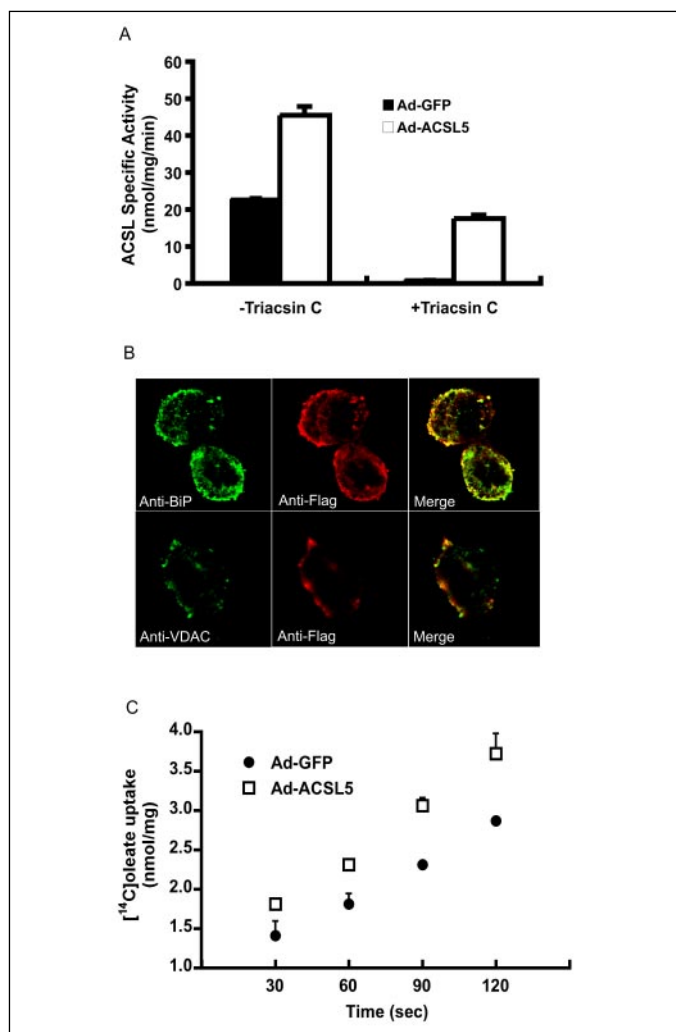
FIGURE 1. **Ad-ACSL5 increases total and triacsin C-resistant ACSL activity, co-localizes with endoplasmic reticulum and mitochondria, and increases fatty acid uptake.** *A*, ACSL activity was determined with 0.5, 1.0, and 2.0 μg of McArdle-RH7777 cell homogenate with or without 20 μM triacsin C using palmitate as the substrate. Assay conditions are described under "Experimental Procedures." Data are means ± S.D. from a representative experiment performed in triplicate and repeated four times. *B*, McArdle-RH7777 cells were fixed, permeabilized, and incubated with antibodies against FLAG, Grp78 (endoplasmic reticulum marker), or VDAC (mitochondria marker). *C*, initial rates of 500 μM [1-^{14}C]oleic acid uptake were measured 24 h after infection and are shown as means ± S.D. from three dishes.

inhibited by triacsin C, is not expressed in McArdle-RH7777 cells. If we assume that ACSL5 is solely responsible for the triacsin C-resistant activity, it accounts for ~6% of total ACSL activity in Ad-GFP-infected cells and 40% in Ad-ACSL5-infected cells.

ACSL5 Colocalizes with Mitochondria and Endoplasmic Reticulum— Our previous studies that used cell fractionation techniques have shown that ACSL5 is present in both microsomes and mitochondria in rat hepatocytes (6). We employed confocal microscopy to identify the subcellular location of FLAG-tagged Ad-ACSL5 in McArdle-RH7777 cells (Fig. 1*B*). The FLAG localization pattern showed substantial colocalization with the anti-Grp78 antibody, an endoplasmic reticulum marker. Similarly, FLAG also colocalized with the anti-VDAC antibody, a mitochondria marker. Immunofluorescence did not show the FLAG marker on the cell surface or the nuclear membrane. Therefore, overexpression of FLAG-tagged ACLS5 followed a localization pattern similar to that of endogenous ACSL5.

Ad-ACSL5 Increases Fatty Acid Uptake and Partitioning to Cellular TAG— To determine the effects of Ad-ACSL5 on fatty acid metabolism, we incubated McArdle-RH7777 cells with 20, 100, or 500 μM oleic acid for 3 h and measured [1-^{14}C]oleic acid incorporation into cellular and medium lipids and oxidation products. When compared with Ad-GFP-infected controls, Ad-ACSL5 did not affect total oleic acid metabolism, as measured by incorporation into cell lipids and medium TAG and ASM, at 20 or 100 μM but increased metabolism 28% at 500 μM oleic acid (Fig. 2*A*), a physiological concentration during short term fasts or a preprandial state (23, 24). To verify these results, we measured initial rates of fatty acid uptake (Fig. 1*C*). In close agreement with the increased fatty acid metabolism, we observed a 30% increase in rates of fatty acid uptake when cells were incubated with 500 μM oleic acid.

Cellular TAG represents the predominant storage form of exogenous fatty acids in most cell types. Incorporation of [1-^{14}C]oleic acid into cellular TAG increased 22 and 21% at 20 and 100 μM oleic acid, respectively, in both Ad-GFP- and Ad-ACSL5-infected cells (Fig. 2*B*). However, with 500 μM oleic acid in the medium, 42% more [1-^{14}C]oleic acid was incorporated in TAG in the Ad-ACSL5-infected cells when compared with control cells. Similar to cell TAG, medium TAG increased with increasing oleic acid concentrations in the media, but there were no differences between Ad-GFP- and Ad-ACSL5-infected cells at any fatty acid concentration (Fig. 2*C*). Although with 500 μM oleic acid, Ad-ACSL5 significantly increased [1-^{14}C]oleic acid incorporation into medium ASM (Fig. 2*D*), an indicator of ketone body production, the 13% increase is minor when compared with the increases in fatty acid uptake and partitioning to TAG, thus disproving our original hypothesis that ACSL5 activates fatty acids destined for β-oxidation. There were no differences in incorporation of [1-^{14}C]oleic acid into cellular phospholipids, fatty acids, or cholesterol esters, but Ad-ACSL5 increased [1-^{14}C]oleic acid incorporation into cellular DAG 2-fold when compared with Ad-GFP-infected cells (Fig. 3*A*).

To further substantiate the role of ACSL5 in TAG synthesis, we incubated cells with triacsin C, an inhibitor of ACSL1, ACSL3, and ACSL4, but not ACSL5. Previous data in hepatocytes and fibroblasts showed that triacsin c inhibits TAG synthesis more than ASM production (10, 25). In Ad-GFP-treated cells, triacsin C decreased TAG and ASM synthesis similarly (Fig. 3*B*). However, Ad-ACSL5 infection blocked triacsin C-mediated inhibition of TAG synthesis and attenuated ASM synthesis. Therefore, when other ACSL isoforms are inhibited, overexpressed ACSL5 can fully compensate for impaired TAG synthesis but can only partially restore ASM production.

Ad-ACSL5 Increases TAG Synthesis through Both de Novo and Reacylation Pathways— With the changes in partitioning of fatty acids, we considered whether the increase of oleic acid incorporation into cellular TAG in Ad-ACSL5-infected cells was due to *de novo* synthesis or to reacylation pathways. We labeled cells with 250 μM [1,2,3-^{3}H]glycerol plus 500 μM [1-^{14}C]oleic acid to elucidate the mechanism of enhanced TAG synthesis. Ad-ACSL5-infected cells incorporated 35% more [^{14}C]oleic acid and 28% more [^{3}H]glycerol into cellular TAG relative to Ad-GFP controls (Fig. 4*A*). The ratio of [^{14}C]oleic acid to [^{3}H]glycerol incorporation was also similar between Ad-GFP (4.6) and Ad-ACSL5 (4.8) groups. In phospholipids, [1-^{14}C]oleic acid incorporation did not differ between treatment groups, but [1,2,3-^{3}H]glycerol incorporation into phospholipids was suppressed 22% in the Ad-ACSL5-infected cells. This led to an increase in the [^{14}C]oleic acid to [^{3}H]glycerol ratio (3.1 for Ad-GFP *versus* 3.8 for Ad-ACSL5), suggesting that overexpression of ACSL5 suppresses *de novo* phospholipid synthesis more than reacylation.

FIGURE 2. **ACSL5 overexpression increases [1-^{14}C]oleic acid metabolism and partitioning to cellular TAG at 500 μm.** McArdle-RH7777 cells were labeled with various concentrations of [1-^{14}C]oleic acid from 24 to 27 h after infection with Ad-GFP or Ad-ACSL5. Cells were harvested; lipids were extracted, separated, and quantified; and ASM was measured as described under "Experimental Procedures." Total fatty acid (*Total FA*) metabolism includes cellular lipids, medium TAG, and medium ASM. Data are means ± S.D. from a representative experiment performed in triplicate and repeated three times. *A*, total [1-^{14}C]oleic acid metabolized; *B*, [1-^{14}C]oleic acid incorporation into cellular TAG; *C*, [1-^{14}C]oleic acid incorporation into medium TAG; *D*, [1-^{14}C]oleic acid incorporation into medium ASM. *, $p < 0.05$.
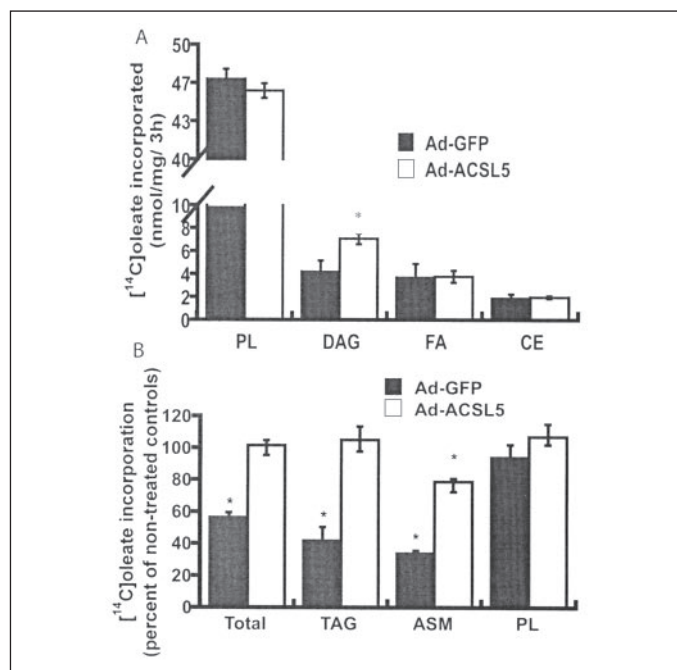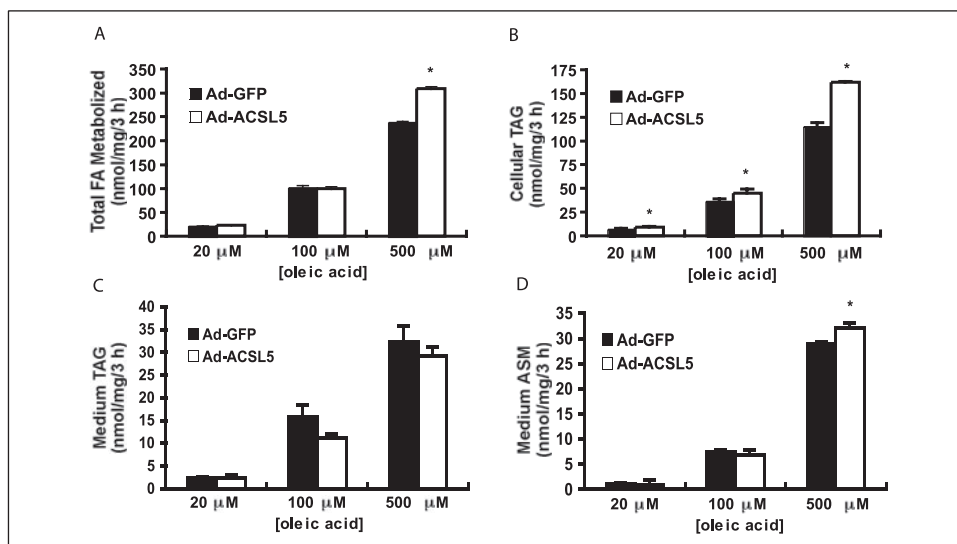

FIGURE 3. **In addition to TAG, Ad-ACSL5 increases incorporation of [1-^{14}C]oleic acid into DAG and confers resistance to triacsin C-mediated effects on fatty acid (*FA*) metabolism. McArdle-RH7777 cells were labeled with 500 μm** [1-^{14}C]oleic acid from 24 to 27 h after infection with Ad-GFP or Ad-ACSL5. Cells were harvested and lipids were extracted, separated, and quantified as described under "Experimental Procedures." *A*, [1-^{14}C]oleic acid incorporation into cellular lipids. Data are means ± S.D. from a representative experiment performed in triplicate and repeated at least three times. *, $p < 0.01$ when compared with Ad-GFP-infected cells. *B*, incorporation of [1-^{14}C]oleic acid into cellular lipids, medium ASM, or total [1-^{14}C]oleic acid metabolized in the presence of 10 μm triacsin C or Me$_2$SO as the carrier during the 3-h labeling period. Data are expressed as a percentage of Ad-GFP- or Ad-ACSL5-infected cells incubated with 10 μm Me$_2$SO. Data are means ± S.D. from a representative experiment performed in triplicate and repeated twice. *PL*, phospholipids; *CE*, cholesterol ester. *, $p < 0.01$ when compared with cells non-treated with triacsin C.

FIGURE 4. **Ad-ACSL5 increases incorporation of [1-^{14}C]oleic acid and [1,2,3-^{3}H]glycerol into TAG similarly and decreases [1,2,3-^{3}H]glycerol into cellular phospholipids (*PL*) but does not activate fatty acids (*FA*) derived from *de novo* synthesis.** From 24 to 27 h after infection with Ad-GFP or Ad-ACSL5, McArdle-RH7777 cells were labeled with 500 μm [1-^{14}C]oleic acid and 250 μm [1,2,3-^{3}H]glycerol (*A*) or 2.5 mm [1-^{14}C]acetate (*B*). Cells were harvested and lipids were extracted, separated, and quantified as described under "Experimental Procedures." Values for [1-^{14}C]oleic acid incorporation into TAG and phospholipid in Ad-GFP-infected cells were 102 ± 2.3 and 48.7 ± 1.4 nmol/mg/3 h, respectively; values for [1,2,3-^{3}H]glycerol incorporation into TAG and phospholipid in Ad-GFP-infected cells were 22.4 ± 1.2 and 15.7 ± 1.3 nmol/mg/3 h, respectively. Data are means ± S.D. from a representative experiment performed in triplicate and repeated twice. *, $p < 0.01$ when compared with Ad-GFP-infected cells.

The *de novo* synthesis of fatty acids can make a major contribution to cellular fatty acid pools. To determine whether ACSL5 activates fatty acids derived from *de novo* synthesis, we followed the incorporation of 2.5 mm [1-^{14}C]acetic acid into cellular lipids. Although the metabolic fate of acetic acid differed greatly from exogenous oleic acid, we observed no differences between Ad-GFP- and Ad-ACSL5-infected cells in the metabolism of acetic acid to any cellular lipid (Fig. 4*B*). Thus,
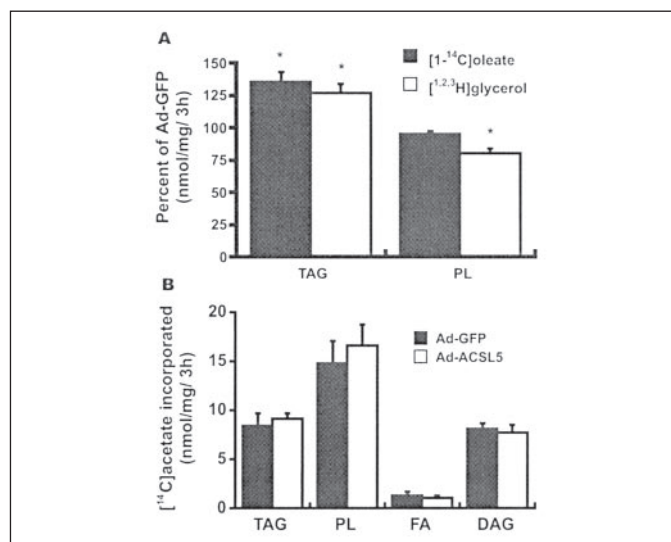
it appears that ACSL5 exclusively activates fatty acids from exogenous sources and does not use fatty acids that are produced endogenously.

ACSL5 mRNA Abundance Changes in Response to Fasting and Refeeding Support an Anabolic Role—Our *in vitro* data obtained from overexpressing ACSL5 in McArdle-RH7777 cells suggest that ACSL5 partitions fatty acids to TAG synthesis. To gain insight into the *in vivo* regulation of ACSL5, we measured changes in ACSL5 mRNA levels in rats that were chow-fed, fasted for 48 h, or fasted for 48 h and refed a 70% sucrose diet for 24 h. Abundance of ACSL5 mRNA decreased 54% in response to a 48-h fast, but when rats were refed a high sucrose diet after the fast, ACSL5 mRNA increased 26% above chow-fed levels (Fig. 5). These *in vivo* changes in ACSL5 mRNA abundance further support the *in vitro* data showing that ACSL5 functions in an anabolic capacity.
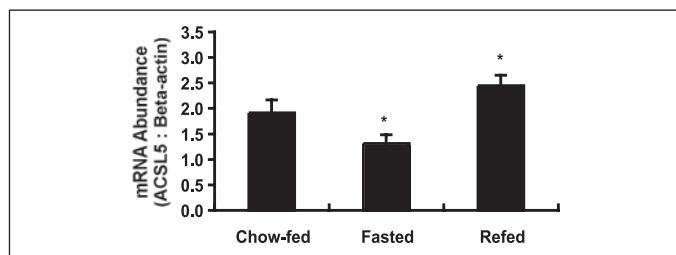
FIGURE 5. **Fasting decreases and refeeding increases hepatic ACSL5 mRNA abundance in rats.** Male Sprague-Dawley rats ($\sim$130 g) were fed a chow diet, fasted for 48 h, or refed a diet containing 70% sucrose for 24 h following a 48-h fast. Tissue harvesting, RNA extraction, and quantitative real-time-PCR were carried out as described under "Experimental Procedures." Data are means $\pm$ S.D. from four animals per group. *, $p <$ 0.05 when compared with chow-fed rats.

DISCUSSION

ACSL is the first enzyme required for fatty acid activation in almost all known pathways of fatty acid metabolism. Given that five isoforms have been identified in the ACSL family, as well as splice variants of several of the isoforms, we suggest that there are distinct roles for the individual isoforms and variants (26). Numerous studies have advanced our understanding of the kinetic properties, tissue distribution and, to some degree, intracellular localization of the ACSL isoforms, but they have not directly shown that each isoform has a specific role in lipid metabolism.

The current study was designed to directly test the effect of an individual ACSL isoform, ACSL5, on the partitioning of fatty acids in the rat hepatoma McArdle-RH7777 cell line. These cells are useful because their fatty acid metabolism is similar to that of hepatocytes and, unlike many hepatoma cell lines, McArdle-RH7777 cells secrete TAG in lipoprotein-containing particles (27). We have shown that overexpression of ACSL5 increases fatty acid metabolism and primarily partitions exogenous fatty acids to TAG that is destined for intracellular storage but not secretion. This increase in TAG synthesis was only observed at a high fatty acid concentration (500 μM) that would be typical of preprandial concentrations or a short term fast (23, 24). This suggests that at lower fatty acid concentrations, sufficient endogenous ACSL activity is present to metabolize exogenous fatty acids to acyl-CoAs. However, when higher concentrations of fatty acids are present in the medium, cellular ACSL activity may become limiting, thus hindering fatty acid uptake. By overexpressing ACSL5 and increasing total cellular ACSL activity 2-fold, intracellular fatty acid can be metabolized more rapidly, and fatty acid uptake increases.

To date, most studies monitoring fatty acid uptake have focused on plasma membrane proteins such as fatty acid translocase/CD36 or fatty acid transport proteins. Although these proteins undoubtedly enhance fatty acid uptake, the exact mechanism is not clear. If overexpressing an intracellular enzyme involved in activating fatty acids can increase the flux of fatty acids through a given pathway, this would suggest that uptake might be regulated by intracellular concentrations of fatty acids. Pownall and Hamilton (28) propose that increased intracellular conversion of fatty acids to acyl-CoAs and their subsequent metabolism may "pull" fatty acids into the cell by altering the fatty acid gradient across the plasma membrane. In support of this hypothesis, 1-acyl-glycerol-3-phosphate acyltransferase overexpression increases oleic acid uptake in both adipocytes and myotubes (29). Although plasma membrane proteins may catalyze part of the transport process, metabolism of the incoming fatty acids may be more critical in regulation.

The 40% increase in fatty acid incorporation into TAG, without substantial increases in any other lipid besides DAG, gives credence to the hypothesis that individual ACSL isoforms partition fatty acids toward distinct pathways. Surprisingly, the increase in fatty acid incorporation into cellular TAG did not coincide with increased TAG secretion. Since medium TAG increased proportionately with increasing amounts of fatty acid added to the medium, we can eliminate the possibility that the capacity for very low density lipoprotein synthesis and secretion was limiting. Therefore, fatty acids activated by ACSL5 may be destined for an intracellular TAG pool with a low turnover rate. It is not known whether DAG synthesized via enzymes located on mitochondria is more or less prone to intracellular storage or secretion when compared with endoplasmic reticulum-located enzymes, which include the terminal enzyme in TAG synthesis, diacylglycerol acyltransferase. Overexpressing mitochondrial glycerol-3-phosphate acyltransferase increases cellular TAG without increasing very low density lipoprotein secretion in rat primary hepatocytes and thus argues that initiating the TAG synthetic pathway in mitochondria predisposes the resulting TAG to storage (30).

In agreement with the cell radiolabel studies, rat liver ACSL5 mRNA decreased following a 48-h fast and increased upon refeeding a high sucrose diet for 24 h. This pattern of ACSL5 message abundance suggests that it encodes an enzyme involved in a TAG synthetic pathway. Additional studies have also shown that ACSL5 mRNA is regulated in a manner concordant with an anabolic pathway. Hepatic ACSL5 message abundance increases in SREBP-1c transgenic mice (15) and in zinc-deficient mice that have fatty livers (17). In addition, hepatic ACSL5 mRNA levels do not respond to peroxisomal proliferator-activated receptor α agonists (14) and decrease in models with suppressed lipogenic gene expression such as SREBP cleavage-activating protein knock-out mice (15), estrogen-related receptor knock-out mice (18), and C57BL/6J obese *ob/ob* mice following leptin administration (16). In contrast to mRNA changes, rat hepatic mitochondrial ACSL5 protein increases after a 48-h fast and decreases when rats are refed a high sucrose diet (6). Discordant regulation of ACSL5 mRNA and protein or ACSL5 abundance on mitochondria and microsomes may occur.

Studies using triacsin C, an inhibitor of ACSL1, ACSL3, and ACSL4 but not ACSL5, have shown more pronounced decreases in glycerolipid synthesis than in fatty acid β-oxidation (10, 25). Based on these data, ACSL5 was hypothesized to activate fatty acids for β-oxidation. Therefore, it was surprising that Ad-ACSL5-infected cells did not substantially alter ASM synthesis. It is possible that ACSL5 activates fatty acids for both anabolic and catabolic pathways and that the supply of acyl-CoAs derived from ACSL5 may be rate-limiting for TAG synthesis but not for ASM synthesis. This rationale could explain the observed increase in cellular TAG without a corresponding change in ASM synthesis. To further explore this hypothesis, we inhibited ACSL1, ACSL3, and ACSL4, the other known ACSL isoforms in McArdle-RH7777 cells with triacsin C. Interestingly, overexpression of ACSL5 conferred resistance to the suppressive effects of triacsin C on cellular TAG synthesis and the total amount of fatty acid metabolized. However, in Ad-ACSL5-infected cells treated with triacsin C, ASM synthesis decreased 21%. It should be noted that this decrease was attenuated when compared with the 66% decrease in ASM synthesis in Ad-GFP-infected cells treated with triacsin C. Thus, some of the acyl-CoAs derived from ACSL5 were likely used for ASM synthesis when the other ACSL isoforms were inhibited. Although we hypothesize that ACSL isoforms have unique roles in partitioning fatty acids, it is unlikely that an individual isoform activates fatty acids exclusively for a given pathway. More likely, a given ACSL isoform may activate fatty acids that are predominantly metabolized into a specific pathway. Some acyl-CoAs, however, may "escape" and be metabolized by alternate pathways.

In contrast to the effects of triacsin C in primary hepatocytes and

fibroblasts, in Ad-GFP-infected McArdle-RH7777 cells, triacsin C had similar inhibitory effects on both TAG and ASM synthesis. Although the triacsin C data further substantiate a major role for ACSL5 in TAG synthesis, there are many caveats when using triacsin C. Many of the ACSL isoforms have splice variants, most of which have not been tested for triacsin C sensitivity. Very long chain acyl-CoA synthetases (ACSVL) also possess some activity toward long chain fatty acids. Similar to the ACSL splice variants, triacsin C sensitivity has not been tested in many of the ACSVL isoforms. Additional research is needed to further characterize other ACSL variants or ACSVL isoforms, their splice variants, subcellular locations, and sensitivity to triacsin C.

In summary, adenoviral overexpression of ACSL5 in McArdle-RH7777 cells increased fatty acid uptake into cells that were exposed to fatty acid concentrations typical of a short term fast or preprandial state. Although high fatty acid concentrations were needed to observe the increase in fatty acid uptake, fatty acid incorporation into cellular TAG was increased at all fatty acid concentrations tested. Despite the increase in cellular TAG synthesis, Ad-ACSL5-infected cells did not increase the amount of TAG secreted into the medium and did not use endogenously synthesized fatty acids. Taken together with changes in hepatic mRNA levels in response to fasting and refeeding, we showed that, in contrast to our original hypothesis, ACSL5 is predominantly involved in synthetic rather than catabolic pathways. Although it is possible that the function of overexpressed ACSL5 may differ from that of the endogenous enzyme, these data suggest a unique role for ACSL5 in fatty acid and TAG metabolism in McArdle-RH7777 cells.

REFERENCES

1. Oikawa, E., Iijima, H., Suzuki, T., Sasano, H., Sato, H., Kamataki, A., Nagura, H., Kang, M. J., Fujino, T., Suzuki, H., and Yamamoto, T. T. (1998) *J. Biochem.* (*Tokyo*) **124,** 679–685
2. Kang, M. J., Fujino, T., Sasano, H., Minekura, H., Yabuki, N., Nagura, H., Iijima, H., and Yamamoto, T. T. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 2880–2884
3. Fujino, T., Kang, M. J., Suzuki, H., Iijima, H., and Yamamoto, T. (1996) *J. Biol. Chem.* **271,** 16748–16752
4. Fujino, T., and Yamamoto, T. (1992) *J. Biochem.* (*Tokyo*) **111,** 197–203
5. Suzuki, H., Kawarabayasi, Y., Kondo, J., Abe, T., Nishikawa, K., Kimura, S., Hashimoto, T., and Yamamoto, T. (1990) *J. Biol. Chem.* **265,** 8681–8685
6. Lewin, T. M., Kim, J. H., Granger, D. A., Vance, J. E., and Coleman, R. A. (2001) *J. Biol. Chem.* **276,** 24674–24679
7. Rusinol, A. E., Cui, Z., Chen, M. H., and Vance, J. E. (1994) *J. Biol. Chem.* **269,** 27494–27502
8. Kim, J. H., Lewin, T. M., and Coleman, R. A. (2001) *J. Biol. Chem.* **276,** 24667–24673
9. Van Horn, C. G., Caviglia, J. M., Li, L. O., Wang, S., Granger, D. A., and Coleman, R. A. (2005) *Biochemistry* **44,** 1635–1642
10. Muoio, D. M., Lewin, T. M., Wiedmer, P., and Coleman, R. A. (2000) *Am. J. Physiol.* **279,** E1366–E1373
11. Yu, X. X., Lewin, D. A., Forrest, W., and Adams, S. H. (2002) *FASEB J.* **16,** 155–168
12. Trotter, P. J., and Storch, J. (1991) *J. Lipid Res.* **32,** 293–304
13. Darnley, A. C., Carpenter, C. A., and Saggerson, E. D. (1988) *Biochem. J.* **253,** 351–355
14. Lewin, T. M., Van Horn, C. G., Krisans, S. K., and Coleman, R. A. (2002) *Arch. Biochem. Biophys.* **404,** 263–270
15. Horton, J. D., Shah, N. A., Warrington, J. A., Anderson, N. N., Park, S. W., Brown, M. S., and Goldstein, J. L. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100,** 12027–12032
16. Liang, C. P., and Tall, A. R. (2001) *J. Biol. Chem.* **276,** 49066–49076
17. tom Dieck, H., Doring, F., Fuchs, D., Roth, H. P., and Daniel, H. (2005) *J. Nutr.* **135,** 199–205
18. Luo, J., Sladek, R., Carrier, J., Bader, J. A., Richard, D., and Giguere, V. (2003) *Mol. Cell. Biol.* **23,** 7947–7956
19. He, T. C., Zhou, S., da Costa, L. T., Yu, J., Kinzler, K. W., and Vogelstein, B. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 2509–2514
20. Bligh, E. G., and Dyer, W. J. (1959) *Can. J. Biochem. Physiol.* **37,** 911–917
21. Pei, Z., Fraisl, P., Berger, J., Jia, Z., Forss-Petter, S., and Watkins, P. A. (2004) *J. Biol. Chem.* **279,** 54454–54462
22. Banis, R. J., and Tove, S. B. (1974) *Biochim. Biophys. Acta* **348,** 210–220
23. Bortz, W. M., and Steele, L. A. (1973) *Biochim. Biophys. Acta* **306,** 85–94
24. Fuller, R. W., and Diller, E. R. (1970) *Metabolism* **19,** 226–229
25. Igal, R. A., Wang, P., and Coleman, R. A. (1997) *Biochem. J.* **324,** 529–534
26. Mashek, D. G., Bornfeldt, K. E., Coleman, R. A., Berger, J., Bernlohr, D. A., Black, P., DiRusso, C. C., Farber, S. A., Guo, W., Hashimoto, N., Khodiyar, V., Kuypers, F. A., Maltais, L. J., Nebert, D. W., Renieri, A., Schaffer, J. E., Stahl, A., Watkins, P. A., Vasiliou, V., and Yamamoto, T. T. (2004) *J. Lipid Res.* **45,** 1958–1961
27. Hansson, P. K., Asztely, A. K., Clapham, J. C., and Schreyer, S. A. (2004) *Biochim. Biophys. Acta* **1684,** 54–62
28. Pownall, H. J., and Hamilton, J. A. (2003) *Acta Physiol. Scand.* **178,** 357–365
29. Ruan, H., and Pownall, H. J. (2001) *Diabetes* **50,** 233–240
30. Lewin, T. M., Wang, S., Nagle, C. A., Van Horn, C. G., and Coleman, R. A. (2005) *Am. J. Physiol.* **288,** E835–E844