MOLECULAR AND CELLULAR BIOLOGY, Sept. 2006, p. 6786–6798 Vol. 26, No. 18
0270-7306/06/$08.00+0 doi:10.1128/MCB.00077-06

Stearoyl-Coenzyme A Desaturase 1 Deficiency Protects against Hypertriglyceridemia and Increases Plasma High-Density Lipoprotein Cholesterol Induced by Liver X Receptor Activation

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Received 12 January 2006/Returned for modification 16 February 2006/Accepted 29 June 2006

Stearoyl-coenzyme A desaturase (SCD) is the rate-limiting enzyme necessary for the biosynthesis of monounsaturated fatty acids. In this study, we investigated the regulation of mouse SCD1 by liver X receptor (LXR) and its role in plasma lipoprotein metabolism upon LXR activation. In vivo, the SCD1 gene remained induced upon LXR activation in the absence of sterol regulatory element-binding protein 1c (SREBP-1c), a known transcriptional regulator of SCD1. Serial deletion and point mutation analyses in reporter gene assays, as well as a gel mobility shift assay, identified an LXR response element in the mouse SCD1 promoter. In addition, SCD1 deficiency prevented the hypertriglyceridemic effect and reduced hepatic triglyceride accumulation associated with LXR activation despite induced hepatic expression of SREBP-1c protein and several SREBP1c and LXR target genes involved in lipoprotein metabolism. Unlike wild-type mice, SCD1-deficient mice failed to elevate the hepatic triglyceride monounsaturated acid (MUFA)/saturated fatty acid (SFA) ratio despite induction of the SCD2 gene. Together, these findings suggest that SCD1 plays a pivotal role in the regulation of hepatic and plasma triglyceride accumulation, possibly by modulating the MUFA-to-SFA ratio. In addition, SCD1 deficiency also increased plasma high-density lipoprotein cholesterol levels induced by LXR activation.

Stearoyl-coenzyme A (CoA) desaturase (SCD) is a central lipogenic enzyme that catalyzes the Δ9-*cis* desaturation of saturated fatty acyl-CoAs used in the biosynthesis of monounsaturated fatty acids (MUFAs). Its major substrates are palmitoyl- and stearoyl-CoA, which are converted to palmitoleoyl- and oleoyl-CoA, respectively (7). Four SCD isoforms have been found in mice (19, 31, 37, 62), and two have been characterized in humans (2, 60). Palmitoleate ($C_{16:1}$) and oleate ($C_{18:1}$) constitute the major MUFAs in membrane phospholipids, triglycerides (TGs), and cholesterol esters. An improper ratio of saturated to monounsaturated fatty acids in these lipids may affect membrane fluidity and lipoprotein metabolism and thus has been implicated in various disease states, including diabetes, atherosclerosis, cancer, and obesity (36, 38, 39).

Recent studies of a mouse model with a targeted disruption in the SCD1 gene have provided evidence that SCD1 plays an important role in lipid homeostasis and lipoprotein metabolism. SCD1-deficient ($SCD1^{-/-}$) mice showed reduced synthesis of lipids, especially triglycerides (32–35), resistance to diet-induced weight gain, and reduced leptin deficiency-induced obesity (6, 40). In addition, $SCD1^{-/-}$ mice have low levels of triglycerides in very-low-density lipoprotein (VLDL) (1, 6, 34). In human as well as in mouse models, the desaturation index (plasma, 18:1/18:0 ratio), a marker for SCD activity, is strongly correlated with plasma triglyceride levels (1). Therefore, SCD1 may be a potential target for lowering triglyceride levels.

Activated by oxysterols, liver X receptor α (LXRα) and LXRβ belong to the nuclear hormone receptor superfamily that is involved in the regulation of cholesterol and lipid homeostasis in multiple tissues, including liver, intestine, and macrophages (41). Studies have implicated LXRs in the development of metabolic disorders and the pathogenesis of atherosclerosis (18, 22). LXR agonists, such as T0901317, were shown to be effective antiatherogenic agents by increasing high-density lipoprotein (HDL) cholesterol levels and promoting reverse cholesterol transport (RCT) (8, 43, 54, 55). However, T0901317-induced LXR activation led to undesirable side effects, specifically hypertriglyceridemia and hepatic steatosis (17). This accumulation of lipids is explained by the increase in the expression of sterol regulatory element-binding protein 1c (SREBP-1c) by LXR (42, 45). SREBPs are important transcription factors that regulate lipogenesis and cholesterol metabolism (13). SREBP-1a is the predominant isoform in cultured cells and a stronger activator of transcription of genes controlling lipogenesis and cholesterol synthesis (51). In vivo, SREBP-1c preferentially stimulates transcription of hepatic lipogenic genes in response to insulin and high-carbohydrate feeding, whereas SREBP-2 preferentially activates genes involved in cholesterol synthesis (47–49). The induction of SREBP-1c by LXR consequently activates the lipogenic pathway by transcriptionally activating genes involved in lipid synthesis, including the SCD1 gene.

Therefore, to further understand the role of SCD1 in triglyceride metabolism, we investigated the effect of SCD1 deficiency on the hypertriglyceridemic and liver steatotic effect of LXR activation by T0901317. First, we found the presence of an LXR response element in the SCD1 promoter and demon-

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strated that SCD1 is a direct transcriptional target of LXR. Feeding of T0901317 to SCD1$^{-/-}$ mice demonstrated that the lack of SCD1 prevented the hypertriglyceridemic effect and reduced the liver steatotic effect of LXR activation in mice. SCD1 deficiency also enhanced the ability of LXR to elevate HDL cholesterol level. This novel regulatory mechanism of SCD1 by LXR has provided us more insight into the metabolic consequences of LXR activation.

MATERIALS AND METHODS

Materials. Radioactive [^{32}P]dCTP, [^{14}C]stearoyl-CoA, and [^{14}C]palmitoyl-CoA were purchased from American Radiolabeled Chemicals (St. Louis, MO) and PerkinElmer Life Sciences (Boston, MA). Thin-layer chromatography (TLC) plates (TLC silica gel G60) were from Merck (Darmstadt, Germany). Pregnenolone 16α-carbonitrile (PCN) was purchased from Sigma-Aldrich (St. Louis, MO). SCD1 and SREBP-1 antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Mice and diets. All mice were housed in colony cages in a pathogen-free barrier facility operating a 12-h light/12-h dark cycle. The breeding and care of these animals were in accordance with the protocols approved by the Animal Care Research Committee of the University of Wisconsin—Madison. The generation of *SCD1*$^{-/-}$ mice has been described previously (35). Prebred homozygous (*SCD1*$^{-/-}$) and wild-type (WT) (*SCD1*$^{+/+}$) male mice on a pure 129/Sv background were used for LXR agonist treatment. The *SREBP-1c*$^{-/-}$ mice were purchased from the Jackson Laboratory and were bred into 129S6/SvEv background for at least six generations. At 12 weeks of age, mice were fed ad libitum a cereal-based powdered diet (Teklad 7001; Harlan Teklad) with or without 0.025% (wt/wt) LXR agonist T0901317 (Cayman Chemical, Ann Arbor, MI) for 2 days. For PXR agonist treatment, 10-week-old 129S6/SvEv male mice were treated for 28 h with either vehicle (olive oil) or PCN (100 mg/kg body weight) in olive oil. Two intraperitoneal injections were administered at 0 h and 24 h, and the mice were sacrificed 4 h after the last injection (27).

Plasmid construction. The 5′ flanking region of the mouse SCD1 gene (position −1537/+155, relative to the transcription start site) was prepared by PCR using bacterial artificial chromosome DNA as a template, a forward primer tailed with a KpnI restriction site (5′-GTGGTACCAGGCAGGCAGAAG AAAAGC GAGAAGA-3′), and a reverse primer tailed with an MluI restriction site (5′-G GACGCGTCATGATGATAGTCAGTTGCTCGCC-3′). The PCR product was digested with KpnI and MluI and subcloned into the KpnI/MluI-digested pGL3-Basic luciferase reporter vector (Promega), generating pGL3/−1537+155mSCD1. Similarly, for the construction of pGL3/−981+155mSCD1, KpnI site-tailed forward primer (5′-GTGGTACCGCACACTCAGGCCCTTTGCTTCCT-3′) and the same reverse primer used for generating pGL3/−1537+155mSCD1 were used. For pGL3/−589+81mSCD1, the plasmid was generously provided by P. A. Edwards (UCLA). LXRE-mutant-pGL3/−1537+155mSCD1 was generated using the QuikChange site-directed mutagenesis kit (Stratagene) and two pairs of 42-mer oligonucleotides containing mutations corresponding, respectively, to nucleotide −1258(A→T)/−1261(A→T)/−1263(T→A) and nucleotide −1248(T→A)/−1251(A→T)/−1253(T→A) of the DR4 element in the mouse SCD1 promoter. For the construction of pGL3p/−1537−563mSCD1 and LXRE-mutant-pGL3p/−1537−563mSCD1, pGL3/−1537+155mSCD1 and LXRE-mutant-pGL3/−1537+155mSCD1 were used as template, respectively, with KpnI site-tailed forward primer (5′-GTGGTACCAGGCAGGCAGAAG AA AAGCGAGAAGA-3′) and MluI site-tailed reverse primer (5′-GGACGCG TTGGTCTGGCGCTTAGC-3′). The PCR products were digested with KpnI and MluI and subcloned into the KpnI/MluI-digested pGL3-promoter luciferase reporter vector (Promega).

Transfections and luciferase assays. HepG2 cells were grown at 37°C in an atmosphere of 5% CO_2 in Dulbecco's modified Eagle's medium containing 25 mM glucose, 100 U/ml penicillin, and 100 μg/ml streptomycin and supplemented with 10% fetal bovine serum or 10% lipoprotein-deficient fetal bovine serum. Transient transfections of HepG2 cells were performed in triplicate in 24-well plates. Cells were transfected with 150 ng of the firefly luciferase reporter plasmids, 4 to 40 ng of receptor plasmids, and 15 ng of pRL-TK vector (Promega) for the control *Renilla* luciferase by using TransIT-LT1 transfection reagent (Mirus). Six hours after transfection, cells were incubated in Dulbecco's modified Eagle's medium containing 10% lipoprotein-deficient fetal bovine serum and vehicle (dimethyl sulfoxide [DMSO]) or 1 μM T0901317 for 24 h. Luciferase activities were measured using the Dual-Luciferase assay system (Promega). Firefly luciferase activity was divided by the *Renilla* activity to obtain a normalized value: relative luciferase unit. Expression plasmids for mouse LXRα and mouse RXRα were kindly provided by D. J. Mangelsdorf and J. Repa (University of Texas Southwestern Medical Center).

Isolation and analysis of RNA. Total RNA was isolated from liver using TRIzol reagent (Invitrogen). The isolated RNA from the livers of four to six mice in each group were pooled, and 15 μg of total RNA was separated by 1.0% agarose-2.2 M formaldehyde gel electrophoresis and transferred onto nylon membranes. After UV cross-linking, the membrane was hybridized with cDNA probes labeled with [^{32}P]dCTP by a random primer labeling kit (Promega). After being washed, the membranes were exposed to X-ray film at −80°C, and signals were quantified by densitometry. The cDNA probes for SCD2, fatty acid synthase (FAS), and glycerol-3-phosphate acyltransferase (GPAT) have been described previously (21, 32). Real-time quantitative PCR analysis was performed using an ABI 7500 fast machine (Applied Biosystems). DNase-treated total RNA (1 μg) from individual mice was used to generate cDNA using SuperScript III reverse transcriptase (Invitrogen). Equal amounts of cDNA were used and amplified with the SYBR green PCR master mix (Applied Biosystems). Levels of various mRNAs were normalized to those of cyclophilin. The real-time quantitative PCR primers used were as follows: G6PDH, 5′-GAACGCAAAGCTGA AGTGAGACT-3′ (forward) and 5′-TCATTACGCTTGCACTGTTGGT-3′ (reverse); PGC-1b, 5′-CCTCCAAGTGCTGTCAGTCG-3′ (forward) and 5′-AAG GAAGTCAGTCGGGTGGG-3′ (reverse); MTTP, 5′-AAGCAGAGCGGAGA CAGAGG-3′ (forward) and 5′-GCCTTGTCCATCTGCATGC-3′ (reverse); LPL, 5′-AGACTCGCTCTCAGATGCCC-3′ (forward) and 5′-GTTGCTTGCC ATTCTCAGTCC-3′ (reverse); Angptl3, 5′-TCAGTGCCAATCGACTCACG-3′ (forward) and 5′-TGTGTGACCTTGTGGAACAGGA-3′ (reverse); PLTP, 5′-CG CTTTCTGGAACAAGAGCTG-3′ (forward) and 5′-TGTGACCCTCACGTCCG AGA-3′ (reverse); and LDLR, 5′-TCAGTGCCAATCGACTCACG-3′ (forward) and 5′-TGTGTGACCTTGTGGAACAGGA-3′ (reverse). Primer sequences for SCD1, SCD2, mouse and human SREBP-1a, mouse and human SREBP-1c, mouse SREBP-2, FAS, Cyp7A1, ATP-binding cassette G5 (ABCG5), Cyp2b10, Cyp3a11, Gsta1, Oatp2, and cyclophilin have previously been described (24, 27, 31, 51).

In vitro transcription/translation and electrophoretic mobility shift assay. Mouse LXRα and RXRα proteins were synthesized in vitro from pCMX-mLXRα and pCMX-mRXRα using the TNT quick-coupled transcription/translation system (Promega) as recommended by the manufacturer's protocol. Double-stranded oligonucleotides corresponding to the LXR response element of the mouse SCD1 gene promoter (SCD1 WT LXRE, 5′-TAAGCGTGACCAC AGGTAACCTCAACTC-3′) were ^{32}P radiolabeled with polynucleotide kinase. Protein-DNA binding assays were performed by incubating the protein and radiolabeled probes at room temperature for 20 min, followed by 15 min on ice. Salmon sperm DNA (0.5 μg/μl) was added in the binding assays to reduce nonspecific binding of labeled oligonucleotides. A 50-fold molar excess of unlabeled double-stranded SCD1wt, the mutant LXR response element (LXRE) of the mouse SCD1 gene promoter wild-type LXRE (SCD1 Mut LXRE, 5′-TAA GCGAGTCCACAGGAATCCTCAACTC-3′), or the LXRE of SREBP-1c (SREBP-1c LXRE, 5′-ACAGTGACCGCCAGTAACCCCAGC-3′) (58) was used for the competition experiments. The DNA-protein complexes were resolved on a 5% polyacrylamide gel in 1× Tris-borate-EDTA buffer. The gels were dried and autoradiographed at −80°C.

Western blot analysis. For the immunoblotting of the SREBP-1 proteins, nuclear extracts and membrane fractions of mice livers were prepared according to the methods of Shimomura et al. (50). Proteins (30 μg) were resolved on an 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel and then transferred and immobilized onto an Immobilon-P nitrocellulose membrane. After blocking with 3% bovine serum albumin in Tris-buffered saline buffer (pH 8.0) plus Tween 20 (final concentration, 0.05%), the membrane was immunoblotted with polyclonal anti-SREBP-1, followed by anti-rabbit immunoglobulin G horseradish peroxidase conjugate. For the immunoblotting of SCD proteins, isolated membrane fractions (30 μg) as described above were resolved on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel and transferred as stated above. After blocking with 10% nonfat milk in Tris-buffered saline buffer (pH 8.0) plus Tween 20 (final concentration, 0.05%), the membrane was immunoblotted with polyclonal anti-SCD1, followed by anti-goat immunoglobulin G horseradish peroxidase conjugate. The proteins were visualized with a chemiluminescence detection system (Pierce) and quantified by densitometry.

SCD activity assay. Liver microsomes were isolated as previously described (31). Briefly, microsomes were isolated from livers by differential centrifugation and suspended in a 0.1 M potassium phosphate buffer (pH 7.2). Stearoyl-CoA desaturase activity was assayed at 23°C with 3 μM [^{14}C]stearoyl-CoA or [^{14}C]palmitoyl-CoA, 2 mM NADH, and 100 μg of microsomal protein. After 5 min of incubation, 200 μl of 2.5 M KOH in 75% ethanol was added, and the reaction mixture was saponified at 85°C for 1 h. The samples were cooled and

acidified with 280 μl of formic acid. Free fatty acids were extracted with 700 μl of hexane and separated on a 10% $AgNO_3$-impregnated TLC plate using chloroform-methanol-acetic acid-H_2O (90:8:1:0.8, vol/vol/vol/vol). The TLC plates were analyzed with Instant Imager (Packard, Meriden, CT) overnight.

Plasma lipid and lipoprotein analysis. Mice were fasted for a minimum of 4 h and sacrificed by CO_2 asphyxiation and/or cervical dislocation. Blood samples were collected aseptically by direct cardiac puncture; EDTA was added as an anticoagulant and centrifuged at 13,000 × *g* for 5 min at 4°C to collect plasma. Total plasma triglyceride and cholesterol were measured using commercially available enzymatic kits (Roche Diagnostics, Indianapolis, IN). Plasma samples for lipoprotein analysis were prepared as described previously (21). Lipoproteins were fractionated on a Superose 6 10/300 GL fast protein liquid chromatography (FPLC) column (Amersham Biosciences). Fractions (500 μl) were collected and used for total triglyceride and cholesterol measurements. The values reported are μg per fraction of total triglycerides or cholesterol.

Lipid analysis. Total lipids were extracted from liver and plasma according to the method of Bligh and Dyer (3). Briefly, tissues were homogenized and lipids were extracted with 3 ml of chloroform-methanol (2:1, vol/vol). After centrifugation, the organic phase was collected and dried under nitrogen and then dissolved in 50 μl chloroform-methanol (2:1). The lipid extracts were separated by silica gel TLC using heptane-isopropyl ether-acetic acid (60:40:3, vol/vol/vol) as a solvent system. The bands were scraped from the plates, methylated, and analyzed by gas-liquid chromatography as described previously (33).

Statistical analysis. All values are represented as means ± standard errors (SE), unless otherwise indicated. Statistical analysis was performed with Student's *t* test, with statistical significance set at a *P* value of <0.05.

RESULTS

Synthetic LXR agonist increases hepatic SCD expression in SREBP-1c$^{-/-}$ mice. Treatment of mice with a synthetic LXR agonist, T0901317, has been shown to induce expression of lipogenic genes through activation of SREBP-1c, followed by elevation of plasma and liver triglycerides (45). Therefore, the ability of LXR to regulate SCD1 has always been presumed to act through SREBP-1c. However, studies with SREBP-1c$^{-/-}$ mice have suggested the ability of LXR to regulate SCD1 in an SREBP-1c-independent manner (24), although the precise mechanism has yet to be determined. In order to elucidate the mechanism by which LXR regulates SCD1, we first investigated the ability of LXR to regulate hepatic SCD1 in an SREBP-1c-independent manner. (To note, SCD1 is also highly expressed in white adipose tissue in addition to the liver. Although adipose SCD1 may be highly induced by LXR, the physiological role of SCD1 regulation by LXR in adipose tissue remains to be investigated in another study.) Wild-type and SREBP-1c$^{-/-}$ mice were fed a diet containing the synthetic LXR agonist T0901317 for 2 days. At the end of 2 days, the level of the mature and active form of SREBP-1 protein (mSREBP-1) was robustly induced in the liver of wild-type mice upon LXR agonist treatment (Fig. 1A). However, in SREBP-1c$^{-/-}$ mice, the basal mSREBP-1 protein level was dramatically reduced compared to that of their wild-type counterpart (Fig. 1A). Upon T0901317 treatment, total SREBP1 protein level appeared to be slightly increased but comparable to that under basal conditions and highly reduced compared to that of T0901317-treated wild-type mice (Fig. 1A). This suggests that SREBP-1c, not SREBP-1a, is the major isoform responding to the LXR agonist. This was consistent with mRNA expressions of hepatic SREBP1 in which only the SREBP-1c isoform increases upon T0901317 treatment (Fig. 1B). Following the induction of mSREBP-1 protein upon LXR agonist treatment, mRNA expression levels of hepatic lipogenic genes, including the SCD1, SCD2, FAS, GPAT, and glucose-6-phosphate dehydrogenase (G6PDH) genes, were strongly induced in wild-type animals, supporting the ability of LXR to transcriptionally activate genes of lipogenesis through SREBP-1c (Fig. 1B). On the other hand, the gene expression of SREBP-2, the isoform that preferentially activates genes of cholesterol metabolism, was not affected by T0901317 treatment as expected, since the SREBP-2 gene is not an LXR target gene. In SREBP-1c$^{-/-}$ mice, strictly SREBP-1c target genes, GPAT and G6PDH genes, were not induced after T0901317 treatment, but the FAS gene, a known direct target of LXR (16), was induced (Fig. 1B). The inability of many lipogenic genes to be induced upon LXR activation is consistent with a previous study by Liang et al. (24). Despite the lack of SREBP-1c, the gene expression of the SCD1 isoform was also induced by T0901317 in the SREBP-1c$^{-/-}$ mice, which was also observed in the study by Liang et al. (24). However, the extent of induction was slightly smaller than that of their wild-type counterpart. Surprisingly, the expression of the SCD2 isoform was induced in the SREBP-1c$^{-/-}$ mice and this induction was further increased by T0901317 treatment (Fig. 1B). The expression of SCD3 and SCD4 isoforms was not induced (data not shown).

The increase in mRNA expression of lipogenic genes was accompanied by the accumulation of plasma and hepatic triglycerides. Plasma VLDL triglyceride levels were elevated in both wild-type and SREBP-1c$^{-/-}$ mice after T0901317 treatment (Fig. 1C). However, in SREBP-1c$^{-/-}$ mice, the VLDL triglyceride level after T0901317 treatment did not rise to the level of their wild-type counterpart (Fig. 1C). Figure 1D shows that the triglyceride content was elevated in the liver of T0901317-treated wild-type and SREBP-1c$^{-/-}$ mice as well, although not to the same extent as that in wild-type mice (Fig. 1D). The MUFA/saturated fatty acid (SFA) ratio (desaturation index) in total hepatic lipid increased 3.98- and 2.67-fold in wild-type and SREBP-1c$^{-/-}$ mice, respectively (Fig. 1E). Also, the MUFA-to-SFA ratio in hepatic triglycerides was increased 1.8-fold in both wild-type and SREBP-1c$^{-/-}$ mice. Together, these results suggest that in the absence of SREBP-1c, LXR can sufficiently increase MUFAs due to increased SCD gene expression in the liver.

Identification of an LXRE in the mouse SCD1 promoter. The ability of T0901317 to induce hepatic SCD1 gene expression in SREBP-1c$^{-/-}$ mice suggests that LXR can regulate SCD1 gene transcription by a mechanism that is independent of SREBP-1c. We proceeded to locate the LXR consensus DR4 element in the promoter region of SCD1 by examining the abilities of LXR and LXR ligand T0901317 to regulate the SCD1 promoter in transient transfection assays. We first generated SCD1 reporter constructs in which the luciferase reporters contained different lengths of the 5′ flanking region of the SCD1 gene, allowing the SCD1 promoter to drive the luciferase expression. These SCD1 reporter constructs were cotransfected with cytomegalovirus promoter-driven expression vectors for LXRα and retinoid X receptor α (RXRα) into HepG2 cells and treated with or without T0901317. Neither the LXR-RXR expression vectors nor T0901317 had any effect on the control pGL3 luciferase reporter plasmid (pGL3-Basic) lacking SCD1 promoter sequences (data not shown). On the other hand, coexpression of LXRα and RXRα in the presence of T0901317 led to an approximately 14.8-fold induction in

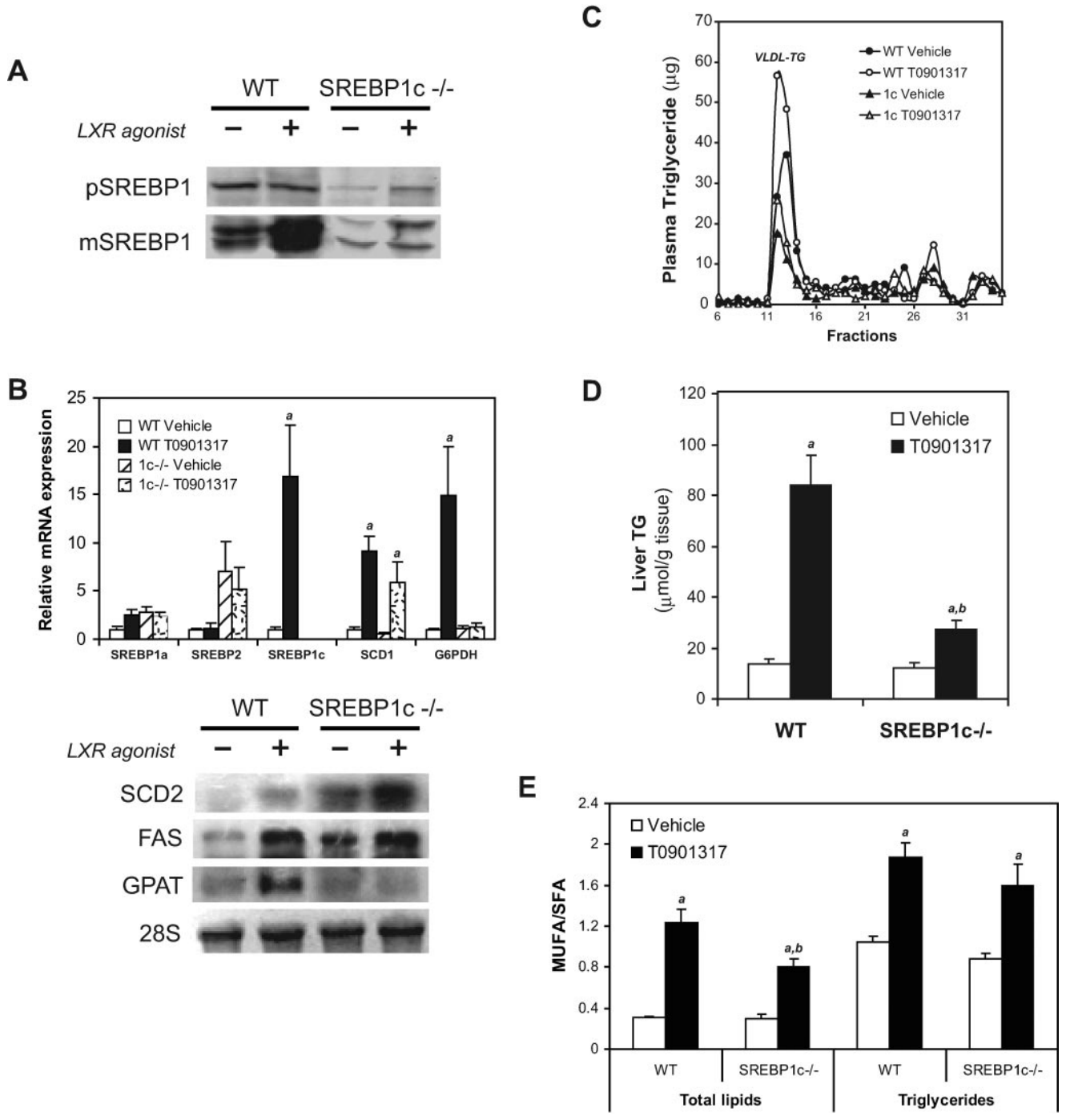
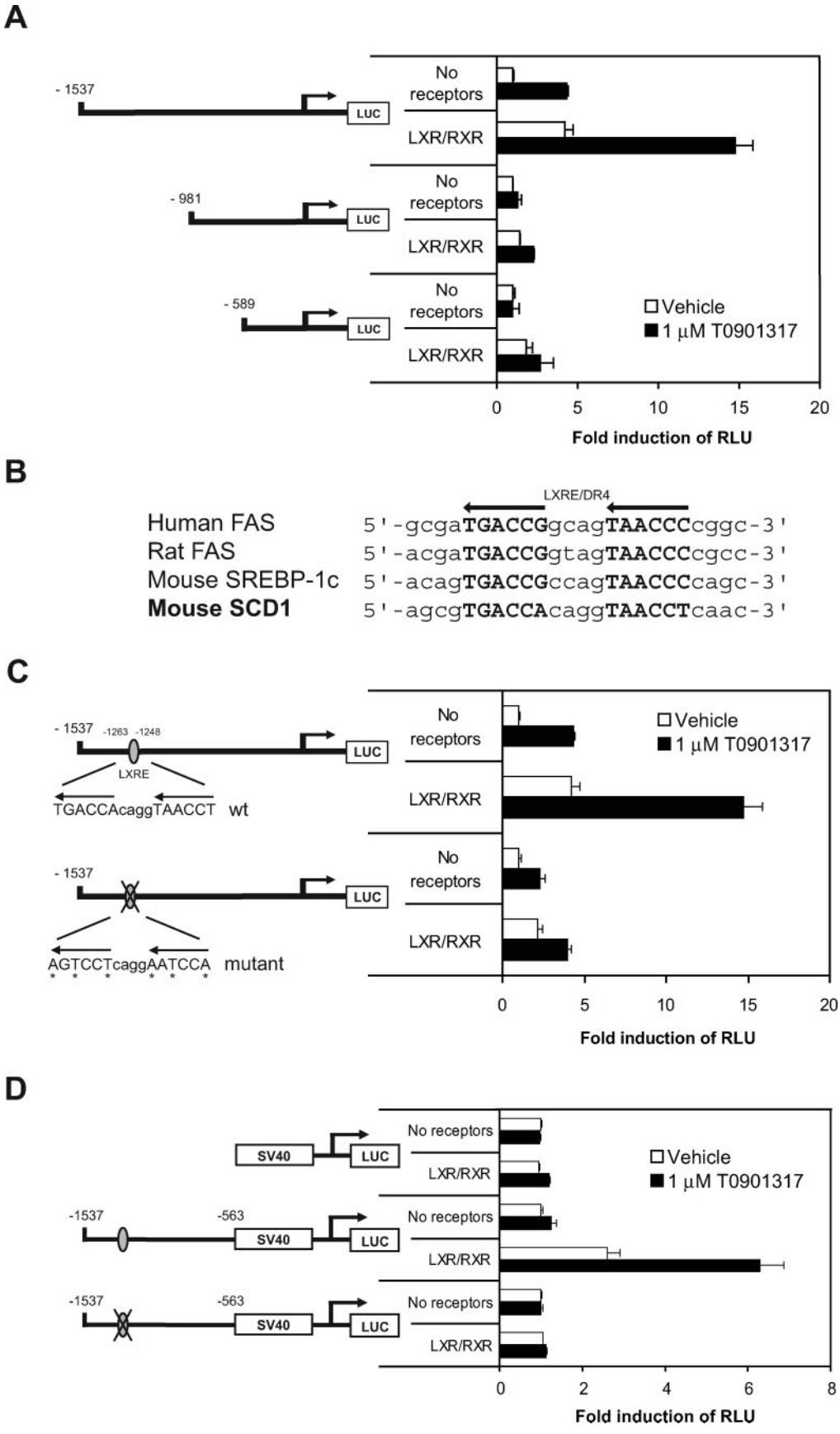


FIG. 1. In vivo regulation of SCD1 independent of SREBP-1c. Wild-type and SREBP-1c$^{-/-}$ mice (four to six mice/group) were fed a chow diet supplemented with either vehicle (DMSO) or 0.025% T0901317 for 2 days before sacrifice. (A) Nuclear and membrane proteins were isolated from the liver and subjected to immunoblot analysis with anti-SREBP1, which detects both SREBP-1a and -1c isoforms. pSREBP-1, precursor SREBP-1 protein. (B) Measurement of hepatic gene expression by real-time quantitative PCR and Northern blot analysis. (C) FPLC fractionation of plasma triglycerides. (D) Measurement of liver TG content. (E) The MUFA-to-SFA ratio was measured for hepatic triglycerides and total hepatic lipids. a, $P < 0.05$ compared with the control group; b, $P < 0.05$ compared with wild-type mice fed an LXR diet.

luciferase activity with the −1537/+155 SCD1 promoter construct (Fig. 2A). However, the T0901317-mediated increases of luciferase activity were decreased to 2.3- and 2.7-fold in −981/+155 and −589/+81 SCD1 promoter constructs, respectively. This minimal response to T0901317 in these two shorter SCD1 promoter constructs is presumably mediated through the activation by LXR of endogenous SREBP1s present in the HepG2 cells. We observed five- and twofold induction of SREBP-1a


A
- 1537
LUC
No receptors
LXR/RXR
- 981
LUC
No receptors
LXR/RXR
- 589
LUC
No receptors
LXR/RXR
Vehicle
1 μM T0901317
0
5
10
15
20
Fold induction of RLU
B
LXRE/DR4
Human FAS 5'-gcgaTGACCGgcagTAACCCcggc-3'
Rat FAS 5'-acgaTGACCGgtagTAACCCcgcc-3'
Mouse SREBP-1c 5'-acagTGACCGccagTAACCCcagc-3'
Mouse SCD1 5'-agcgTGACCAcaggTAACCTcaac-3'
C
- 1537
-1263
-1248
LUC
LXRE
TGACCAcaggTAACCT wt
- 1537
LUC
AGTCCTcaggAATCCA mutant
No receptors
LXR/RXR
No receptors
LXR/RXR
Vehicle
1 μM T0901317
0
5
10
15
20
Fold induction of RLU
D
SV40
LUC
No receptors
LXR/RXR
-1537
-563
SV40
LUC
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LXR/RXR
-1537
-563
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No receptors
LXR/RXR
Vehicle
1 μM T0901317
0
2
4
6
8
Fold induction of RLU

and SREBP-1c gene expression, respectively, upon T0901317 treatment, as well as induction in total SREBP1 protein (data not shown). Nonetheless, the T0901317-mediated induction of luciferase activity was highly maintained only in the −1537/+155 SCD1 promoter construct, suggesting that the region between positions −1537 and −981 contains regulatory sequences that mediate induction by LXR.

To further define the regulatory sequences responsible for LXR regulation, a homology search was performed using the consensus DR4 T(G/A)A(C/A)C(T/C)XXXXT(G/A)A(C/A)C(T/C) (57). Computational analysis of the mouse SCD1 promoter revealed a putative LXRE at positions −1263 to −1248 in the upstream regulatory region showing extensive homology to LXREs previously identified in mouse SREBP-1c and human and rat FAS genes (Fig. 2B) (16, 42, 58). This high conservation of the DR4 element suggested that it is likely to be an important regulatory sequence.

To definitively demonstrate that the effect of LXR on the SCD1 promoter is mediated through this putative LXRE regulatory sequence, point mutations in the LXRE were generated in the −1537/+155 SCD1 promoter construct and subjected to the same transfection assay (Fig. 2C). The mutations in the LXRE of the SCD1 promoter diminished the response to T0901317 treatment from 15-fold to 4-fold induction, similar to those of the shorter SCD1 promoter constructs that flanked the LXRE region (Fig. 2A and C). Since the minimal induction in the mutant LXRE SCD1 promoter may be due to induction in SREBP1, mutations in the SREBP response element (SRE) located at position −423/−413 were also introduced to eliminate SREBP1-mediated induction of SCD1 by T0901317. However, mutations in the SRE resulted in >90% loss of promoter activity (data not shown). To circumvent this caveat, the region between positions −1537 and −563 of the SCD1 promoter, which contains the LXRE but excludes the SRE, was cloned into the pGL3-promoter vector, where the luciferase expression is driven on its own through the simian virus 40 promoter. Figure 2D shows that the pGL3-promoter vector alone does not respond to T0901317 treatment. However, when the vector contained the region between positions −1537 and −563 of the SCD1 promoter, T0901317 treatment led to an approximately sixfold induction in luciferase activity. This induction, however, was completely abolished when mutations in the LXRE were introduced. Together, these results indicate that an LXR response element is located in the mouse SCD1 promoter between positions −1263 and −1248.

LXR-RXR complex binds to the SCD1 LXRE. To determine whether LXR binds to the SCD1 LXRE as a complex with RXR, gel mobility shift assays were performed using in vitro-translated LXRα and RXRα proteins and ^{32}P-radiolabeled oligonucleotides corresponding to the SCD1 LXRE (Fig. 3). Double-stranded radiolabeled oligonucleotides corresponding to the LXRE of the SREBP-1c promoter were used as a control (Fig. 3, lanes 1 to 3). Neither LXRα nor RXRα alone bound to the LXRE of the SCD1 promoter (Fig. 3, lanes 5 and 6), but when both were present, there was a shift in the band of radiolabeled oligonucleotides (lane 7), indicating the binding of the LXR-RXR heterodimer to the SCD1 WT LXRE. However, the addition of a 50-fold molar excess of unlabeled SREBP-1c or SCD1 WT LXRE oligonucleotides was able to compete the radiolabeled band (Fig. 3, lanes 8 and 9). On the other hand, unlabeled mutant SCD1 LXRE oligonucleotides were not able to compete the radiolabeled band (Fig. 3, lane 10). These results demonstrated the specific binding of the LXR-RXR complex to the SCD1 LXRE.

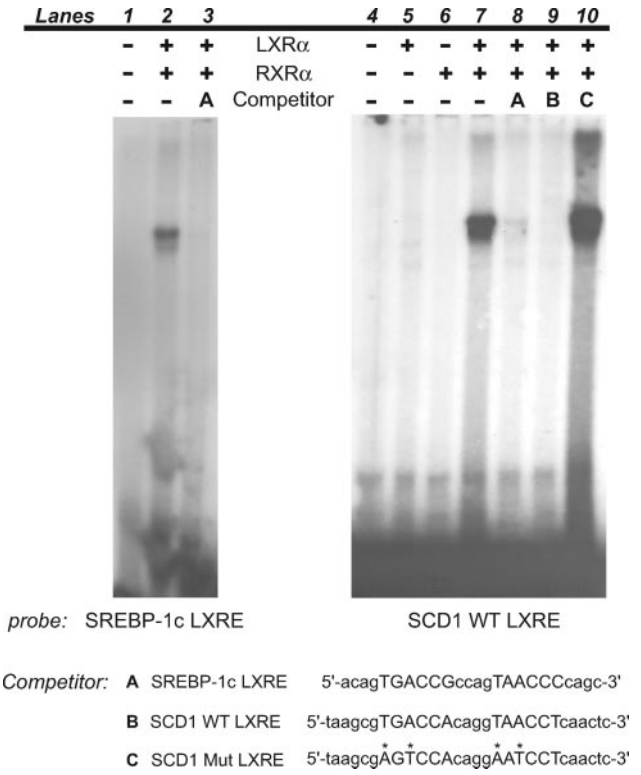

FIG. 3. LXR-RXR complex specifically binds to the mouse SCD1 DR4 element. Electrophoretic mobility shift assays were performed using ^{32}P-radiolabeled double-stranded oligonucleotides corresponding to either the LXR response element of the mouse SCD1 promoter (SCD1 WT LXRE) or the LXR response element of the mouse SREBP-1c promoter (SREBP-1c LXRE). Competitive assays were performed using a 50-fold molar excess of unlabeled oligonucleotides corresponding to the LXR response element of the mouse SREBP-1c promoter (A), the wild-type LXR response element of the SCD1 promoter (B), or the mutant LXR response element of the SCD1 promoter (C) as indicated. The asterisks indicate the specific base pairs mutated in the LXR response element of the SCD1 promoter.

FIG. 2. Mapping of LXR response element in the mouse SCD1 gene promoter. (A) Deletion analysis of the SCD1 promoter using a luciferase reporter gene assay. The schematic illustrations represent the serially deleted SCD1 luciferase reporter constructs. (B) Sequence comparison of the putative LXRE-DR4 in the promoter region of the SCD1 gene with the LXRE-DR4 in LXR target genes reported previously. (C) Mutational analysis of the SCD1 promoter pGL3/−1537+155mSCD1. The asterisk indicates the mutation made in the LXRE of the SCD1 gene promoter. (D) Mutational analysis of pGL3p/−1537−563mSCD1. Control cells were treated with 0.1% DMSO in the absence of LXR-RXR receptors. Results are expressed as the relative luciferase units (RLU) of induction (*n*-fold) over the control value for each construct and represent the means ± SE of three independent transfection experiments of duplicates.

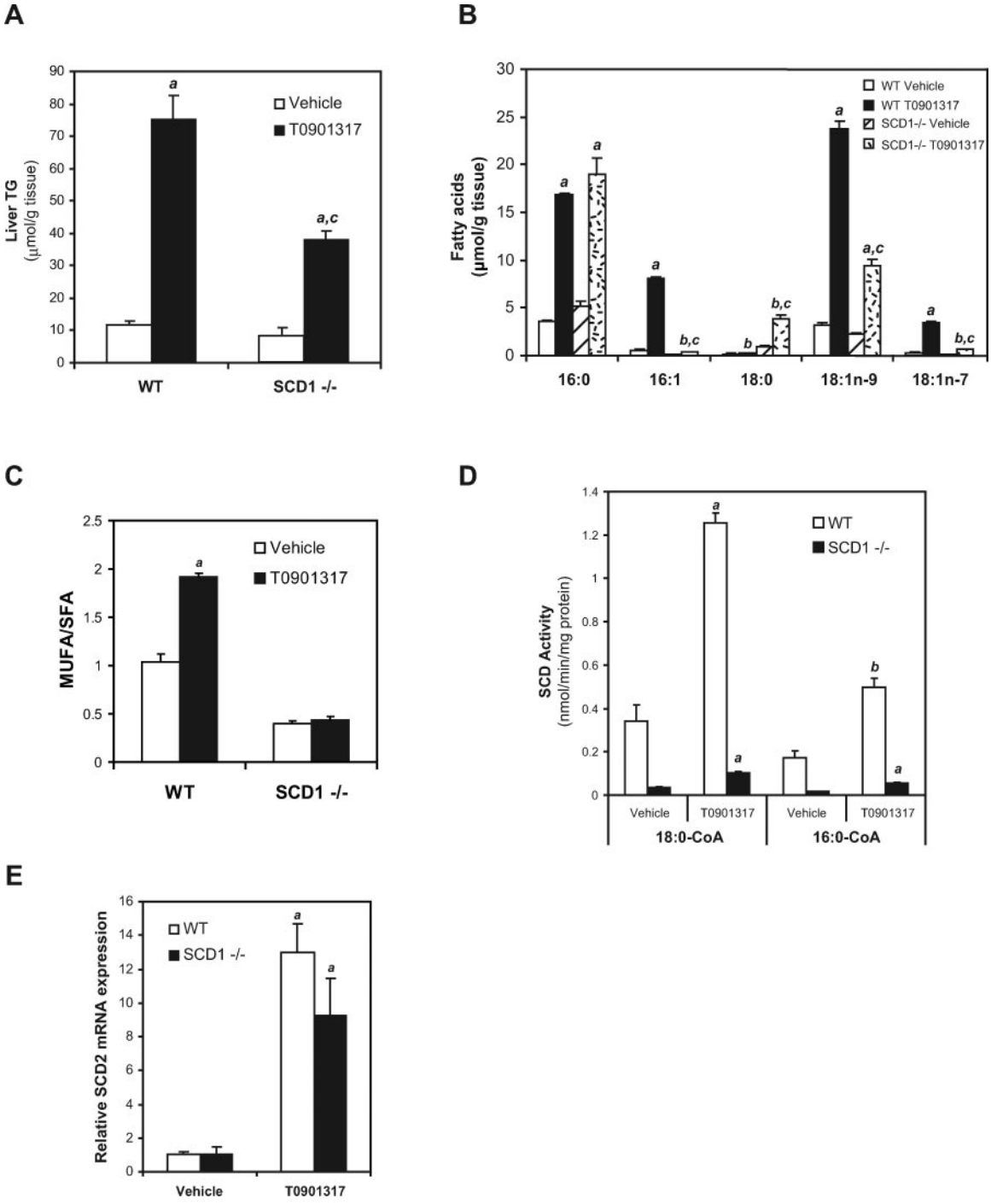

FIG. 4. Hepatic response to T0901317 treatment in wild-type and SCD1$^{-/-}$ mice. Mice (four mice/group) were fed a chow diet supplemented with either vehicle or 0.025% T0901317 for 2 days. The total content of triglyceride (A) and the fatty acid composition in hepatic triglyceride fractions (B) in the liver of wild-type and SCD1$^{-/-}$ mice were determined. (C) MUFA-to-SFA ratios in hepatic triglycerides. (D) Aliquots of microsome fractions (100 μg) from the liver of each mouse were subjected to SCD enzyme activity analysis. (E) SCD2 mRNA expression levels were determined by real-time quantitative PCR and normalized to those of cyclophilin. Each value represents the mean ± SE. a, $P < 0.01$ compared with the control group; b, $P < 0.05$ compared with the control group; c, $P < 0.01$ compared with wild-type mice fed an LXR diet.

T0901317-induced hepatic TG accumulation was reduced in SCD1$^{-/-}$ mice. To investigate the potential significance of transcriptional activation of SCD1 by direct LXR activation in vivo, we fed wild-type and SCD1$^{-/-}$ mice the LXR agonist diet for 2 days. T0901317 treatment in mice has been shown to lead to hepatic steatosis (42, 45). Indeed, when hepatic triglyceride levels in wild-type mice were measured, T0901317 treatment resulted in a 6.5-fold increase in triglyceride content (Fig. 4A).

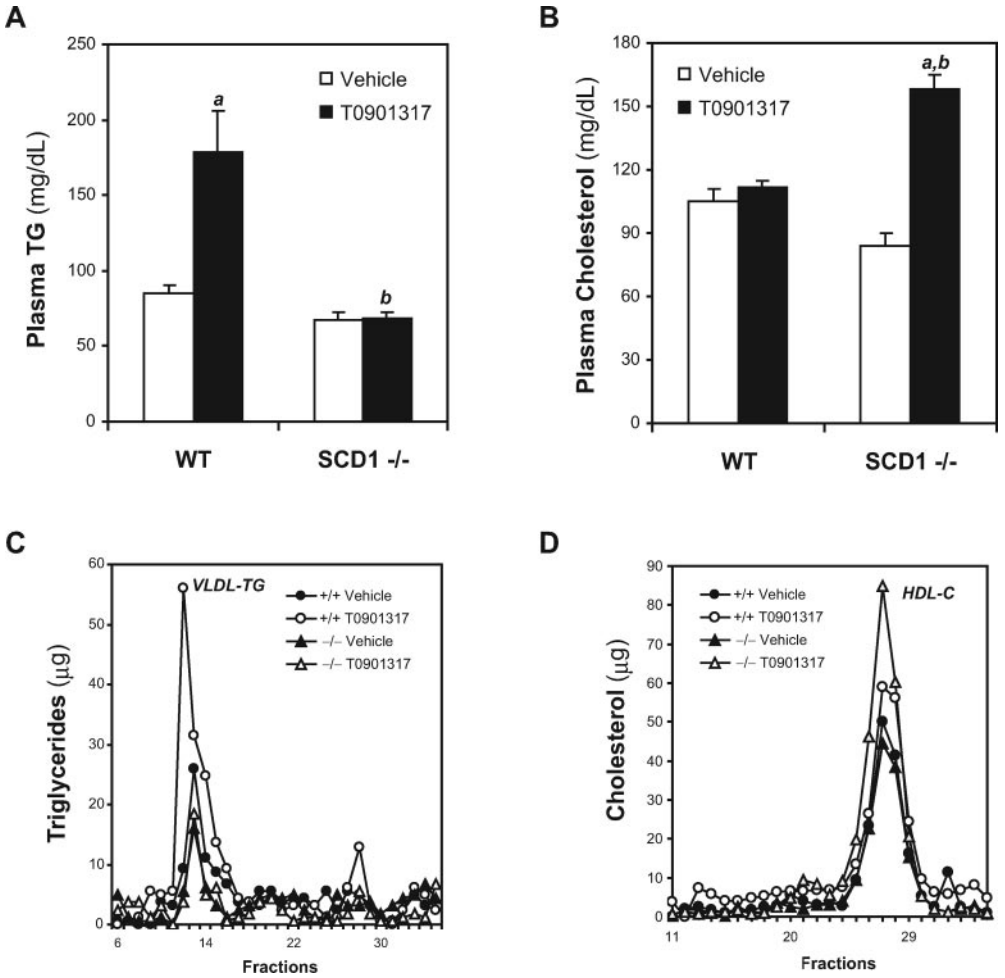

FIG. 5. Plasma lipid levels after LXR agonist T0901317 treatment for 2 days. Wild-type and SCD1$^{-/-}$ mice were fed a chow diet supplemented with either vehicle or 0.025% T0901317 for 2 days and fasted for 4 h before sacrifice. Plasma triglyceride (A) and cholesterol (B) levels were measured. FPLC fractionation of triglycerides (C) and cholesterol (D). Each data point in the lipoprotein profile represents the total triglyceride or cholesterol mass per fraction. The lipoprotein peaks for VLDL and HDL are indicated.

However, triglyceride accumulation was reduced by approximately 50% in T0901317-treated SCD1$^{-/-}$ mice (Fig. 4A). Oil Red O staining in liver tissues also confirmed reduction in hepatic lipid accumulation in T0901317-treated SCD1$^{-/-}$ mice compared to that in their wild-type counterpart (data not shown). In the hepatic triglyceride fractions of T0901317-treated SCD1$^{-/-}$ mice, we observed a 95% and 63% decrease in the levels of palmitoleic (16:1) and oleic (18:1) acids, respectively, compared to those of the T0901317-treated wild-type mice (Fig. 4B). There was a corresponding increase in SFAs, in particular stearic (18:0) acid, which increased 14.4-fold in the triglyceride fractions of T0901317-treated SCD1$^{-/-}$ livers, compared to 1.4-fold in wild-type livers. Consequently, the MUFA-to-SFA ratio was dramatically altered in T0901317-treated SCD1$^{-/-}$ mice (0.44 ± 0.04) compared to that of their wild-type counterpart (1.94 ± 0.04) (Fig. 4C).

Due to differences in the fatty acid compositions of liver triglycerides, hepatic SCD activity was measured. SCD activity was also increased 3.7- and 2.9-fold in wild-type mice when stearoyl-CoA and palmitoyl-CoA were used as substrates, respectively (Fig. 4D). Although SCD activity in the SCD1$^{-/-}$ mice was drastically reduced in the liver—0.04 ± 0.002 (18:0-CoA) and 0.02 ± 0.001 (16:0-CoA) nmol/min/mg protein—compared to that in wild-type mice under basal conditions, upon T0901317 treatment, SCD activity was increased 2.5-fold with both substrates. This increase in SCD activity in SCD1$^{-/-}$ mice is due solely to the presence of the SCD2 isoform in the liver. When SCD2 gene expression in the liver was measured, we saw a 12- and 9.3-fold induction upon T0901317 treatment in wild-type and SCD1$^{-/-}$ mice, respectively (Fig. 4E). Thus, the minimal increase in MUFA content in the triglycerides of SCD1$^{-/-}$ liver is due to SCD2.

SCD1 deficiency alters plasma lipid response to T0901317-mediated LXR activation. To further investigate the effect SCD1 deficiency may have on LXR activation, plasma lipids were measured after 2 days of T0901317 treatment. Figure 5A and B show plasma triglyceride and cholesterol concentration in T0901317-treated mice. T0901317 treatment significantly elevated total plasma triglyceride levels in wild-type mice but failed to increase these levels in SCD1$^{-/-}$ mice. This effect is also reflected in VLDL triglyceride levels, as shown in the lipoprotein profiles (Fig. 5C). HDL cholesterol levels were

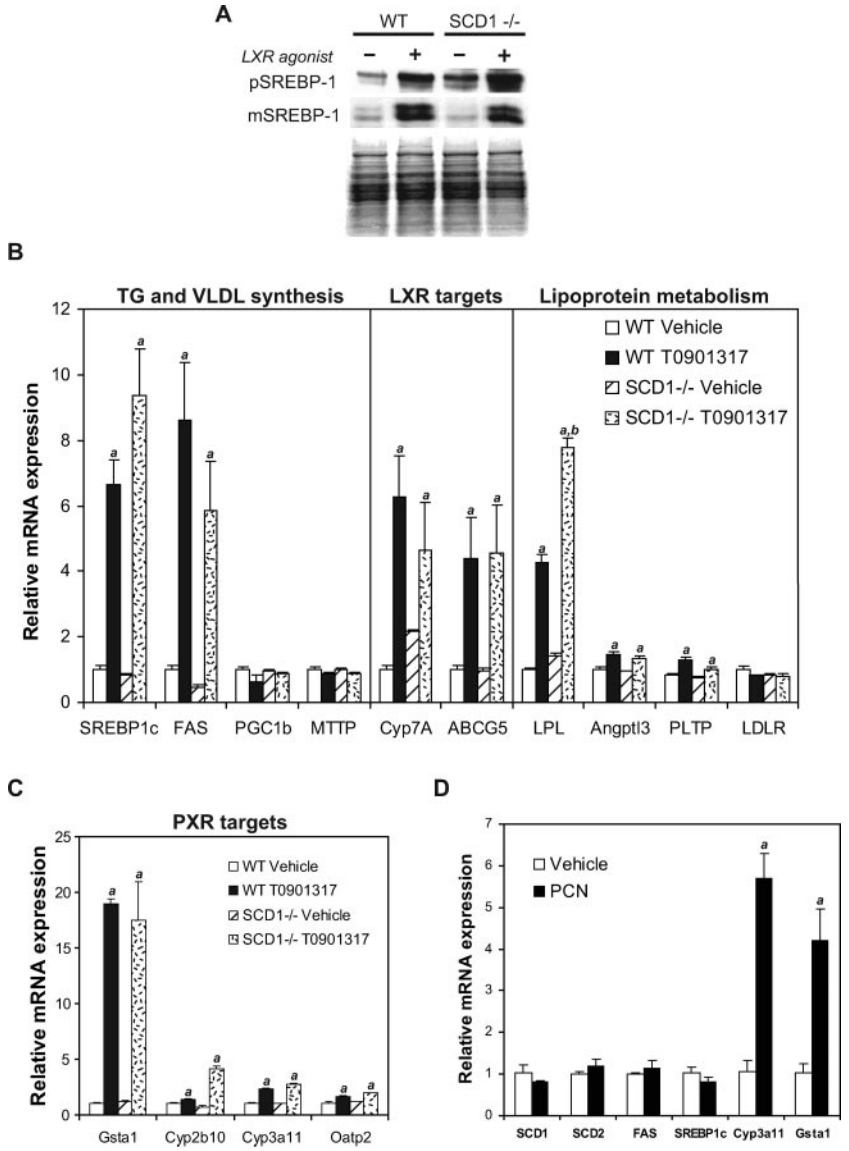

FIG. 6. Analysis of SREBP-1 protein, hepatic gene expressions, and the PXR pathway. Mice (four mice/group) were fed a chow diet supplemented with either vehicle or 0.025% T0901317 for 2 days. (A) Nuclear and membrane proteins were isolated from the liver and subjected to immunoblot analysis with antibody against mouse SREBP1 (upper panels). Ponceau S staining of the membrane used for immunodetection served as a loading control (lower panel). pSREBP-1, precursor SREBP-1 protein. (B) Measurement of various hepatic genes involved in lipid regulation by real-time quantitative PCR. (C) Measurement of PXR target genes under T0901317 treatment. (D) Measurement of hepatic gene expressions upon PCN treatment in wild-type mice. The expression levels of various genes were normalized to those of cyclophilin, with mean expression of the control wild-type vehicle group normalized to 1. Each value represents the mean ± standard deviation. a, $P < 0.01$ compared with the control group; b, $P < 0.001$ compared with wild-type mice fed an LXR diet.

increased in wild-type mice (Fig. 5D), although T0901317 treatment did not have an apparent effect on total plasma cholesterol levels, consistent with published results (4, 11, 45). In contrast, total plasma cholesterol and HDL cholesterol levels were significantly elevated (~2-fold) after T0901317 treatment in SCD1$^{-/-}$ mice.

Analysis of genes involved in lipid and lipoprotein metabolism. To explore the molecular mechanisms responsible for the effects observed on hepatic triglyceride and plasma lipids, we examined a spectrum of genes of enzymes and proteins involved in hepatic and plasma lipid modulation in the liver. One key transcription factor involved in lipogenesis that is induced by LXR activation is SREBP-1c. Upon T0901317 treatment, premature and mature SREBP-1 protein levels were robustly induced in both wild-type and SCD1$^{-/-}$ mice to comparable levels in the liver (Fig. 6A), reflecting the relative hepatic SREBP-1c mRNA expression levels, 6.64 ± 0.75 and 9.35 ± 1.42, respectively, in T0901317-treated wild-type and SCD1$^{-/-}$ mice (Fig. 6B). FAS, a downstream component of the lipogenic pathway, was also strongly induced in both wild-type and SCD1$^{-/-}$ mice upon LXR activation, contributing to the increased hepatic triglyceride synthesis. Other LXR target genes involved in lipid modulation, such as those encoding lipoprotein lipase (LPL) and angiopoietin-like protein 3 (Angptl3),

were also measured. LPL is an enzyme that hydrolyzes triglycerides on VLDL and thus participates in the clearance pathway of plasma VLDL triglyceride. Interestingly, LPL expression was higher ($P < 0.001$) in T0901317-treated SCD1$^{-/-}$ mice (7.79 ± 0.30) than in their wild-type counterpart (4.25 ± 0.27) (Fig. 6B). However, basal LPL mRNA expression was slightly higher in SCD1$^{-/-}$ mice (1.43 ± 0.05) than in wild-type mice (1.00 ± 0.03). Angptl3 is another LXR target which inhibits LPL activity, and induction of Angptl3 has been shown to contribute to the hypertriglyceridemic effect upon LXR activation (15). However, hepatic Angptl3 gene expression was induced to the same level in both wild-type and SCD1$^{-/-}$ mice upon LXR activation (Fig. 6B). Recently, an increase in peroxisome proliferator-activated receptor-gamma coactivator 1β (PGC-1β) gene expression was shown to contribute to hyperlipidemia by activating LXR and promoting VLDL triglyceride secretion (25), but PGC-1β gene expression was not altered upon T0901317 treatment in both wild-type and SCD1$^{-/-}$ mice (Fig. 6B). The gene expressions of microsomal triglyceride transfer protein and LDL receptor, which are involved in secretion and clearance of VLDL particles, respectively, were not affected by T0901317 treatment.

The phospholipid transfer protein (PLTP) gene is also an LXR target gene and is involved in HDL remodeling by mediating the transfer of phospholipids from triglyceride-rich lipoproteins to HDL and the exchange of phospholipids between HDL particles (20). This exchange in phospholipids yields small lipid-poor pre-β-HDL particles which are the preferred acceptors of cholesterol from peripheral cells (14, 44). Induction of PLTP by LXR activation plays a role in mediating the elevation of HDL cholesterol (4). We observed no change in the induction of the PLTP gene in the livers of wild-type and SCD1$^{-/-}$ mice upon LXR activation (Fig. 6B), although HDL cholesterol levels were higher in T0901317-treated SCD1$^{-/-}$ mice. The RCT pathway involves the efflux of cholesterol from peripheral tissues to the liver, the secretion of cholesterol into bile, and the excretion of sterols in feces. One mediator of RCT is cholesterol 7α-hydroxylase, which is the rate-limiting enzyme in the conversion of cholesterol to bile acids and is strongly induced upon LXR activation (23). ABCG5 is also another LXR target that mediates the final step in the RCT pathway by increasing sterol excretion (59). We observe that upon LXR activation, both cholesterol 7α-hydroxylase and ABCG5 gene expressions are induced similarly in both wild-type and SCD1$^{-/-}$ mice (Fig. 6B). Taken together, upon examining expression of a battery of genes to elucidate the molecular mechanisms responsible for the decrease in VLDL triglyceride levels and the increase in HDL cholesterol levels in SCD1$^{-/-}$ mice upon LXR activation, the results do not explain the phenotype observed in SCD1$^{-/-}$ mice.

Analysis of PXR activation due to T0901317. The dose at which T0901317 was used in the study has been shown to accumulate in the liver at concentrations sufficient to activate pregnane X receptor (PXR) (46). To determine whether the effects of T0901317 on SCD1 may be mediated through PXR as opposed to, or in addition to, LXR, several hepatic PXR target genes were measured under LXR activation. The gene expressions of glutathione *S*-transferase α1 (Gsta1), cytochrome P450 2b10 (Cyp2b10), cytochrome P450 3a11 (Cyp3a11), and organic anion transporting peptide 2 (Oatp2) were all induced upon T0901317 treatment in both wild-type and SCD1$^{-/-}$ mice, suggesting activation of the PXR pathway under the LXR-activated condition in this study (Fig. 6C). Recently, the SCD1 gene has been shown to be induced in livers of transgenic mice overexpressing human PXR (63). Therefore, to determine whether the observed induction of SCD1 is due in part to PXR activation, wild-type mice were treated with PXR-specific agonist PCN. However, in our study, neither the SCD1 nor the SCD2 gene was induced upon PCN treatment (Fig. 6D). Other genes, such as those encoding FAS, SREBP-1c, Cyp3a11, and Gsta1, responded as expected, which was consistent with published results (63). Also, sequence analysis of the −1537/+155 SCD1 promoter did not suggest the presence of a DR3 element. Therefore, in our study, the inability of PCN to induce the SCD1 gene suggests that the contribution of the observed PXR activation to the effect of T0901317 on SCD1 is minimal.

DISCUSSION

Many studies have demonstrated that the primary mechanism by which LXR regulates lipogenic genes is via SREBP-1c. To date, the effects of LXR activation on hepatic SCD1 expression have been presumed to be mainly SREBP-1c dependent, although some evidence indicate an alternative mechanism (24, 45). In addition, some evidence indicates the ability of LXR to directly target lipogenic genes, such as the FAS (16) and acetyl-CoA carboxylase (24, 61) genes. In this study, we have shown that LXR regulates SCD1 gene expression through a direct transcriptional mechanism via an LXR response element in the SCD1 promoter, suggesting that LXR-mediated regulation of lipogenesis in the liver involves direct targeting to the SCD1 promoter.

Recently, the SCD1 gene was shown to be induced in livers of transgenic mice overexpressing human PXR (63), and the T0901317 dosage used in this study has been shown to accumulate in the liver at concentrations sufficient to activate PXR (28, 46). Therefore, the effects of T0901317-mediated LXR activation on SCD1 may be mediated through PXR. However, the SCD1 gene was not induced upon PXR activation through PCN administration despite the induction of PXR target genes upon T0901317 treatment. The discrepancy in this study of the SCD1 gene in response to PXR activation compared to that of human PXR transgenic mice (63) may be due to differences in the genetic backgrounds of the mice used and in the methods of PXR activation. In addition, sequence analysis of the SCD1 promoter did not reveal a DR3 type of PXR response element. Therefore, the effect of T0901317 on SCD1 is mediated mainly through LXR activation as opposed to PXR activation.

Increasing evidence indicates that hypertriglyceridemia, especially from triglyceride-rich lipoproteins, is a risk factor for coronary heart disease (9, 12). Therefore, the lowering of plasma triglycerides has been considered as a therapeutic strategy in reducing the risk of cardiovascular disease (8, 10). Previously, LXR ligands, one of which is a synthetic LXR agonist, T0901317, have been shown to induce hypertriglyceridemia in several mouse models (5, 11, 17, 45, 53). The ability of this synthetic ligand to elevate plasma triglycerides or how LXR activation promotes pathways of VLDL TG secretion is not well understood. However, studies in the field have uncovered

several important proteins that attribute to the LXR-mediated hypertriglyceridemic effect, including SREBP-1c and downstream lipogenic enzymes and lipoprotein remodeling enzymes, such as LPL and Angptl3. However, similar to that observed with wild-type mice, SREBP-1c and downstream components remained highly induced in the SCD1$^{-/-}$ mice upon LXR activation by T0901317 despite abolished hypertriglyceridemia. Although Angptl3 gene expression was induced to similar levels in both wild-type and SCD1$^{-/-}$ mice upon LXR activation, LPL gene expression was higher in SCD1$^{-/-}$ mice, suggesting increased VLDL TG hydrolysis. However, previous work has demonstrated that SCD1 deficiency dramatically reduced VLDL secretion in leptin-deficient *ob*/*ob* mice when VLDL hydrolysis was inhibited (6), suggesting that SCD1 deficiency may also play a part in VLDL TG secretion. Oleate in triglycerides is a major constituent of VLDL particles, and perturbation in the availability of this MUFA may affect VLDL production. Our previous work of investigating the role of SCD1 in lipid and carbohydrate metabolism documented the importance of SCD1-derived oleate. We demonstrated that endogenous oleate produced by SCD1 is necessary for fructose-mediated induction of lipogenic gene expression and suggests that oleate plays an important role in lipid metabolism (30). Indeed, the fatty acid composition of triglycerides that accumulated in the liver of SCD1$^{-/-}$ mice upon LXR activation had dramatically lower palmitoleate and oleate content and increased stearate content. This change in the MUFA-to-SFA ratio may have significantly affected some processes of VLDL production despite some oleate contribution from the induction of SCD2 upon LXR activation, thus leading to moderate triglyceride accumulation in T0901317-treated SCD1$^{-/-}$ liver. The inability of SCD2 to compensate for SCD1 may be due to very low basal expression in adult liver (19, 36) and the more crucial role it plays during early development than in the adult stage (29). On the other hand, in SREBP-1c$^{-/-}$ mice where SCD1 is present, the MUFA-to-SFA ratio in hepatic triglycerides was increased to the same level as that in wild-type mice upon LXR activation. Under such circumstances, hepatic triglyceride accumulation was much less in SREBP-1c$^{-/-}$ mice than in SCD1$^{-/-}$ mice upon LXR activation. Consequently, unlike in SCD1$^{-/-}$ mice, plasma VLDL triglyceride accumulation persisted in SREBP-1c$^{-/-}$ mice, although to a smaller extent. Therefore, fatty acids high in the monounsaturated-to-saturated ratio may serve as better substrates for triglyceride synthesis and VLDL packaging and secretion. Importantly, our study strongly suggests that SCD1 plays a crucial role in the plasma hypertriglyceridemic effect associated with LXR activation, although the precise mechanism by which SCD1 deficiency prevents the hypertriglyceridemic effect remains to be investigated.

The ability of LXR activation to raise HDL levels is considered antiatherogenic due to its ability to promote reverse cholesterol transport (8, 43, 54, 55). Pharmacological activation of LXR has been shown to promote such a process and elevate HDL cholesterol levels (26, 55). Interestingly, SCD1 deficiency enhanced the ability of T0901317 to elevate plasma HDL cholesterol to a level higher than that of LXR-activated wild-type mice, while no difference in the induction of the PLTP gene was observed between them. Alternatively, studies have shown that overexpression of SCD in cell culture inhibits ABCA1-mediated cholesterol efflux, possibly by modulating membrane domain structures through changes in MUFA-to-SFA ratios (52). Sun et al. suggested that SCD changes membrane organization and depletes a specific pool of membrane cholesterol supporting ABCA1-mediated efflux (52). In addition, another group has demonstrated that LXR-mediated activation of macrophage SCD generates unsaturated fatty acids that destabilize ABCA1 (56). Taken together, these findings suggest an inhibitory role of SCD in ABCA1-mediated cholesterol efflux in macrophages, and a decrease in unsaturated or monounsaturated fatty acids may explain the elevation of HDL cholesterol we observed in SCD1$^{-/-}$ mice upon LXR activation. However, further experiments need to be conducted to examine the role of SCD1 in cholesterol efflux and its impact on atherosclerosis.

In summary, we report here that SCD1 is a direct transcriptional target of LXR and propose that in the liver, SCD1 plays a pivotal role in the regulation of hepatic and plasma triglyceride by modulating the ratio of monounsaturated to saturated fatty acids. In addition, SCD1 deficiency appears to promote the beneficial aspect of the reverse cholesterol pathway upon LXR activation by increasing HDL cholesterol. Taken together, inhibition of SCD1 under LXR activation may be beneficial due to the antihypertriglyceridemic, antihepatosteatotic, and antiatherogenic traits and thus reveals SCD1 to be a promising therapeutic target in the treatment of atherosclerosis and dyslipidemia.

ACKNOWLEDGMENTS

We thank Peter Edwards for the 0.6-kb SCD1 promoter construct and David Mangelsdorf and Joyce Repa for the LXR and RXR expression plasmids.

This work was supported by the National Institute of Diabetes and Digestive and Kidney Diseases, National Institute of Health grant RO162388 (to J.M.N.).

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