SREBP2 Activation Induces Hepatic Long-chain Acyl-CoA Synthetase 1 (ACSL1) Expression in Vivo and in Vitro through a Sterol Regulatory Element (SRE) Motif of the ACSL1 C-promoter

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Long-chain acyl-CoA synthetase 1 (ACSL1) plays a key role in fatty acid metabolism. To identify novel transcriptional modulators of ACSL1, we examined ACSL1 expression in liver tissues of hamsters fed a normal diet, a high fat diet, or a high cholesterol and high fat diet (HCHFD). Feeding hamsters HCHFD markedly reduced hepatic Acsl1 mRNA and protein levels as well as acyl-CoA synthetase activity. Decreases in Acsl1 expression strongly correlated with reductions in hepatic Srebp2 mRNA level and mature Srebp2 protein abundance. Conversely, administration of rosvastatin (RSV) to hamsters increased hepatic Acsl1 expression. These new findings were reproduced in mice treated with RSV or fed the HCHFD. Furthermore, the RSV induction of acyl-CoA activity in mouse liver resulted in increases in plasma and hepatic cholesterol ester concentrations and reductions in free cholesterol amounts. Investigations on different ACSL1 transcript variants in HepG2 cells revealed that the mRNA expression of C-ACSL1 was specifically regulated by the sterol regulatory element (SRE)-binding protein (SREBP) pathway, and RSV treatment increased the C-ACSL1 abundance from a minor mRNA species to an abundant transcript. We analyzed 5′-flanking sequence of exon 1C of the human ACSL1 gene and identified one putative SRE site. By performing a promoter activity assay and DNA binding assays, we firmly demonstrated the key role of this SRE motif in SREBP2-mediated activation of C-ACSL1 gene transcription. Finally, we demonstrated that knockdown of endogenous SREBP2 in HepG2 cells lowered ACSL1 mRNA and protein levels. Altogether, this work discovered an unprecedented link between Acsl1 and SREBP2 via the specific regulation of the C-ACSL1 transcript.

In mammals, the predominant long-chain fatty acids (LCFAs) 3 have 16 and 18 carbons with different degrees of saturation. Prior to entering various metabolic pathways, free LCFAs must be esterified with coenzyme A by members of the long-chain acyl-CoA synthetase (ACSL) family (1, 2). This activation process requires ATP, CoA, and LCFAs. Activated fatty acids can then undergo degradation through β-oxidation, be utilized for cellular lipid synthesis, or serve as lipid anchors for protein modifications (3–8).

The mammalian ACSL family consists of five distinct members, including ACSL1, ACSL3, ACSL4, ACSL5, and ACSL6. These isoforms differ substantially in their substrate preference, tissue distribution, and subcellular compartmentation as well as in their responses to nutritional status (9), all of which contributes to the unique functions of each isoform in a particular tissue or a cell type.

ACSL1 has been the most extensively studied isoform of this family since it was cloned from rat liver in 1990 (10) and has broad substrate specificity for saturated FAs of 16 and 18 chain lengths and unsaturated FAs of 16–20 carbon atoms. ACSL1 is the abundant isomeric in major metabolic tissues, including liver, heart, adipose, and muscle. Whereas in vitro studies carried out in different cell lines have reported similar as well as conflicting functions of ACSL1 in mediating FA oxidation, FA uptake, and triglyceride (TG) synthesis (9, 11, 13), recent animal studies with tissue-specific knock-out of Acsl1 revealed some definitive functions of this enzyme. In heart and adipose tissues, Acsl1 deficiency generated a profound decrease in FA oxidation without an impact on [14C]oleate incorporation into TG or phospholipid (14, 15). In skeletal muscle, Acsl1 deficiency caused a 91% loss of ACSL specific activity and 60–85% decreases in muscle FA oxidation. Furthermore, systemic glucose homeostasis became severely compromised in mice lacking Acsl1 in skeletal muscle (16).

Despite the recent advance in our understanding of the tissue-specific functions of ACSL1, to date, only limited information is available regarding the regulation of ACSL1 at transcriptional levels. It is reported that rat Acsl1 is regulated by the PPAR family members through a PPAR-responsive element (PPRE) embedded in the C-promoter region of the Acsl1 gene (17, 18). In particular, treating rats with fenofibrate, an activator of PPARα, increased Acsl1 expression in liver and kidney but not in heart or muscle.
Regulation of ACSL1 Expression by SREBP Pathway

In mammals, both FA and cholesterol biosynthetic processes are controlled by a common family of transcription factors designated sterol regulatory element-binding proteins (SREBPs). The family consists of three different SREBP proteins: SREBP1a, SREBP1c, and SREBP2. Together, SREBPs transcriptionally activate a cascade of enzymes required for endogenous cholesterol, FA, TG, and phospholipid synthesis (19, 20). The activity of SREBPs is regulated by cellular sterol levels. SREBPs are synthesized as inactive precursors localized to the membrane of the endoplasmic reticulum. In the inactive state within the endoplasmic reticulum, the C-terminal domains of the SREBPs interact with another membrane protein, SREBP cleavage-activating protein, which functions as a sterol sensor. Under low sterol conditions, SREBP cleavage-activating protein escorts the precursor forms of SREBPs from the endoplasmic reticulum to the Golgi, where they are processed by two membrane-associated proteases, the site 1 and site 2 proteases, which release the NH2-terminal transcriptionally active mature form of the SREBPs from the precursor proteins (21, 22). The mature SREBPs translocate to the nucleus, where they bind to the SRE motifs and E-boxes within promoters of SREBP target genes (23). Interestingly, despite the key roles of ACSL1 in FA metabolism, an intrinsic relationship between ACSL1 and SREBPs has not been firmly established.

In this current study, we utilized normolipidemic and hypercholesterolemic hamsters and mice as in vivo models to examine ACSL1 expression levels in liver tissues under conditions of suppression of SREBP pathways by dietary cholesterol and activation of SREBP2 by treating animals with a statin. These in vivo studies revealed a strong correlation of ACSL1 expression and activity with SREBP2. By using human hepatoma-derived HepG2 as a model system, we identified ACSL1 transcript variant C-ACSL1 as a novel molecular target of SREBP2 and further mapped the SREBP2 binding site to a novel SRE motif located in the proximal region of the C-ACSL1 promoter. Finally, by using a specific siRNA targeting SREBP2, we demonstrated that knockdown of endogenous SREBP2 in HepG2 cells directly reduced ACSL1 mRNA and protein levels.

Experimental Procedures

Cells and Reagents—HepG2 cells were obtained from ATCC. Cholesterol and 25-hydroxycholesterol were purchased from Sigma, and rosuvastatin (RSV) was purchased from AK Scientific Inc. (Mountain View, CA).

Animals, Diets, and RSV Treatment—All animal experiments were performed according to procedures approved by the Veterans Affairs Palo Alto Health Care System Animal Care and Use Committee. Ten-week-old male golden Syrian hamsters were purchased from Harlan. Hamsters were housed (2 animals/cage) under controlled temperature (72°F) and lighting (12-h light/dark cycle). Animals had free access to autoclaved water and food. For the diet study, 18 hamsters were divided into three diet groups of a normal diet (ND), a high fat diet (HFD) (TD.88137 from Harlan; 42% of total calories from fat, 0.15% cholesterol), and a high cholesterol and high fat diet (HCHFD) (D12336, 1.25% cholesterol; Research Diets, Inc., New Brunswick, NJ) for 2 weeks. The main ingredients of these diets are summarized in Table 1. At the experimental termination, after a 16-h fast, hamsters were anesthetized, and terminal blood samples and tissue samples were collected.

For the RSV treatment, 18 hamsters were fed a high fructose diet (60% fructose; Diets, Inc., Bethlehem, PA) for 3 weeks. While continuously on the fructose diet, hamsters were randomly divided into two groups (n = 9/group) and were given a daily dose of 20 mg/kg RSV or the vehicle (0.5 ml of 10% 2-hydroxypyrol-β-cyclodextrin in autoclaved water) by oral gavage. The drug treatment lasted 7 days. Twenty-four h after the last dosing, all animals were sacrificed.

For the mouse study, 18 adult male C57BL/6J mice were divided into three groups; group one (n = 6) were fed the HCHFD for 2 weeks, group two (n = 6) were fed with an ND and orally administered with vehicle, and group three (n = 6) were fed an ND and were orally administered with RSV at a daily dose of 20 mg/kg for 2 weeks. At the end of the experiment, mice were fasted for 4 h, and blood samples were collected for the analysis of serum total cholesterol (TC) and free cholesterol (FC). Liver tissue samples were collected and stored at −80°C for the analysis of mRNA and protein expression and lipid contents.

Measurement of Serum and Hepatic Lipid Levels—Serum was isolated at room temperature and stored at −80°C. Extraction of lipids from liver tissues was performed as described (24). Standard enzymatic methods were used to determine TC, TG, and FC levels of serum and hepatic lipids using commercially available kits purchased from Stanbio Laboratory (Boerne, TX). Each sample was assayed in duplicate. Cholesterol ester (CE) and ester ratio were calculated by using the following formulas provided in the kit manual.

\[
\text{CE(mg/dl)} = \text{TC} - \text{FC} \quad (\text{Eq. 1})
\]

\[
\text{Ester ratio (\%)} = \frac{\text{CE(mg/dl)}/\text{TC(mg/dl)}}{100} \times 100 \quad (\text{Eq. 2})
\]

RNA Isolation and Quantitative RT-PCR (qRT-PCR)—Total RNA isolation, generation of cDNA, and qRT-PCR were conducted as reported previously (24). Each cDNA sample was assayed in duplicate. The correct size of the PCR product and the specificity of each primer pair were validated by examination of PCR products on an agarose gel. Species-specific primer sequences used in qRT-PCR are listed in Table 2. Target mRNA expression in each sample was normalized to the housekeeping gene GAPDH. The 2−ΔΔCt method was used to calculate relative mRNA expression levels.

Western Blot Analysis—Approximately 50 mg of frozen tissue was homogenized in radioimmune precipitation assay buffer containing 1 mM PMSF and protease inhibitor mixture (Roche Applied Science). After protein quantitation using BCA protein assay reagent (Pierce), 50 μg of homogenate proteins from individual tissue samples were resolved by SDS-PAGE and transferred to nitrocellulose membranes. The anti-human ACSL1 antibody (catalog no. ab76702) was obtained from Abcam (Cambridge, MA). The rabbit anti-SREBP2 polyclonal antibodies recognizing both the precursor form and the nuclear mature form of SREBP2 have been described in our previous studies (26). The membranes were reprobed with an anti-β-actin (catalog no. A1978, Sigma) antibody or with anti-GAPDH (catalog no. AM4300, Thermo Fisher). Immunoreactive bands
of predicted molecular mass were visualized using a SuperSignal West Femto Maximum Sensitivity Substrate ECL kit (Thermo Scientific) and quantified with Alpha View software with normalization by signals of β-actin.

Measurement of Tissue Acyl-CoA Synthetase Activity—Approximately 50 mg of frozen tissue were homogenized in a buffer containing 20 mM HEPES, 1 mM EDTA, and 250 mM sucrose, pH 7.4. After a centrifugation at 16,000 rpm, cell lysates were collected, and protein concentrations of cell lysates were determined by the BCA method (Pierce) prior to the ACSL activity assay. The activity assay incubation mixture contained 175 mM Tris-HCl, pH 7.4, 8 mM MgCl₂, 5 mM dithiothreitol, 1 mM ATP, 0.2 mM CoASH, 0.5 mM Triton X-100, 10 μM EDTA, and 50 μM palmitate mixed with 0.1 μCi of [³H]palmitic acid (25). The reaction was initiated by adding the homogenized sample and terminated by adding 1 ml of Dole's reagent as described previously (24). Generated [³H]acyl-CoA was extracted, and the radioactivity was determined in a scintillation counter. The radioactivity in the reaction that contained all components but omitted homogenate was included as a negative control.

ACSL1 Promoter Cloning and Reporter Construction—A 2.5-kb DNA fragment covering the 5′-flanking region from −2215 to +300, relative to the 5′ end of exon 1C of the human

### TABLE 1

**Diet comparisons**

| Product # | D10012G (NCD*) | TD.88137 (HFD*) | D12336 (HCHFD*) |
|-----------|-----------------|-----------------|-----------------|
|           | gm% kcal%       | gm% kcal%       | gm% kcal%       |
| Protein   | 20 20.3         | 17.3 15.3       | 21 20           |
| Carbohydrates | 64 63.9    | 48.5 42.7       | 46 45           |
| Fat       | 7 15.8          | 21.2 42.0       | 16 35           |
| Total     | 100             | 100             | 100             |
| kcal/gm   | 3.9             | 4.5             | 4.1             |

### Ingredients

|                | gm kcal | gm kcal | gm kcal |
|----------------|---------|---------|---------|
| Casein         | 200 800 | 195 780 | 75 300  |
| L-Cystine      | 3 12    | -       | -       |
| Soy protein    | - -     | - -     | 130 520 |
| DL-Methionine  | - -     | 3 12    | 2 8     |
| Corn Starch    | 397 1590| 50 200  | 275 1100|
| Maltodextrin   | 132 528 | 100 400 | 150 600 |
| Sucrose        | 100 400 | 341 1364| 30 120  |
| Cellulose      | 50 0    | 50 0    | 90 0    |
| Soybean Oil    | 70 630  | - -     | 50 450  |
| Milk fat       | - -     | 200 1800| - -     |
| Corn Oil       | - -     | 10 90   | - -     |
| Cocoa Butter   | - -     | - -     | 75 675  |
| Coconut Oil    | - -     | - -     | 35 315  |
| Mineral Mix    | 35 0    | 35 0    | 35 0    |
| Sodium Chloride| - -     | - -     | 8 0     |
| Calcium Carbonate| - -   | 4 0     | 5.5 0   |
| Potassium Citrate| - -  | - -     | 10 0    |
| Vitamin Mix    | 10 40   | 10 40   | 10 40   |
| Choline Bitartrate| 2.5 | 2 0     | 2 0     |
| Ethoxyquin     | 0.04 0  | - -     | - -     |
| Cholesterol    | 1.5 0   | 12.5 0  | - -     |
| Sodium Cholic acid| - - | - 5     | - -     |
| FD&C Red Dye #40| - - | - 0.01  | - -     |
| Total          | 1000 4000| 4686 1000.1| 4128    |
Regulation of ACSL1 Expression by SREBP Pathway

| Table 2 | qRT-PCR primer, EMSA probes, and ChIP primer sequences |
|---------|--------------------------------------------------|
| **Gene name** | **Forward** | **Reverse** |
| **Mouse primers** | | |
| Acsl1 | ATCTGGTTGGAACGAGGCAAG | TCCTTTGGGGTTGCCTGTAG |
| Srebp1 | CAGGCGATTGCACTCTACG | OCACTTTGGGGTTGCCTTAG |
| Srebp2 | CAAAGAGGAAGAGAGACGG | CAGGAGTTGCACTCTAGTG |
| Acat1 | CAGGTCATAGAGCTGGACAC | TGGGATGTCATAGAGGACAA |
| Acat2 | CCGGTCATAGAGCTGGACAC | TGGGATGTCATAGAGGACAA |
| Ldlr | CCGGTCATAGAGCTGGACAC | TGGGATGTCATAGAGGACAA |
| Gapdh | AATCTGGTTGGAACGAGGCAAG | TCCTTTGGGGTTGCCTGTAG |
| **Human primers** | | |
| ACSL1 | CTGCTTGTCGCTGCAAGAAC | GTCTCTTTGGGGTTGCCTGTAG |
| A-ACSL1 | AGCGCGGTTTTCATTGAGCA | ATGAGCTGCTGCTGGCTGCC |
| C-ACSL1 | GTCAACCCGGCTCAGTCCGCC | GTCCGACGAGGAAAGGCTC |
| GAPDH | CTGGTTGGCTGTCGGTTCGG | GTCCGACGAGGAAAGGCTC |
| LDLR | GACGGCGGCTGCAAGACCTC | CTGGTTGGCTGTCGGTTCGG |
| SREBP1 | CTTCTGGTTGGAACGAGGCAAG | TCCTTTGGGGTTGCCTGTAG |
| SREBP2 | CAGGCGATTGCACTCTACG | OCACTTTGGGGTTGCCTTAG |
| **Hamster primers** | | |
| Ppara | AAAGTGGGTCATAGAGGACAA | TGGGATGTCATAGAGGACAA |
| Ppara | CTTCTGGTTGGAACGAGGCAAG | TCCTTTGGGGTTGCCTGTAG |
| Ldlr | GACGGCGGCTGCAAGACCTC | CTGGTTGGCTGTCGGTTCGG |
| Srebp1 | GACGGCGGCTGCAAGACCTC | CTGGTTGGCTGTCGGTTCGG |
| Srebp2 | GACGGCGGCTGCAAGACCTC | CTGGTTGGCTGTCGGTTCGG |
| Gapdh | AATCTGGTTGGAACGAGGCAAG | TCCTTTGGGGTTGCCTGTAG |
| **ACSL1 promoter cloning primers** | | |
| ACSL1 promoter C 2.5 kb | GGAGGTGGCTGCTGCTGGCTGCC | CCACTGACGAGGAAAGGCTC |
| ACSL1 promoter C-SRE-Mut 2.5 kb | CAAAGTGGGTCATAGAGGACAA | TGGGATGTCATAGAGGACAA |
| **EMSA probes** | | |
| ACSL1 promoter C-SRE-WT | GAGGGCGGCTGCAAGACCTC | CTGGTTGGCTGTCGGTTCGG |
| ACSL1 promoter C-SRE-Mutant | GAGGGCGGCTGCAAGACCTC | CTGGTTGGCTGTCGGTTCGG |
| **ChIP primer** | | |
| ACSL1 promoter C | CGGGGTCTGCTGCTGGCTGCC | CCACTGACGAGGAAAGGCTC |
| ACSL1 promoter A | CTAAGGTTGGAACGAGGCAAG | TCCTTTGGGGTTGCCTGTAG |

ACSL1 gene was amplified from HepG2 genomic DNA with primers listed in Table 2. The PCR was performed using the Invitrogen High Fidelity PCR system. PCR products were initially cloned into the TOPO-TA cloning vector (Invitrogen). After digestion with SacI and XhoI, the fragment was subcloned into pGL3 basic vector at the SacI and XhoI sites, thereby generating the reporter vector ACSL1 promoter C-Luc.

Luciferase Reporter Activity—HepG2 cells seeded in 96-well culture plate (3 × 10⁴ cells/well) were transfected with 0.1 μg of ACSL1 promoter C-Luc, pGL3-basic vector, or pGL3-basic vector and cotransfected with 10 ng of pCMV-β-Gal as an internal control by using Polyjet transfection reagent (SignaGen Laboratories, Gaithersburg, MD). Twenty-four h after transfection, cells were cultured in medium containing 0.5% FBS for 12 h and then trypsinized, resuspended in PBS at a dilution of 6 × 10⁵ cells/ml, and fixed with 1% formaldehyde (Sigma) for 10 min. To obtain nuclear lysates, fixed cells were resuspended in 500 μl chromatin shearing buffer and sonicated at 4 °C in a Bioruptor 300 instrument (Diagenode, Inc.) for 12 cycles of 30 s on/30 s off at a high setting with intermittent vortex mixing. Chromatin containing nuclear lysates (100 μl) was incubated overnight with 5 μg of plasmid of pCMV-β-Gal and pCMV-β-Gal plasmid was transfected into HEK-293A cells, and the protein was purified using the Protobond purification system (Invitrogen), following the manufacturer’s instructions.
with 3 μg of either mouse anti-His (Ab18184, Abcam) or isotype control IgG antibodies (Santa Cruz Biotechnologies, Inc.), and complexes were immunoprecipitated using protein A magnetic beads. ChIP DNA was eluted, cross-linking was reversed, and protein-free DNA was purified before PCR amplification with site-specific primers covering the ACSL1 promoter C SRE region. Primer sequences for the ChIP assay are listed in Table 2.

**SREBP2 siRNA Transfection**—siRNA duplexes (SREBP2 siRNA (sc-36559) and siRNA control (sc-44231)) were obtained from Santa Cruz Biotechnologies. HepG2 cells were seeded in 6-well plates (3 x 10⁵ cells/well) and transfected with SREBP2 siRNA or control siRNA duplexes at concentrations of 20 nM using siPORT™ NeoFX™ transfection agent (Invitrogen), according to the manufacturer’s instructions. Transfection complexes were performed in serum-free OptiMEM (Invitrogen). Cell medium was changed 24 h after transfection. After 72 h of transfection, cells were harvested for protein and RNA expression analysis.

**Statistical Analysis**—Values are presented as the mean ± S.E. Significant differences between diet groups were assessed by two-tailed Student’s t test (nonparametric Mann–Whitney test) or one-way analysis of variance with Dunnett’ post-test. Statistical significance is displayed as p < 0.05 (*), p < 0.01 (**), or p < 0.001 (***)

**Results**

**Repression of ACSL1 Expression in Hamsters by Dietary Cholesterol**—To determine whether ACSL1 expression could be affected by dietary cholesterol, we fed hamsters three different diets (ND, HFD, or HCHFD) for 2 weeks. Measurement of serum TC and TG as well as hepatic TC and TG levels confirmed the hyperlipidemic status of hamsters in the HCHFD group. The results showed that TC and TG were increased (Table 3). Serum triglycerides (mg/dl) were decreased by HFD but were lowered by ~40% by HCHFD, whereas hepatic triglycerides (mg/g liver) were slightly increased by HFD but were lowered by ~31% (p < 0.001) in the liver homogenates of the three diet groups. The results showed that ACSL1 mRNA levels were reduced in ND and HCHFD groups, whereas the levels of mature SREBP2 in the liver homogenates of the three diet groups were reduced by 31% (p < 0.01) of control and 37% (p < 0.001) of control, respectively, by HCHFD feeding but not by HFD feeding. Additionally, these results provided the first evidence that ACSL1 expression in vivo was down-regulated by dietary cholesterol along with the active form of SREBP2.
Regulation of ACSL1 Expression by SREBP Pathway

We next measured ACSL activity of individual homogenates of liver tissue from ND, HFD, and HCHFD groups. ACSL activity assays using liver homogenate and [3H]palmitic acid (16:0) demonstrated a 16% (p < 0.05) reduction of palmitoyl-CoA synthetase activity in the HCHFD group (Fig. 1C). Collectively, these results demonstrated that dietary cholesterol exerted inhibitory effects on the expression levels of Acsl1 mRNA and protein, thereby leading to a reduced ACSL enzymatic activity.

**Induction of Hepatic ACSL1 Expression in Hamsters by RSV Treatment**—The above dietary studies in hamsters revealed a positive correlation between ACSL1 and SREBP2. To examine the direct effect of SREBP2 activation on Acsl1 gene transcription in vivo, we examined hepatic Acsl1 mRNA and protein expressions in hamsters that were on a fructose diet for 3 weeks and were then treated with 20 mg/kg RSV or vehicle for 7 days (26). Fig. 2A shows that ACSL1 protein levels were 71% (p < 0.01) higher in RSV-treated livers as compared with the vehicle group. qRT-PCR measurement of hepatic Acsl1 mRNA levels corroborated the results of Western blot analysis with a 75% increase over the vehicle control group (Fig. 2B).

ACSL1 Expression Is Down-regulated in Livers of Mice Fed HCHFD and Up-regulated by RSV Treatment—We further examined the regulation of ACSL1 expression and activity in livers of mice that were fed the HCHFD or treated with RSV for 2 weeks. Fig. 3, A and B, shows that the HCHFD significantly suppressed ACSL1 protein expression by more than 50% (p < 0.001) and ACSL specific activity by 16% (p < 0.05), respectively. SREBP2 protein expression was also significantly suppressed by the HCHFD diet (Fig. 3A). In contrast, the amount of ACSL1 protein and specific activity were up-regulated significantly by 160% (p < 0.001) and 21% (p < 0.001) (Fig. 3, A and B) in livers of mice treated with RSV compared with vehicle treatment. The changes in Acsl1 and Srebp2 mRNA levels correspond to their protein expression; HCHFD diet dramatically suppressed Acsl1 and Srebp2 mRNA levels, whereas RSV treat-
ment significantly up-regulated mRNA expression of Acsl1 and Srebp2 (Fig. 3C).

We next examined the hepatic cholesterol distributions in all groups of mice (Fig. 4, A–D). Feeding mice the HCHFD for 2 weeks resulted in massive accumulations of liver TC, CE, and FC contents without changing the hepatic CE ratio as compared with the liver of the ND group. Interestingly, under normal diet conditions, 2-week RSV treatment significantly increased the hepatic content of CE, reduced FC, and increased liver CE ratio by 57% as compared with the control mice. Next, we examined the TC and FC in serum samples of all groups and observed changes similar to hepatic cholesterol contents (Fig. 4, E–H). These new findings suggest that increased ACSL1 activity by RSV treatment led to enhanced CE synthesis.

In mammals, endogenous CEs are synthesized by acyl-CoA cholesterol acyltransferase (ACAT) that catalyzes the formation of cholesteryl esters from cholesterol and long-chain fatty acids. There are two known genes (Acat1 and Acat2) that encode the two ACAT enzymes, and both enzymes are present in the liver tissue of adult mice. Utilizing qRT-PCR, we measured the mRNA levels of hepatic Acat1 and Acat2 in all three groups. The results showed that the mRNA expressions of Acat1 and Acat2 were coordinately regulated with Acsl1 mRNA by RSV and HCHFD (Fig. 3C). Considering the functional roles of ACAT1 and ACAT2 in CE formation and the function of ACSL1 to provide the substrate acyl-CoAs for CE synthesis, the coordinated up-regulation of ACSL1 and ACAT might provide an underlying mechanism for the observed elevations of CE levels in liver and plasma of RSV-treated animals.

Identification of ACSL1 Transcript Variant 3 (C-ACSL1) as the Molecular Target of SREBP2—Previous studies of rat Acsl1 gene structure revealed three ACSL1 transcript variants derived from the Acsl1 gene through alternative splicing. These transcripts are under the control of its own promoter, and the C-promoter contained a PPRE sequence that was responsive to fibrate stimulation (17, 18). Similar to the rat ACSL1 gene, the human ACSL1 gene gives rise to three transcript variants that all encode the full-length ACSL1 protein with 698 amino acids. The three transcripts (v1, v2, and v3) contain the same coding exon but have different 5′-UTRs (Fig. 5A). To be consistent with the rat ACSL1 mRNA nomenclature, we designated human ACSL1 v1 as A-ACSL1, v2 as B-ACSL1, and v3 as C-ACSL1. Using HepG2 as a model system, we sought to identify the ACSL1 mRNA species that is regulated by the SREBP pathway.

HepG2 in triplicate dishes were treated with RSV or cholesterol for 24 h. Fig. 5B shows that cholesterol repressed and RSV stimulated the formation of mature SREBP2, resulting in the suppression and up-regulation of the protein levels of ACSL1 and LDLR, a well known SREBP2 target gene. We performed qRT-PCR using a common ACSL1 primer set that amplifies the same coding exon but have different 5′-UTRs (Fig. 5A). To be consistent with the rat ACSL1 mRNA nomenclature, we designated human ACSL1 v1 as A-ACSL1, v2 as B-ACSL1, and v3 as C-ACSL1. Using HepG2 as a model system, we sought to identify the ACSL1 mRNA species that is regulated by the SREBP pathway.

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Regulation of ACSL1 Expression by SREBP Pathway

A

Promoter A

Promoter B

Promoter C

ATG

Exon 1A

Exon 1B

Exon 1C

Exon 2

3’

DMSO

CHO

RSV

B

ACSL1

LDLR

mSREBP2

β-actin

C

Promoter C

ACSL1

SREBP2

SREBP1

LDLR

Normalized mRNA levels

(Fold of DMSO)

D

ACSL1 mRNA levels

(%) of A-ACSL1 control

ACSL1

A-ACSL1

C-ACSL1

DMSO

+CHO

+RSV

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obtained from liver tissues of hamsters and mice. Next, we designed three sets of PCR primers targeting the 5’-UTR sequence of individual ACSL1 mRNA species and performed qRT-PCR analysis. In untreated HepG2 cells, A-ACSL1 mRNA was highly expressed; B-ACSL1 mRNA could not be detected, and C-ACSL1 mRNA was expressed at a low level of ~5% of A-ACSL1 abundance. However, the low abundance C-ACSL1 mRNA levels were dramatically changed in cells treated by cholesterol and RSV. Its mRNA levels declined more than 90% in sterol-treated cells and were increased 11.2-fold by RSV treatment (Fig. 5D). In contrast to C-ACSL1, A-ACSL1 mRNA expression was slightly reduced by cholesterol treatment, and it was not up-regulated by RSV. We repeated the qRT-PCR assays using another set of exon-specific primers to individually amplify each transcript variant and obtained identical results. These results provided the first evidence to demonstrate the specific regulation of the C-ACSL1 transcript by the SREBP pathway in hepatic cells.

Transactivation of ACSL1 Promoter C Activity by SREBP2—To further demonstrate the regulation of C-ACSL1 mRNA levels by SREBP pathway at the transcriptional level, we analyzed the nucleotide sequence within the 2.5-kb 5’-flanking sequence of exon 1C of the human ACSL1 gene and found one consensus SRE site along with several Sp1, E-box, and PPRE motifs immediately upstream of the 5’-end of exon 1C. The SRE site, 5’-GTCACCCCAC-3’ (5’-GTCACCCCAC-3’ in the complementary strand), contains sequences identical to those of the SRE-1 motif found in the LDLR promoter and is located at positions −7 to +1, relative to the 5’-end of exon 1C (Fig. 6A). Using HepG2 genomic DNA, we cloned the 2.5-kb DNA fragment covering the human C-ACSL1 proximal promoter region from −2215 to +300 relative to the 5’-end of exon 1C to pGL3-basic luciferase (Luc) vector to generate Promoter C-Luc reporters (promoter C) with SRE-1 intact (WT) or mutated. The ACSL1 promoter C constructs along with a LDLR promoter reporter (pLDLR234) were transfected into HepG2 cells and treated with RSV or cholesterol. Fig. 6B shows that the wild-type ACSL1 promoter C activity was strongly induced by RSV and was repressed by cholesterol to extents similar to the changes in LDLR promoter activity. Mutation of the SRE site in promoter C luciferase constructs rendered the promoter C totally unresponsive to either RSV or cholesterol treatment. These results strongly suggest that the SRE site mediates the cholesterol and RSV-dependent regulation of the ACSL1 promoter C activity.

Next, we co-transfected promoter C wild-type and SRE mutated constructs with plasmid pCMV-His-nSREBP2, encoding the His-tagged active nuclear form of SREBP2 (Fig. 6C). In this set of experiments, again we included pGL3-basic and LDLR promoter reporters as negative and positive controls. Compared with the mock transfection control (pCMV-His), luciferase activities of promoter C WT plasmid and pLDLR234 were increased 42- and 31-fold in HepG2 cells overexpressing nSREBP2. In contrast, promoter C mutant constructs did not respond to SREBP2 overexpression at all.

To determine whether SREBP2 could bind directly to the SRE site in the ACSL1 promoter C, first we used purified recombinant nSREBP2 protein to perform EMSA. Fig. 6D shows the result of EMSA that detected a single shifted band responding to SREBP2 overexpression at all. In contrast, promoter C mutant constructs did not respond to SREBP2 overexpression at all.

To further examine the in vivo interaction of SREBP2 with the ACSL1 promoter C, we performed a ChIP assay in HepG2 cells. Because the anti-SREBP2 antibody does not work in an
immunoprecipitation assay, we expressed the His-nSREBP2 in HepG2 cells and used anti-His antibody to immunoprecipitate the SREBP2-bound chromatin. The empty vector pCMV-His-transfected HepG2 cells were included in this experiment as the mock control. Isolated DNA fragments were used as templates to perform the PCR with one set of primers that amplify a 181-bp fragment of the human ACSL1 promoter C region from -116 to +65, encompassing the SRE site, and another set of primers to amplify a 147-bp fragment within the promoter A region of the human ACSL1 gene as a negative control. Fig. 6E shows that a strong binding signal of His-nSREBP2 to ACSL1 promoter C was detected using immunoprecipitation materials obtained from pCMV-His-nSREBP2-transfected cells, but very little background binding signal was detected using the ChIP materials of the empty vector-transfected HepG2 cells (pCMV-His). Furthermore, this strong binding was not observed in the promoter A region of the ACSL1 gene. We also did not observe specific signals using a control antibody (anti-IgG). These
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![Graph A](image)

**FIGURE 7.** siRNA-mediated knockdown of SREBP2 decreases ACSL1 mRNA and protein levels in HepG2 cells. SREBP2 siRNA and scrambled siRNA were transfected into HepG2 cells in 6-well plates with duplicate wells per treatment condition. Two days after transfection, whole protein lysates and total RNA were isolated, and cDNA was synthesized from total RNA. A, hepatic mRNA levels of SREBP2, ACSL1, and GAPDH were assessed by qRT-PCR using specific primers with triplicate measurement of each cDNA sample. After normalization with GAPDH mRNA levels, the relative levels are presented. The data shown are the summarized results of duplicate wells. Error bars, S.E. B, Western blotting was performed using the indicated antibodies after SREBP2 siRNA or control siRNA transfection. Results are representative of two independent transfections with similar results.

Results demonstrated the direct binding of SREBP2 to the ACSL1 promoter C under in vivo conditions. Altogether, these results provided a molecular basis for increased mRNA levels of C-ACSL1 transcript by RSV treatment and further demonstrate that C-ACSL1 gene transcription in liver cells is activated by SREBP2 through the SRE site of promoter C.

Finally, we examined the functional role of SREBP2 in ACSL1 expression by transfecting HepG2 cells with a SREBP2 siRNA or a control nonspecific siRNA. Transfection of SREBP2 siRNA resulted in a marked decrease in SREBP2 mRNA levels (~67%) and also lowered total ACSL1 mRNA levels by 30% in HepG2 cells (Fig. 7A). Consistent with the quantitative PCR results, the protein levels of SREBP2 and ACSL1 were all reduced in SREBP2 siRNA-transfected cells as compared with the cells transfected with the scrambled control siRNA (Fig. 7B).

**Discussion**

Abnormality in LCFA metabolism is increasingly recognized as an underlying causal factor for cardiovascular diseases, type II diabetes mellitus, insulin resistance, obesity, nonalcoholic fatty liver disease, and metabolic syndrome (27, 28). Because ACSL1 is the dominant isoform of the ACSL family in a majority of metabolic tissues, variations in ACSL1 levels of expression and activity will inevitably impact the LCFA metabolism at cellular levels and at the whole body level. In particular, changes in the upstream signaling pathway and transcriptional networks that govern ACSL1 expression might influence the entry of fatty acyl-CoAs into different metabolic pathways.

Previous studies have demonstrated a primary role of PPARα in FA β-oxidation (29, 30). Thus, it is conceivable that activation of PPARα led to the increased expression of ACSL1 to provide more conjugated acyl-CoAs to be utilized as fuels through the FA oxidative pathway. On the other hand, several in vitro studies have demonstrated the activity of ACSL1 in promoting TG and phospholipid synthesis (11). Until this current study, it is unknown whether the activity of ACSL1 in promoting the entry of FA into these anabolic pathways is regulated by different upstream signals that do not involve the transcriptional activation of PPARα.

We set out in this study to identify transcriptional networks that might regulate ACSL1 expression under hyperlipidemic conditions with excessive amounts of LCFA and dietary cholesterol. By profiling Acsl1 mRNA and protein expressions in liver tissues of hamsters fed a cholesterol-enriched HCHFD, an HFD, and a normal diet, our studies revealed a strong inhibitory effect of dietary cholesterol on hepatic Acsl1 mRNA and protein expressions; however, there still remains the possibility that different fat compositions between these diets could also be an influence on Acsl1 expression. A previous study using adenovirus-mediated overexpression of SREBP1 has shown a positive effect of SREBP1c on Acsl5 expression in livers of diabetic mice (11). In our study, we did not observe a correlation of SREBP1 with Acsl1 in the mRNA level in vivo or in hepatic cells. Instead, we found a strong correlation of down-regulation of Acsl1 with reduced SREBP2 in the liver of hamsters.

We further confirm the correlation of Acsl1 regulation by SREBP2 in C57BL/6j mice in which HCHFD dramatically suppressed Acsl1 and SREBP2 expression. In addition, Acsl1 specific activity was also reduced significantly in livers of hamsters and mice. To seek additional evidence to support the role of SREBP2 in Acsl1 expression, we examined Acsl1 expression in liver tissues of hamsters and mice that were treated with RSV, the activator of the SREBP pathway. Compared with the vehicle group, Acsl1 mRNA and protein were both elevated in RSV-treated liver samples. These results from two different rodent models strongly suggest a positive role of SREBP2 in Acsl1 gene transcription.

The above new findings brought up an important question as to which Acsl1 transcript is the target of SREBP pathway. In rats, it has been demonstrated that the Acsl1 gene generates three transcripts (A-ACSL1, B-ACSL1, and C-ACSL1) by alternative transcription from three different promoters. The C-promoter was shown to be highly inducible by the PPARα agonist fenofibrate in rat liver. To identify the Acsl1 transcript variants that are responsive to SREBP activation, we decided to use HepG2 cells as a model system because in HepG2 cells, Acsl1 mRNA and protein levels were repressed by sterols and induced by RSV to degrees similar to what we have observed in liver tissues. Our analysis of human Acsl1 gene structure revealed three transcripts (v1, v2, and v3) that contain the same exon 2 but different first exons that are preceded by specific 5’-upstream regulatory sequences. We named these three...
variants A-ACSL1, B-ACSL1, and C-ACSL1. By conducting qRT-PCR with variant-specific primers (Fig. 5D), we demonstrated that A-ACSL1 is highly abundant in HepG2 cells and attributes to ~95% of total ACSL1 mRNA species in HepG2 cells under uninduced conditions. Transcript B-ACSL1 could not be detected in HepG2 cells, and transcript C-ACSL1 was expressed at a low level, ~5% of the A-ACSL1 mRNA amount. However, in RSV-treated HepG2 cells, mRNA levels of A-ACSL1 did not change, but C-ACSL1 mRNA abundance increased >10-fold, which probably accounted for the ~2-fold increase in total ACSL1 mRNA levels when measured using the primers that recognize all ACSL1 transcripts.

Our sequence analysis of the 5′-flanking region of human C-ACSL1 promoter revealed a single SRE site adjacent to the 5′-end of exon 1C. The role of the SRE site in the sterol-dep-endent regulation of the ACSL1 promoter C was supported by the observation that in HepG2 cells, treatment with RSV and over-expression of the nuclear forms of SREBP-2 significantly activated the ACSL1 promoter C activities, whereas mutation in SRE resulted in a complete loss of activation by SREBP2 and statin treatment. By performing EMSA and ChIP assays, we provided direct evidence for the interaction of SREBP2 with the SRE site in the ACSL1 promoter C specifically. Finally, by utilizing siRNA targeting SREBP2, we provided functional evidence that depletion of endogenous SREBP2 led to direct inhibition of ACSL1 C expression.

Although more comprehensive lipidomic analysis is needed in future studies in order to thoroughly determine the outcomes of ACSL1-produced fatty acyl-CoAs under SREBP-activated (RSV treatment) and repressed (HCHFD feeding) conditions, in the current mice study, we have detected a strong association of higher ACSL enzyme activity with increased CE and decreased free cholesterol in liver as well as in plasma of RSV-treated animals (Fig. 4). Because SREBP2 is a master transcription activator for many genes encoding enzymes in the cholesterol de novo biosynthetic pathway, the transcriptional activation of ACSL1 by SREBP2 through a SRE site embedded in the C-ACSL1 gene promoter could provide a molecular mechanism to explain the function of ACSL1 in channelling acyl-CoAs into an anabolic pathway including cholesterol ester synthesis.

Statins have been the cornerstone to treat hypercholesterolemia to reduce the risk of heart disease. The identification of ACSL1 as a new molecular target of SREBP2 may unravel a new aspect of statin efficacy, a potential benefit of statin therapy beyond the LDL cholesterol lowering.

Author Contributions—A. B. S. performed in vitro and in vivo experiments and contributed to the manuscript writing. C. F. K. performed enzyme assays and performed in vivo experiments. B. D. conducted the statin treatment study in hamsters. J. W. L. conceived and coordinated the study and wrote the paper. All authors reviewed the results and approved the final version of the manuscript.

References
1. Soupene, E., and Kuypers, F. A. (2008) Mammalian long-chain acyl-CoA synthetases. Exp. Biol. Med. (Maywood) 233, 507–521
2. Lopes-Marques, M., Cunha, J., Reis-Henriques, M. A., Santos, M. M., and Castro, L. F. (2013) Diversity and history of the long-chain acyl-CoA synthetase (Acs) gene family in vertebrates. BMC. Evol. Biol. 13, 271
3. Currie, E., Schulze, A., Zechner, R., Walther, T. C., and Farre, R. V., Jr. (2013) Cellular fatty acid metabolism and cancer. Cell Metab. 18, 153–161
4. Ellis, J. M., Frahm, J. L., Li, L. O., and Coleman, R. A. (2010) Acyl-coenzyme A synthetases in metabolic control. Curr. Opin. Lipidol. 21, 212–217
5. Coleman, R. A., Lewin, T. M., Van Horn, C. G., and Gonzalez-Baro, M. R. (2002) Do acyl-coA synthetases regulate fatty acid entry into synthetic versus degradative pathways? J. Nutr. 132, 2123–2126
6. Watkins, P. A., and Ellis, J. M. (2012) Peroxisomal acyl-CoA synthetases. Biochim. Biophys. Acta 1822, 1411–1420
7. Grevengoo, T. J., Klett, E. L., and Coleman, R. A. (2014) Acyl-CoA metabolism and partitioning. Annu. Rev. Nutr. 34, 1–30
8. Yan, S., Yang, X. F., Liu, H. L., Fu, N., Ouyang, Y., and Qing, K. (2015) Long-chain acyl-CoA synthetase in fatty acid metabolism involved in liver and other diseases: an update. World J. Gastroenterol. 21, 3492–3498
9. Mashek, D. G., Li, L. O., and Coleman, R. A. (2006) Rat long-chain acyl-CoA synthetase mRNA, protein, and activity vary in tissue distribution and in response to diet. J. Lipid Res. 47, 2094–2100
10. Suzuki, H., Kawarabayasi, Y., Kondo, J., Abe, T., Nishikawa, K., Kimura, S., Hashimoto, T., and Yamamoto, T. (1990) Structure and regulation of rat long-chain acyl-CoA synthetase. J. Biol. Chem. 265, 8681–8685
11. Achouri, Y., Hegarty, B. D., Allanic, D., Bécard, D., Hainault, L., Frèru, P., and Foufelle, F. (2005) Long chain fatty acyl-CoA synthetase 5 expression is induced by insulin and glucose: involvement of sterol regulatory element-binding protein-1c. Biochimie 87, 1149–1155
12. Deleted in proof
13. Li, L. O., Mashek, D. G., An, J., Doughman, S. D., Newgard, C. B., and Coleman, R. A. (2006) Overexpression of rat long chain acyl-CoA synthetase 1 alters fatty acid metabolism in rat primary hepatocytes. J. Biol. Chem. 281, 37246–37255
14. Ellis, J. M., Mentock, S. M., Depetillo, M. A., Koves, T. R., Sen, S., Watkins, S. M., Muoio, D. M., Cline, G. W., Taegtmeyer, H., Shulman, G. I., Willis, M. S., and Coleman, R. A. (2011) Mouse cardiac acyl coenzyme A synthetase 1 deficiency impairs Fatty Acid oxidation and induces cardiac hypertrophy. Mol. Cell Biol. 31, 1252–1262
15. Ellis, J. M., Li, L. O., Wu, P. C., Koves, T. R., Ilkayeva, O., Stevens, R. D., Watkins, S. M., Muoio, D. M., and Coleman, R. A. (2010) Adipose acyl-CoA synthetase-1 directs fatty acids toward β-oxidation and is required for cold thermogenesis. Cell Metab. 12, 53–64
16. Li, L. O., Grevengoed, T. J., Paul, D. S., Ilkayeva, O., Koves, T. R., Pascual, F., Newgard, C. B., Muoio, D. M., and Coleman, R. A. (2015) Compart-mentalized acyl-CoA metabolism in skeletal muscle regulates systemic glucose homeostasis. Diabetes 64, 23–35
17. Suzuki, H., Watanabe, M., Fujino, T., and Yamamoto, T. (1995) Multiple promoters in rat acyl-CoA synthetase gene mediate differential expres-sion of multiple transcripts with 5′-flanking heterogeneity. J. Biol. Chem. 270, 9676–9682
18. Schoonjans, K., Watanabe, M., Suzuki, H., Mahfoudi, A., Krey, G., Wahl, W., Grimoldi, P., Staels, B., Yamamoto, T., and Auwerx, J. (1995) Induction of the acyl-coenzyme A synthetase gene by fibrates and fatty acids is mediated by a peroxisome proliferator response element in the C promoter. J. Biol. Chem. 270, 19269–19276
19. Horton, J. D., Goldstein, J. L., and Brown, M. S. (2002) SREBP: activators of the complete program of cholesterol and fatty acid synthesis in the liver. J. Clin. Invest. 109, 1125–1131
20. Hua, X., Yokoyama, C., Wu, J., Briggs, M. R., Brown, M. S., Goldstein, J. L., and Wang, X. (1993) SREBP-2, a second basic-helix-loop-helix-leucine zipper protein that stimulates transcription by binding to a sterol regulatory ele-ment. Proc. Natl. Acad. Sci. U.S.A. 90, 11603–11607
21. Brown, M. S., and Goldstein, J. L. (1997) The SREBP pathway: regulation of cholesterol metabolism by proteolysis of the membrane bound transcription factor. Cell 89, 331–340
22. Brown, M. S., and Goldstein, J. L. (1999) A proteolytic pathway that controls the cholesterol content of membranes, cells, and blood. Proc. Natl. Acad. Sci. U.S.A. 96, 11041–11048
23. Eberlé, D., Hegarty, B., Bossard, P., Ferré, P., and Foufelle, F. (2004) SREBP transcription factors: master regulation of lipid homeostasis. Biochimie 86, 839–848
Regulation of ACSL1 Expression by SREBP Pathway

24. Dong, B., Kan, C. F., Singh, A. B., and Liu, J. (2013) High-fructose diet downregulates long-chain acyl-CoA synthetase 3 expression in liver of hamsters via impairing LXR/RXR signaling pathway. J. Lipid Res. 54, 1241–1254

25. Wang, Y. L., Guo, W., Zang, Y., Yaney, G. C., Vallega, G., Getty-Kaushik, L., Pilch, P., Kandror, K., and Corkey, B. E. (2004) Acyl coenzyme A synthetase regulation: putative role in long-chain acyl coenzyme A partitioning. Obes. Res. 12, 1781–1788

26. Dong, B., Wu, M., Li, H., Kraemer, F. B., Adeli, K., Seidah, N. G., Park, S. W., and Liu, J. (2010) Strong induction of PCSK9 gene expression through HNF1α and SREBP2: mechanism for the resistance to LDL-cholesterol lowering effect of statins in dyslipidemic hamsters. J. Lipid Res. 51, 1486–1495

27. Ruderman, N. B., Saha, A. K., Vavvas, D., and Witters, L. A. (1999) Malonyl-CoA, fuel sensing, and insulin resistance. Am. J. Physiol. 276, E1–E18

28. Scorletti, E., and Byrne, C. D. (2013) Omega-3 fatty acids, hepatic lipid metabolism, and nonalcoholic fatty liver disease. Annu. Rev. Nutr. 33, 231–248

29. Burri, L., Thoresen, G. H., and Berge, R. K. (2010) The role of PPARα activation in liver and muscle. PPAR Res. 10.1155/2010/542359

30. Glosli, H., Gudbrandsen, O. A., Mullen, A. J., Halvorsen, B., Røst, T. H., Wergedahl, H., Prydz, H., Aukrust, P., and Berge, R. K. (2005) Down-regulated expression of PPARα target genes, reduced fatty acid oxidation and altered fatty acid composition in the liver of mice transgenic for hTNFα. Biochim. Biophys. Acta 1734, 235–246