Oxysterol Binding Protein Induces Upregulation of SREBP-1c and Enhances Hepatic Lipogenesis

Daoguang Yan, Markku Lehto, Laura Rasilainen, Jari Metso, Christian Ehnholm, Seppo Ylä-Herttuala, Matti Jauhiainen, Vesa M. Olkkonen

Background—Oxysterol binding protein (OSBP) has previously been implicated as a sterol sensor that regulates sphingomyelin synthesis and the activity of extracellular signal-regulated kinases (ERK).

Methods and Results—We determined the effects of adenovirus-mediated hepatic overexpression of OSBP and its homologues ORP1L and ORP3 on mouse serum lipids. Whereas ORP1L and ORP3 had no effect on serum lipids, OSBP induced a marked increase of VLDL triglycerides (TG). Also, the liver tissue TG were elevated in the AdOSBP-injected mice, and their TG secretion rate was increased by 70%. The messenger RNAs for enzymes of fatty acid synthesis and their transcriptional regulator, SREBP-1c, as well as the Insig-1 mRNA, were upregulated two-fold in the OSBP-expressing livers. No change occurred in the messages of liver X receptor target genes ABCA1, ABCG5, and CYP7A1, and the Insig-2a mRNA was reduced. The phosphorylation of ERK was decreased in AdOSBP-infected liver and cultured hepatocytes. Importantly, silencing of OSBP in hepatocytes suppressed the induction of SREBP1-c by insulin and resulted in a reduction of TG synthesis.

Conclusion—Our results demonstrate that OSBP regulates hepatic TG metabolism and suggest the involvement of OSBP in the insulin signaling pathways that control hepatic lipogenesis. (Arterioscler Thromb Vasc Biol. 2007;27:1108-1114.)

Key Words: OSBP ■ oxysterol binding protein ■ lipogenesis ■ liver ■ SREBP-1c ■ triglyceride synthesis ■ VLDL secretion

The liver plays a central role in triglyceride (TG) and cholesterol homeostasis. Complex regulatory circuits within hepatocytes maintain the body lipid homeostasis under varying environmental conditions. Hepatic lipid syntheses and fluxes are controlled by transcription factors that respond to signals from a variety of lipidous ligands. The synthesis of cholesterol and fatty acids as well as the uptake of cholesterol to signals from a variety of lipidous ligands. The synthesis of cholesterol and fatty acids as well as the uptake of cholesterol and hepatic glucose use are controlled by sterol regulatory element binding proteins, SREBP.1,2 A two-step proteolytic cleavage of SREBP precursors occurs within the Golgi complex and releases a basic helix-loop-helix-leucine zipper (bHLH-LZ) transcription factor denoted nuclear SREBP (nSREBP). The cleavage is controlled by the endoplasmic reticulum (ER) cholesterol content, which is sensed by SREBP cleavage activating protein (SCAP). SCAP, together with Insig proteins, retains SREBP within the ER when cholesterol is abundant but escorts it to the Golgi complex on cholesterol depletion. In addition to cholesterol, exogenously added oxysterol 25-hydroxycholesterol (25OH) is a potent inducer of SREBP activation, suggesting that also endogenous cellular oxysterols regulate the SREBP machinery.2 Of the 3 SREBPs, SREBP-1c is particularly abundant in the liver where its expression is regulated by insulin and glucagon, and it plays a major role in controlling hepatic lipogenesis and glucose use.1,3 SREBP-2, also expressed at relatively high levels in the liver, is responsible for control of cholesterol metabolism. The third family member, SREBP-1a, functions in both cholesterol and TG metabolism. In cultured cells, SREBP-1a is expressed at much higher levels than SREBP-1c.4 The cleavage of SREBP-1a and -2 precursors is regulated by cholesterol status, whereas the expression and maturation of SREBP-1c are primarily regulated by nutritional factors. SREBP-1c expression in liver, white adipose tissue, and skeletal muscle is depressed during fasting but increases when animals are re-fed a high carbohydrate diet, whereas such manipulations induce only minor effects on the other SREBP isoforms.1,3

Liver X receptors (LXR), central oxysterol-responsive nuclear receptors, form heterodimers with retinoid X receptor (RXR) and regulate a number of genes involved in hepatic functions such as bile acid synthesis, biliary sterol secretion, and synthesis of nascent high-density lipoproteins. LXRs also impact on hepatic lipogenesis by controlling the expression of SREBP-1c.5

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Proteins displaying homology to the C-terminal ligand binding domain of oxysterol binding protein (OSBP) are present in practically all eukaryotic organisms. The OSBP-related proteins (ORP) have been implicated in diverse aspects of cellular physiology, including sterol and phospholipid metabolism, vesicle transport, and cell signaling. The founder member of the family, OSBP, was suggested to act as a sterol sensor that regulates the transport of ceramides from the ER to the Golgi apparatus for sphingomyelin synthesis. On the other hand, OSBP was reported to act as a sterol-dependent scaffolding protein that controls the activity of extracellular signal regulated kinases, ERK. The present study provides evidence that OSBP impacts on the expression/maturation of SREBP-1c, the insulin responsiveness of hepatic lipogenesis, and the serum levels of VLDL.

Materials and Methods

Reagents
The antibodies and other special reagents used are specified in the online supplement (available at http://atvb.ahajournals.org).

Construction of Recombinant Adenoviruses
The rabbit OSBP (acc. No. J05056) and human ORP1L and ORP3 cDNAs (acc. Nos. AF323726 and NM_015550) were inserted into the BgIII site of pAdenovector-CMV5-ires-GFP (QbioGene, Illkirch, France), and recombinant adenoviruses (AdOSBP, AdORP1L, AdORP3) generated in HEK293 cells as described in the online supplement.

Intravenous Injections of C57B/6 Mice
Female C57B/6OlaHsd mice were injected with recombinant adenoviruses as described (please see the online supplement).

Analysis of Plasma Lipids/Lipoproteins and Hepatic Lipids
Plasma and liver lipid concentrations were determined and lipoprotein fractionation performed using standard methodology (for details see the online supplement).

Quantitative RT-PCR
Messenger RNAs in liver tissue or cultured mouse Hepa1–6 hepatoma cells were quantified by real-time RT-PCR as described in the online supplement. Sequences of the primers used are listed in supplemental Table I.

Analysis of Nuclear and Precursor SREBPs in Liver Tissue
Nuclear extracts were prepared from liver tissue as described. Forty μg of nuclear protein was analyzed by Western blotting (see the online supplement) with monoclonal antibodies against SREBP, ORP1L, ORP3 protein levels in mouse liver were under detection threshold by Western blotting with the available antibodies.

Silencing of OSBP Expression and Insulin Stimulation of Hepa-1 to -6 Cells
Mouse Hepa1–6 cells were transfected with OSBP-specific siRNAs (siOSBP1, sense strand AGGCUUACACGCGCCGAUGdTdT; siOSBP2, sense strand GAUCCCAUGCGGUAACACdTdT) or a scrambled control siRNA (sense strand UAGCGACUAAACACAUCAAAddTdT; Sigma-Aldrich/Proliog) for 36 hours using the HiPerFect reagent (Qiagen), followed by incubation in serum-free medium in the absence or presence of 100 nmol/L insulin (Sigma-Aldrich) for 6 hours. Total RNA was thereafter isolated and the mRNAs for OSBP, SREBP-1c, and FAS quantified as described (see the online supplement).

Figure 1. Western analysis of Ad-mediated OSBP/ORP overexpression in mouse liver. Equal amounts (20 μg/lane) of liver total protein from mice after 3-day infection with AdGFP, AdOSBP, AdORP1L, or AdORP3 were resolved by SDS-PAGE and Western blotted using antibodies against OSBP, ORP1L, or ORP3 (indicated on the left). The apparent molecular masses of the immunoreactive proteins are indicated.

Assay for Triglyceride Synthesis
Hepa1–6 cells on 12-well plates were treated with control or OSBP siRNAs and with 100 nmol/L insulin as above, and labeled for 3 hours with [3H]oleic acid (7.0 Ci/mmol, GE Healthcare)-bovine serum albumin complexes (4.2 μCi/well) as described. The synthesized TG were quantified according to.

Results
Adenoviral-Mediated Expression of OSBP, ORP1L, and ORP3 in Mouse Liver
The recombinant adenoviruses (AdOSBP, AdORP1L, AdORP3) or a control virus encoding GFP (AdGFP) were injected into female C57B/6 mice through the tail vein at the viral dose 5×10^9 pfu per animal. Each of the 3 OSBP/ORP proteins was efficiently expressed in the liver tissue of injected animals (Figure 1). The endogenous ORP1 and ORP3 protein levels in mouse liver were under detection threshold by Western blotting with the available antibodies. However, the endogenous hepatic OSBP was readily detectable. Densitometric analysis of the OSBP Western signals revealed that the AdOSBP transduction resulted in 4-fold overexpression as compared with the endogenous protein level.

Adenoviral-Mediated Expression of OSBP Increases Plasma VLDL TG Levels
The plasma total cholesterol, TG, and choline-containing phospholipid (PL) concentrations were determined for the mice at 3, 5, and 7 days after Ad injection. The results summarized in the Table revealed a significant increase of plasma TG in AdOSBP-injected animals as compared with AdGFP-injected controls at the 3- and 5-day time points, whereas AdOSBP transduction did not affect plasma cholesterol or PL levels. Expression of ORP1L or ORP3 had no significant effect on plasma lipid parameters. As compared with the values of un.injected C57B/6 mice, the plasma cholesterol and PL levels of all Ad-injected animals increased during the experiments, whereas the TG concentration in the Ad-injected animals, except for the AdOSBP-transduced ones, was lower than in the uninjectected mice. Analysis of the distribution of lipids in fast protein liquid (FPLC)-separated lipoproteins revealed a marked increase of TG in the VLDL fractions for the plasma of AdOSBP-injected animals at all 3 time points, whereas the distribution of cholesterol and PL remained unchanged (shown for 3 days in Figure 2A and 2B). The relative increase of TG in the VLDL fractions was 95%.
at the 3-day time point, 64% at 5 days, and 52% at 7 days (the % values are representative of 2 plasma pools, 3 to 5 animals each). Transduction with AdORP1L or AdORP3 had no effect on the lipoprotein profile (data not shown; Figure 2C). SDS-PAGE analysis of apoB100 and apoB48 from lipoproteins isolated from the plasma of AdGFP- or AdOSBP-transduced animals by ultracentrifugation at density 1.063 g/mL revealed a 50% increase of apoB100 and 40% increase of apoB48 in AdOSBP-injected animals (Figure 2D), demonstrating that not only the lipid but also the protein moiety of VLDL was increased.

Liver Tissue TG Are Increased in AdOSBP-Injected Animals
Prompted by the observed increase of VLDL triglycerides in the plasma of AdOSBP-injected animals, we analyzed lipids in the liver tissue of these animals. A significant increase of liver TG in AdOSBP-injected mice as compared with AdGFP-injected controls was evident at the 3- and 5-day time points (supplemental Table II). Histological analysis of liver tissue on days 3 and 5 after infection revealed lipid droplet accumulation, visualized as large unstained vacuolar-like structures, in the hepatocytes of AdOSBP-transduced animals. These structures were absent in sections of AdGFP-injected liver (supplemental Figure I).

The Rate of TG Secretion Is Enhanced in AdOSBP-Injected Mice
To study whether the observed increase of SREBP-1c mRNA translates into an increase of mature nuclear SREBP-1c, we transduced liver tissue from AdOSBP- and AdGFP-injected animals at the 3-day time point. All 3 mRNAs were found to be significantly upregulated (AcceS, 2.1-fold; FAS, 2.1-fold; SCD-1, 2.0-fold) in AdOSBP-transduced liver as compared with AdGFP-injected controls. Furthermore, the message for the major transcriptional regulator of the lipogenic pathway enzymes, SREBP-1c, was induced 1.8-fold (supplemental Figure II). No difference in the mRNA for apolipoprotein A5, a regulator of TG metabolism, was observed between AdOSBP-transduced and control liver. Furthermore, the mRNA level of medium-chain acyl-CoA dehydrogenase (MCAD), a key enzyme in fatty acid β-oxidation, was not affected by AdOSBP transduction. Because the gene encoding SREBP-1c is target of LXR, we quantified the gene expression of LXRα, LXRβ, and the LXR targets ABCA1, ABCG5, and CYP7A1. All these mRNAs were unaffected by OSBP expression. We also quantified the mRNAs for the major regulator of hepatic cholesterol homeostasis, SREBP-2, and the major rate limiting enzyme of the cholesterol biosynthetic pathway, 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase. The latter was mildly upregulated in OSBP expressing liver, whereas the SREBP-2 mRNA was not significantly altered. The maturation of SREBP1s in the liver is controlled by Insig-2, the mRNA expression of which is subject to downregulation by insulin,12,13 and Insig-1, a variant induced by insulin,14,15 The Insig-2a mRNA was downregulated by 34% that the synthesis of fatty acids and TG is enhanced in the AdOSBP-transduced animals. We therefore quantified the mRNAs for 3 major enzymes of the lipogenic pathway, acetyl-coenzyme A (CoA) synthetase (AceCS), fatty acid synthase (FAS), and stearoyl CoA desaturase 1 (SCD-1) in liver tissue from AdOSBP- and AdGFP-injected animals at the 3-day time point. All 3 mRNAs were found to be significantly upregulated (AcceS, 2.1-fold; FAS, 2.1-fold; SCD-1, 2.0-fold) in AdOSBP-transduced liver as compared with AdGFP-injected controls. Furthermore, the message for the major transcriptional regulator of the lipogenic pathway enzymes, SREBP-1c, was induced 1.8-fold (supplemental Figure II). No difference in the mRNA for apolipoprotein A5, a regulator of TG metabolism, was observed between AdOSBP-transduced and control liver. Furthermore, the mRNA level of medium-chain acyl-CoA dehydrogenase (MCAD), a key enzyme in fatty acid β-oxidation, was not affected by AdOSBP transduction. Because the gene encoding SREBP-1c is target of LXR, we quantified the gene expression of LXRα, LXRβ, and the LXR targets ABCA1, ABCG5, and CYP7A1. All these mRNAs were unaffected by OSBP expression. We also quantified the mRNAs for the major regulator of hepatic cholesterol homeostasis, SREBP-2, and the major rate limiting enzyme of the cholesterol biosynthetic pathway, 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase. The latter was mildly upregulated in OSBP expressing liver, whereas the SREBP-2 mRNA was not significantly altered. The maturation of SREBP1s in the liver is controlled by Insig-2, the mRNA expression of which is subject to downregulation by insulin,12,13 and Insig-1, a variant induced by insulin,14,15 The Insig-2a mRNA was downregulated by 34%
analyzed by Western blotting nuclear fractions and total protein preparations of livers from mice infected for 3 days with AdGFP, AdOSBP, or AdORP3. As compared with AdGFP-transduced liver, the amount of nuclear SREBP-1c was elevated by 128% ($n=4$, $P<0.01$) in the OSBP-expressing liver, whereas no change was observed in nSREBP-2 (Figure 3). ORP3 overexpression had no impact on the level of either nSREBP-1c or nSREBP-2. The quantity of the SREBP precursor forms was not significantly affected in AdOSBP- or AdORP3-transduced liver. These results suggest that not only the expression level but also the proteolytic activation of SREBP-1c or the stability of nSREBP-1c is enhanced by OSBP overexpression.

**OSBP Overexpression Reduces ERK Phosphorylation**

Because OSBP was reported to regulate the dephosphorylation and thus the activity of ERKs, we analyzed by Western blotting the ERK1/2 phosphorylation status in the liver of AdGFP or AdOSBP-infected mice, as well as in the mouse hepatocyte cell line Hepa1–6 transduced with the same adenoviruses at a multiplicity of 100 pfu/cell. Analysis of the ratio of pERK to total ERK revealed a significant 30% reduction of ERK phosphorylation in AdOSBP-transduced liver (Figure 4). In the Hepa1–6 cells, ERK phosphorylation was reduced by almost 70%, supporting the view that OSBP is involved in regulation of the MEK–ERK pathway.

**Silencing of OSBP Expression Disturbs SREBP-1c Regulation by Insulin**

The observed effects of OSBP overexpression on SREBP-1c and its targets, as well as on Insig-1 and Insig-2a, are similar to the responses elicited in hepatocytes by insulin. Furthermore, the ERKs are known to play an important role in insulin signaling. We therefore silenced OSBP expres-
Silencing of OSBP Expression Causes a Reduction of Triglyceride Synthesis

We next determined the effect of OSBP silencing on TG synthesis in cells stimulated with insulin, by metabolic labeling with [3H]oleic acid. The results revealed a significant 30% reduction of [3H]oleic acid incorporation into TG in the cells treated with siOSBP.1, as compared with cells incubated with control siRNA (Figure 5C). This shows that the reduction in SREBP-1c and FAS mRNA expression by OSBP knock-down also translates to a reduced synthesis of TG.

Discussion

We analyzed the effects of hepatic overexpression of OSBP or its homologues ORP1L and ORP3 on plasma lipids, to clarify the function of these proteins in lipid metabolism in vivo. We show that moderate adenovirus-mediated OSBP overexpression promotes hepatic lipogenesis concomitant with enhancement of the expression of SREBP-1c and its target genes responsible for fatty acid synthesis. The effect is specific for OSBP, as it was not observed in mice infected with adenoviruses encoding GFP, ORP1L, or ORP3.

OSBP was isolated as an oxysterol receptor anticipated to be responsible for the transcriptional regulation of genes maintaining cellular sterol homeostasis.19,20 This task was, however, found to be executed by the SREBPs.2 Nishimura et al21 demonstrated that silencing of OSBP expression in HeLa cells has no effect on the 25OH-induced inhibition of HMG-

![Figure 3](image-url) - The nuclear form of SREBP-1c is increased in AdOSBP-transduced liver. Equal amounts of nuclear or total protein (40 μg/lane) from the liver of mice infected for 3 days with AdOSBP, AdGFP, or AdORP3 were Western blotted with anti-SREBP-1 or anti–SREBP-2. Data for 2 animals from each group are shown. nSREBP-1c, nSREBP-2, the nuclear forms of SREBP; pSREBP-1c, pSREBP-2, the precursor forms of SREBP.

![Figure 4](image-url) - ERK phosphorylation is decreased on OSBP overexpression. Protein preparations from AdGFP- or AdOSBP-transduced liver (A) or the Hepa1–6 cell line infected with the recombinant adenoviruses (B) were Western blotted with antibodies against phospho-ERK1/2 (P-ERK), ERK1/2 (ERK), or β-actin. Data for 4 mice and 4 Hepa1–6 cell specimens are shown. The panels at the bottom represent the phospho (P)-ERK/ERK ratio (in arbitrary units) obtained by densitometric quantification of the Western signals (mean±SD). *P<0.05; **P<0.01; Student t test; difference between AdGFP and AdOSBP-infected specimens.
CoA reductase and squalene epoxidase mRNA expression, implying that OSBP is not a major regulator of sterol homeostasis. According to a simplistic scheme, OSBP could act as a buffer for cellular oxysterols, thus tuning down their inhibitory effects on SREBP activation. However, several observations argue against this interpretation. (1) Activation of SREBP-1c is only weakly affected by cellular sterols; (2) Sequestration of oxysterols should lead to a general reduction of LXR transactivation potential, but we observed no changes in the mRNA expression of LXR target genes other than SREBP-1c; (3) OSBP expression had no effect on plasma cholesterol status, and the hepatic cholesterol level was only mildly affected.

We now show that OSBP overexpression increases TG levels in both liver tissue and serum. Furthermore, silencing of OSBP expression in insulin-treated cultured hepatocytes reduces their TG synthesis. These alterations coincide with alterations of SREBP-1c expression and the abundance of nuclear SREBP-1c. Hepatic overexpression of NH2-terminal transcriptionally active SREBP-1a and SREBP-1c fragments in transgenic mice resulted in fatty liver but no elevation of plasma TG, unlike in the present study. In the case of SREBP-1a, the lack of plasma TG elevation was shown to be attributable to simultaneous upregulation of LDL receptor expression, which masked the enhanced hepatic VLDL production. On the other hand, the absence of SREBP-1c was shown to ameliorate fatty liver development in ob/ob mice. The relationship of hepatic TG accumulation and VLDL secretion is complex.

Overexpression of the nuclear forms of SREBP-1 represents an extreme situation leading to gross alterations of hepatic lipid metabolism not comparable to the present study, in which the observed effects on lipogenesis and SREBP-1c expression/maturation were relatively mild.

Insulin induces lipogenesis via phosphoinositide-3-kinase (PI-3-K) and Akt-dependent signaling cascades that upregulate SREBP-1c transcription. Moreover, insulin enhances the proteolytic activation of SREBP-1c and increases the half-life of nSREBP-1c. In addition to the PI-3-K/Akt signaling pathway, the activity of SREBPs is subject to regulation by the MEK–ERK pathway. The present findings provide 3 mechanistic clues to the observed upregulation of SREBP-1c: (1) siRNA-mediated silencing of OSBP attenuates the response of SREBP-1c and FAS mRNA levels to insulin; (2) OSBP decreases the expression of Insig-2a and increases that of Insig-1, resembling the response of these genes to insulin; (3) ERK1/2 phosphorylation is significantly reduced in the liver of AdOSBP-infected animals and in OSBP-expressing cultured hepatocytes.

How could OSBP affect the MEK–ERK signaling pathway? Wang et al showed that, with cholesterol bound, OSBP facilitates the integrity of a cytosolic phosphatase complex that dephosphorylates ERKs, resulting in their inactivation. In the presence of 25OH, the OSBP-phosphatase complex dissociated, leading to the hyperphosphorylation of ERKs. Consistent with the fact that cholesterol is in cells vastly more abundant than oxysterols, our finding suggests that the
overexpressed OSBP tends to adopt a cholesterol-bound conformation facilitating the dephosphorylation of ERKs. Expression of Insig-1 is regulated by insulin in a MEK–ERK-dependent manner. Furthermore, Botolin et al. recently showed that the abundance of nSREBP-1 is regulated by ERKs via control of proteasomal degradation. According to the findings by Botolin et al., the increase of nuclear SREBP-1c in AdOSBP-transduced liver could be at least in part attributable to reduction of phospho-ERK, resulting in increased stability of the nSREBP-1c. However, alteration of signaling through the MEK-ERK pathway could also affect the hepatic TG metabolism via other mechanisms.

The results of both overexpression and silencing of OSBP demonstrate a new function for the protein as a regulator of hepatic TG metabolism. Furthermore, our findings suggest the involvement of OSBP in the insulin signaling pathways that control hepatic lipogenesis and play central roles in the development of insulin resistance and diabetes mellitus.

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Disclosures

None.

References

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Materials and Methods

Reagents
Rabbit polyclonal and mouse monoclonal antibodies against OSBP were kind gifts from Drs. Ignacio Rodriguez (NIH, Bethesda, MD) and Neale Ridgway (Dalhousie Univ., Halifax, Canada). Rabbit antibodies against ORP1L and ORP3 were described in (Johansson et al., Mol Biol Cell. 2005;16:5480-92) and in (Lehto et al., Cell Tissue Res. 2004;315:39-57), respectively. The monoclonal anti-SREBP-1 (clone 2A4) was from BD Biosciences (Erembodegem, Belgium) and anti-SREBP-2 (clone 1C6) from BD Biosciences (San Jose, CA). Antibodies against p44/42 MAP kinase (ERK1/2) and phospho-p44/42 MAP kinase were from Cell Signaling Technology (Danvers, MA). Triton WR1339 (Tyloxapol) and bovine insulin were from Sigma-Aldrich (St. Louis, MO).

Construction of recombinant adenoviruses
The rabbit OSBP (acc. No. J05056) and human ORP1L and ORP3 cDNAs (acc. Nos. AF323726 and NM_015550) were inserted into the BglII site of pAdenovator-CMV5-IRES-GFP (QbioGene, Illkirch, France), and recombinant adenoviruses (AdOSBP, AdORP1L, AdORP3) generated in HEK293 cells using the AdEasy system according to the manufacturer’s instructions. The amino acid identity of rabbit OSBP and human ORP1L and ORP3 with their mouse counterparts is 96%, 92%, and 91%, respectively. A control adenovirus encoding GFP alone (AdGFP) was generated from the plain pAdenovator transfer vector. The recombinant viruses were plaque purified, expanded and purified on CsCl gradients as previously described (Laitinen et al., Hum Gene Ther. 1998;9:1481-6).

Intravenous injections of C57B/6 mice
Female C57B/6JOlaHsd mice were purchased from Harlan Nederland (AD Horst, the Netherlands), housed in a humidity (40-50%) and temperature (21-22 °C) controlled room with a 12:12 h dark/light cycle, and maintained on Rat&Mouse Maintenance Diet 1324 (Altromin, Lage, Germany). For adenovirus injections, 10-week-old animals were
calmed with Hypnorm-Dormicum (Vetapharma, Leeds, UK) and injected through the tail vein with \(5 \times 10^8\) pfu of adenovirus in PBS per mouse. At 3, 5, or 7 days after injection, the animals were fasted for 5 h, and blood and liver tissue samples were collected. One mg/ml EDTA was immediately added in the blood samples and plasma prepared by centrifugation. To determine the hepatic TG secretion rate, animals were fasted overnight and, on day 5 after adenoviral infection, injected through the tail vein with 500 mg/kg body weight of Triton WR1339, and blood samples were withdrawn from the tail at 0, 15, 30, 60, and 90 min. All animal protocols were carried out according to a license (STU 446A) from the regional government of Southern Finland.

**Analysis of plasma lipids and lipoproteins**

Total plasma cholesterol (Kit 1489232, Roche Diagnostics GmbH, Mannheim, Germany), choline-containing phospholipids (Kit 990-54009, Wako Chemicals GmbH, Neuss, Germany) and triglycerides (Kit 1488872, Roche Diagnostics) were measured using fully enzymatic methods. Serum lipoproteins were fractionated by fast-performance liquid chromatography (FPLC) using a Superose 6HR 10/30 size-exclusion chromatography column (GE Healthcare, Buckinghamshire, UK). The column was equilibrated with Tris-buffered saline (10 mM Tris-HCl, 150 mM NaCl, pH 7.4) at a flow rate of 0.5 ml/min and 0.5 ml fractions were collected for lipid analyses. Serum pools from 3-5 mice (vol. 200 µl) were analyzed. The apolipoprotein B (apoB)-containing lipoproteins were isolated from plasma by ultracentrifugation. Briefly, 200 µl of pooled plasma was adjusted to density 1.055g/ml, placed in a 3 ml tube, and overlaid with 1.8 ml of \(\delta=1.063\)g/ml NaBr solution. The specimens were spun at 400,000 x g for 3 hours in Optima TL Ultracentrifuge (Beckman, Brea, CA). The top 0.6 ml of solution, which contains the apoB-rich particles, was concentrated by Nanosep Centrifugal Devices (Pall Life Sciences, Dreieich, Germany). To analyze the apolipoprotein composition, the isolated apoB-rich particles (20 µl, corresponding to 20 µl plasma) were run on 5% SDS-PAGE gels and the proteins visualized by Coomassie Blue Staining.
Liver tissue lipid analysis

Briefly, liver tissue (approximately 100 mg) was homogenized and sonicated in 1 ml 95 % methanol and mixed with 2 ml chloroform. The organic phase was washed with 0.9 % NaCl solution and dried under nitrogen. The residuals were dissolved in 200 µl of tetraethylammoniumhydroxide (diluted 1:28 with 95 % ethanol) and incubated at 60 °C for 30 min with 200 µl of 0.05 M HCl. The formed glycerol was measured enzymatically (Kit 1488872, Roche Diagnostics). Total cholesterol and choline-containing phospholipids (PC, lyso-PC, SM) were measured for the same tissue specimens from the solvent phase after initial chloroform-methanol extraction, using the assays specified above for serum samples.

Histology

Pieces of liver tissue were fixed with 10% formalin and embedded in paraffin using a standard protocol. Tissue sections were stained with haematoxylin/eosin and viewed/photographed with a Zeiss Axioplan 2 microscope equipped with a CCD camera.

Quantitative RT-PCR

Liver tissue or cultured cells were homogenized in RLT buffer (Qiagen, Valencia, CA) and total RNA isolated with RNeasy Mini kit (Qiagen) according to the manufacturer’s instructions. The total RNA (2 µg) was treated with DNase I (Promega, Madison, WI) in the presence of RNase Inhibitor (Promega) and reverse-transcribed by using Superscript II (Invitrogen, Carlsbad, CA) and random hexamer primers (Applied Biosystems, Foster City, CA). Each mouse RNA sample was amplified in triplicate for the genes of interest and two housekeeping markers, 36B4 and 18S rRNA, on a 7000 Sequence Detection System (Applied Biosystems) by using SYBR-green (ABgene, Surrey, UK). Sequences of the primers used are listed in Supplementary Table 1. The threshold was set in the linear range of fluorescence, and a threshold cycle (Ct) was measured for each well. The data was analyzed as described in (Pfaffl, Nucleic Acids Res. 2001; 29: e45).
Western blotting

Proteins specimens for SDS-PAGE were prepared by homogenizing liver tissue or cultured cells in 250 mM Tris-HCl, pH 6.8, 8% SDS, protease inhibitor cocktail (Roche Diagnostics). The crude extracts were cleared by centrifugation at 16,000 x g for 3 min, and protein concentration of the supernatant determined by the $D_c$ assay (BioRad, Hercules, CA). The proteins were electrophoresed and Western blotted using the primary antibodies specified above. The bound antibodies were visualized by using horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit IgG (Bio-rad, Hercules, CA) and enhanced chemiluminescence (ECL; GE Healthcare).
Table I. Oligonucleotide primers used for mRNA quantification by real-time RT-PCR

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<th>mRNA</th>
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<td>SREBP-1c</td>
<td>gga gcc atg gat tgc aca tt</td>
<td>gct tcc aga gag gag gcc ag</td>
</tr>
<tr>
<td>FAS</td>
<td>gct ggc gaa act tca gga aat</td>
<td>aga gac gtg tca ctc ctc gac tt</td>
</tr>
<tr>
<td>AceCS</td>
<td>gct gcc gcc ggg aca ggc ag</td>
<td>tcc aga cacatt gag cat gtc at</td>
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<tr>
<td>SCD-1</td>
<td>cgc gag acc cct tag atc ga</td>
<td>tag cct gta aaa gat ttc tgc aca cc</td>
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<tr>
<td>MCAD</td>
<td>gat cgc aat ggg ctc ctt tga tag aa</td>
<td>agc tga ttc gca atg tct cca gca aa</td>
</tr>
<tr>
<td>apoA5</td>
<td>ctc tgt ccc aca aac tca ctc gac g</td>
<td>agg tag gtg tca tgc cga aca a</td>
</tr>
<tr>
<td>SREBP-2</td>
<td>ggc ttc tgg aga cca cgg a</td>
<td>aca aag ttc ctc tga aca aca aca aca aca a</td>
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<tr>
<td>HMGCoAR</td>
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<td>agc cga ggc ggc aca tga t</td>
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<tr>
<td>LXRα</td>
<td>ggg aag agt gtt tgc tgt cag</td>
<td>ggc cgc ctc tta cac tgt tgc</td>
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<tr>
<td>LXRβ</td>
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<td>tgc att ctc tct ggt ggt t</td>
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<tr>
<td>ABCA1</td>
<td>cgt ttc cgg gaa gtt tcc ta</td>
<td>gct aga gat ggc ggg gag gat gga</td>
</tr>
<tr>
<td>ABCG5</td>
<td>tgg atc cca cac ctc tat gct aca a</td>
<td>ggc agg ttc tct cga tga aca a</td>
</tr>
<tr>
<td>CYP7A1</td>
<td>agc aac taa aca acc tcc cag tac ta</td>
<td>gtc cgg ata ttc aca gat gca</td>
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<tr>
<td>Insig-1</td>
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<td>cct aag cgc taa aac aca aca t</td>
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<tr>
<td>Insig-2a</td>
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<td>cct aag cgc taa aac aca atg</td>
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<td>36B4</td>
<td>cat gct cca cat ctc ccc ctt ctc c</td>
<td>ggg aag gtt taa ttc gtt ctc aca g</td>
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The abbreviations are: SREBP, sterol regulatory element binding protein; FAS, fatty acid synthetase; AceCS, acetyl coenzyme A synthetase; SCD-1, stearoyl coenzyme A desaturase 1; MCAD, medium-chain acyl-coenzyme A dehydrogenase; apoA5, apolipoprotein a5; HMGCoAR, 3-hydroxy-3-methylglutaryl coenzyme A reductase; LXR, liver X receptor; ABC, ATP-binding cassette transporter; CYP7A1, gene encoding cholesterol-7α-hydroxylase; Insig, insulin-induced gene; OSBP, oxysterol binding protein; 36B4, acidic ribosomal phosphoprotein 36B4
Table II. Hepatic lipids in mice transduced with AdGFP or AdOSBP

<table>
<thead>
<tr>
<th>Days post-inf.</th>
<th>Chol(^1) µmol/g</th>
<th>TG(^2) µmol/g</th>
<th>PL(^3) µmol/g</th>
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</thead>
<tbody>
<tr>
<td>AdGFP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>4.32 ± 0.30(^4)</td>
<td>8.76 ± 0.54</td>
<td>6.18 ± 0.48</td>
</tr>
<tr>
<td>5</td>
<td>3.78 ± 0.30</td>
<td>6.12 ± 0.90</td>
<td>10.38 ± 0.24</td>
</tr>
<tr>
<td>7</td>
<td>3.66 ± 0.42</td>
<td>3.90 ± 0.24</td>
<td>12.42 ± 1.08</td>
</tr>
<tr>
<td>AdOSBP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>2.88 ± 0.60</td>
<td>11.10 ± 0.60(^*)</td>
<td>6.42 ± 0.72</td>
</tr>
<tr>
<td>5</td>
<td>2.82 ± 0.30</td>
<td>9.84 ± 0.48(^**)</td>
<td>11.52 ± 0.84</td>
</tr>
<tr>
<td>7</td>
<td>3.06 ± 0.36</td>
<td>5.10 ± 0.36</td>
<td>11.40 ± 0.48</td>
</tr>
</tbody>
</table>

\(^1\)Total cholesterol, \(^2\)Triglycerides, \(^3\)Choline-containing phospholipids per gram tissue
\(^4\)Mean ± SD; n = 4-6

* p<0.05, ** p<0.01; Student’s t-test, comparison with corresponding AdGFP-transduced group
Figure I. Histological observation reveals hepatic steatosis in AdOSBP-infected mice. Liver tissue from mice injected with AdGFP or AdOSBP was, on day 3 post-infection, fixed with formalin, embedded in paraffin and stained with haematoxylin/eosin. Neutral lipid accumulation in hepatocytes is seen as unstained structures in the cytoplasm (arrows).
Figure II. Transcripts of the lipogenic pathway genes are up-regulated in OSBP expressing animals. Total RNA isolated on day 3 post-infection from the liver of mice transduced with AdGFP or AdOSBP was subjected to real-time RT-PCR analysis of selected mRNAs (identified at the bottom) using a SYBR-green assay. Relative mRNA quantity is shown. The value for AdGFP-transduced liver was set at 1. The data represents a mean ± SD from 4 animals. * p<0.05; ** p<0.01; Student’s t-test; difference between AdGFP and AdOSBP-infected mice.
Figure III. Silencing of OSBP expression with siOSBP.2 impairs the insulin response of SREBP-1c expression and activity. Hepa1-6 cells transfected with a control (siNT) or OSBP-specific (siOSBP.2) siRNAs were incubated for 6 h in the presence or absence of 100 nM insulin, and the relative quantity of the mRNAs for SREBP-1c (A) and FAS (B) was determined by real-time RT-PCR. The data shown represents mean ± SD, n=4; * p<0.05; Student’s t-test; difference between siNT- and siOSBP.2-treated cells.