Lipolysis Stimulated Lipoprotein Receptor

A NOVEL MOLECULAR LINK BETWEEN HYPERLIPIDEMIA, WEIGHT GAIN, AND ATHEROSCLEROSIS IN MICE*

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The lipolysis-stimulated lipoprotein receptor, LSR, is a multifunctional protein complex in the liver that undergoes conformational changes upon binding of free fatty acids, thereby revealing a binding site(s) that recognizes both apoB and apoE. Complete inactivation of the LSR gene is embryonic lethal in mice. Here we show that removal of a single LSR allele (LSR inactivation of the LSR gene) is embryonic lethal in mice. Here a binding site(s) that recognizes both apoB and apoE. Complete inactivation of the LSR gene is embryonic lethal in mice. Here a binding site(s) that recognizes both apoB and apoE. Complete inactivation of the LSR gene is embryonic lethal in mice. Here a binding site(s) that recognizes both apoB and apoE. Complete inactivation of the LSR gene is embryonic lethal in mice. Here a binding site(s) that recognizes both apoB and apoE. Complete inactivation of the LSR gene is embryonic lethal in mice. Here a binding site(s) that recognizes both apoB and apoE. 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Complete inactivation of the LSR gene is embryonic lethal in mice. Here a binding site(s) that recognizes bothapoB-containing lipoproteins and represents a novel therapeutic target for the treatment of hyperlipidemia associated with obesity and atherosclerosis.

Triglyceride (TG)²-rich apoB-containing lipoproteins are produced by the liver as VLDL or by the intestine as chylomicrons (1). Both types of particles deliver their TG load to peripheral tissues through interactions with lipoprotein lipase (LpL) anchored to capillary endothelium heparan sulfate proteoglycans (HSPGs) (1). The residual cholesterol enriched inter-mEDIATE density lipoprotein (IDL) and LDL for VLDL, and chylomicrons remnants are then removed from the circulation by hepatocytes. Clearance of these lipoproteins in the liver is a complex process through which the particles enter the space of Disse, bind to HSPGs, acquire apoE, and interact with hepatic lipase and LpL (2). After this, the residual particles are delivered to endocytic receptors for cellular internalization and degradation (3).

The LDL receptor (LDL-R) accounts for most of LDL removal and contributes to part of the uptake of chylomicron remnants (4, 5). This highly informative endocytic receptor prototype nevertheless leaves two unsolved issues regarding apoB-containing lipoprotein clearance. First, in the absence of functional LDL-R, the amount of LDL cleared daily is 2-fold greater than that of subjects with normal LDL-R activity (6) and has been shown to take place in the liver (7). Second, the clearance of intestinally derived chylomicron remnants proceeds at normal rates in human subjects and Watanabe heritable hyperlipidemic rabbits with genetic lesions leading to complete impairment of LDL-R function (8, 9).

Identification of the molecular mechanisms and, thus, of the receptor(s) that cooperates with the LDL-R for the liver’s specific capture of chylomicrons and LDL remains controversial. The LDL receptor-related protein (LRP1) of the LDL-R family (10) has been proposed as the primary candidate for the clearance of chylomicron remnants (11). However, liver inactivation of LRP1 gene alone does not cause hyperlipidemia per se (11). An accumulation of apoB48 lipoproteins is observed only if hepatic LRP1 is inactivated in mice lacking the LDL-R (11), suggesting that LRP1 plays a supporting rather than a principal role in the clearance of remnants in the liver.

Cell studies have shown that HSPGs are directly involved in internalization of apoE-enriched and lipase-associated lipoproteins (12, 13). Reduction of sulfation of hepatic heparan sulfate leads to a delayed clearance of both intestinally derived and hepatic apoB-containing lipoproteins independent of the LDL-R or LRP1, clearly demonstrating that the processing of HDL, high density lipoprotein; siRNA, small interfering RNA; CHD, cyclohexane-dione; HDL, high density lipoprotein; RT, reverse transcription; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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‡ The abbreviations used are: TG, triglyceride; DiI, 1,1’-dioctadecyl-3,3,3’,3’-tetratetramethylindocarbocyanine perchlorate; HSPG, heparan sulfate proteoglycan; LDL, low density lipoprotein; LDL-R, LDL-receptor; VLDL, very LDL; LRP1, LDL-R-related protein; LpL, lipoprotein lipase; LSR, lipolysis-stimulated receptor; PHP, post-heparin plasma; PL, phospholipid; RAP, receptor-associated protein; TC, total cholesterol; FFA, free fatty acid.
the particles in the space of Disse involving cell surface proteoglycans is a critical and necessary step before endocytic uptake of the lipoprotein (14). The final internalization step may be mediated by HSPGs themselves or by another as yet unidentified remnant receptor that would act downstream of HSPG-dependent lipolytic processing of lipoproteins.

We have proposed an alternate mechanism for remnant lipoprotein clearance mediated by a receptor that in cell culture and liver membrane studies was shown to be activated by the products of lipolysis, free fatty acids (FFAs) (15, 16). This lipolysis-stimulated receptor, LSR, binds both apoB- and apoE-containing lipoproteins and displays highest affinity for TG-rich lipoproteins (15, 17, 18). Further in vitro studies showed that LSR activity is inhibited by lactoferrin, a milk protein that inhibits chylomicron remnant uptake in the liver (17, 19), and by the 39-kDa receptor-associated protein (RAP) (20) at concentrations similar to those causing hyperlipidemia after adenovirus-mediated expression in mice (21). Furthermore, LSR binding to TG-rich lipoproteins, but not to LDL, was inhibited by apoCIII (22), an apolipoprotein that causes hypertriglyceridemia when overexpressed (23, 24).

Expression cloning led to identification of a candidate gene encoding three mRNA transcripts, α, α′, and β (18), of which the latter two are alternately spliced products. The α and β subunits are associated as multimeric complexes through disulphide bridges (18). Co-transfection of both subunits is sufficient to reconstitute LSR activity in cultured cells (18).

Inactivation of the LSR gene leads to embryonic lethality in homozygous mice (25). In this study we show delayed postprandial lipid clearance in mice with a single functional LSR allele (LSR+/−) and demonstrate in vivo the role of the LSR gene product in the clearance of both TG-rich particles and LDL.

**EXPERIMENTAL PROCEDURES**

**Materials and Antibodies—**All chemicals and solvents were purchased from Sigma-Aldrich unless otherwise indicated. Rabbit anti-sera recognizing a peptide in the C-terminal of mouse LSR (EEGHYPPAPPYSET) were prepared (Eurogentec) (18). A second LSR antibody was purchased from Sigma-Aldrich unless otherwise indicated. Antibodies recognizing mouse LDL-R and the 85-kDa subunit of mouse LRP1 were obtained from Abcam and Progen, respectively.

**Animals and Diet—**LSR heterozygote mice on a C57Bl/6J background after >4 generations (25) were transferred from Bordeaux and re-derivated into the same strain C57Bl/6J mice (Janvier Breeding) before being introduced into a specific pathogen-free, certified animal facility authorized to house transgenic animals. For these studies, LSR−/− male mice were used with LSR+/− littermates. LDLR−/− mice were obtained from Jackson Laboratories. All animals were provided a normal rodent chow diet and water ad libitum in a room maintained on a 12-h light/dark cycle with a mean temperature of 21 ± 2 °C and relative humidity of 50 ± 20%. Animals were handled in accordance with French State Council guidelines for the use and care of laboratory animals. Body mass composition was measured using the EM-Scan model SA-3000 (EM-SCAN) as described previously (26).

**Postprandial Lipemia and Intralipid Studies—**Blood samples in EDTA were obtained at the indicated times by retro-orbital puncture under light isoflurane anesthesia. After centrifugation at 4 °C (10,000 rpm, 5 min), the plasma fraction was then immediately analyzed or snap-frozen in liquid nitrogen for later analysis. For TG clearance studies, Intralipid was administered as a bolus injection in the tail vein (100 µl of a 2.6% solution in sterile physiological saline) at 9 a.m. in non-fasting animals. Pilot studies showed that the TG peak was achieved at 2 min. For induced postprandial lipemia experiments, overnight-fasted mice were gavaged with 300 µl of olive oil. For time course studies, blood samples were collected at the indicated times into paraoxon coated tubes to inhibit lipase activity (27). Plasma samples were snap-frozen for later analysis.

**Western-type Diet Study—**A high fat (20% w/w, lard) and high cholesterol (0.15% w/w) Western-type diet (824063, Special Diets Services) was provided ad libitum to 8-week-old LSR+/+ and LSR−/− mice for 30 weeks. At the end of the study blood samples were collected from the jugular vein of anesthetized animals. Samples of the liver, heart, skeletal muscle, adipose tissue, and aorta were rinsed in physiological saline, snap-frozen in liquid N₂, and stored at −80 °C. Lipid extractions were performed on pre-weighed lyophilized tissue samples (28) followed by analysis of TG, total cholesterol (TC), and phospholipids (PL).

**Measurement of Lipase Activity in Post-heparin Plasma (PHP)—**PHP was obtained by injection of non-fasting mice at 9 a.m. with 300 IU/kg of sodium heparin followed by blood sampling 10 min after. Total and hepatic lipase activities were measured in PHP based on an established procedure (29). Incubations were performed at 25 °C in a shaker for 60 min followed by immediate analysis for FFAs using a colorimetric enzymatic kit (Wako). LpL activity was calculated as the difference between total and hepatic lipase activities, the latter being measured in the presence of 1 M NaCl.

**Measurement of Plasma and Tissue Lipids—**TC, TG, and PL in plasma or tissue extracts were analyzed using colorimetric enzymatic kits (Biomerieux) according to the manufacturer’s instructions. A serum control (Unitrol, Biomerieux) was included with each assay performed.

**Lipoprotein Profiles—**The plasma samples of 3 mice from the same group were pooled (210 µl total) and then added to 290 µl of 30 mM phosphate buffer containing 150 mM NaCl, 1 mM EDTA, and 0.02% sodium azide, pH 7.4. This was applied to a Superose 6 10–300 GL column (GE Healthcare) equilibrated with the same buffer (0.2 ml/min). Fractions of 500 µl were collected and then analyzed for TC and TG content using the enzymatic kits described above. Preliminary runs with plasma obtained from C57Bl/6J control mice were performed with the same column under identical conditions to determine the location of the VLDL (fractions 1–7), LDL (fractions 8–14), and HDL (fractions 15–22) peaks.

**Hepatic Clearance of Remnant Lipoproteins by LSR**
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**TABLE 1**

Sequences of primers used for real-time PCR

| Gene       | Primer | Sequence (5’–3’)                                      | Location | Amplicon length (bp) |
|------------|--------|------------------------------------------------------|----------|----------------------|
| LSR        | Forward| CAGGAAGATCACCACCTCACAGGAA                           | Exon 2   | 77                   |
|            | Reverse| AGGAATCCCTCCCCGTCCAG                               | Exon 3   | 76                   |
| LDL-R      | Forward| CCTGTTGACCAGAACATCACTCA                           | Exon 11  | 109                  |
|            | Reverse| AATACCCACATAGAGACGCG                                 | Exon 12  | 150                  |
| LRP1       | Forward| GCTGATGACAGAAGACATCACTAAG                           | Exon 33  | 76                   |
|            | Reverse| AGGACGATCTGCTTCTCTGAGG                               | Exon 34  | 109                  |
| GAPDH      | Forward| CTCAATCTACATGGCTCTACATGTCCAG                       | Exon 3   | 150                  |
|            | Reverse| TCTCCCACATTTATGATGTAUG                              | Exon 3   | 109                  |
| HPRT       | Forward| TACAGCTGAGAGATCTACCTGCTGAA                           | Exon 2   | 76                   |
|            | Reverse| AAAGTGGAGATCGATCTCCACCAA                           | Exon 3   | 109                  |

**Aorta Staining**—Tissue cryosections (8 μm) in OCT were prepared using a cryostat (Microm HM550), air-dried on gelatin-covered slides, and fixed in 4% paraformaldehyde. Slides were stained in 0.5% Oil Red O in propylene glycol followed by counterstaining in Harris’ hematoxylin solution and then mounted in Gel/Mount (Microm).

**siRNA Studies**—siRNA targeted toward LSR (Silencer® pre-designed siRNA, ID166924, Genbank™ NM_017405) and an siRNA negative control (Silencer® Negative Control #1) were obtained from Ambion (Applied Biosystems). siRNA were transfected into mouse hepatoma Hepa1–6 cells plated 48 h earlier on coverslips and grown in Dulbecco’s Modified Eagle’s Medium (DMEM) (18) followed by a 2-h incubation at 37 °C with 2 μg/ml DiI-labeled lipoproteins as previously described (18). Briefly, siRNA-transfected Hepa1–6 cells were treated 30 min with 20 ng/ml recombinant mouse leptin (Sigma-Aldrich) (18) followed by a 2-h incubation at 37 °C with 2 μg/ml DiI-CHD-LDL or 5 μg/ml DiI-VLDL in the presence of 0.8 mM oleate. Cells were washed, fixed in 4% paraformaldehyde, stained with 4’,6-diamidino-2-phenylindole to identify nuclei. Digital images were obtained using identical exposure times for cells treated with either control or LSR siRNA. Image analysis of DiI-labeled cells was then performed on the same number of cells (n = 20) in quadruplicate using the ImageJ software (National Institutes of Health). All experiments were performed in duplicate.

**Western Blotting**—Liver total and plasma membranes were prepared as described previously (22). Protein was solubilized directly in sample buffer, and identical amounts of protein (20–50 μg) (32) were applied to Novex 4–12% SDS-PAGE gels. For in vitro experiments, cells were lysed in radioimmune precipitation assay buffer containing anti-proteases before separation by SDS-PAGE. After electrophoresis, separated proteins were transferred onto nitrocellulose membranes for Western blotting as described previously (18). Loading was systematically verified using Ponceau Red staining. Bands were revealed by chemiluminescence (GE Healthcare) using a peroxidase-conjugated secondary antibody and a chemiluminescence kit (GE Healthcare). Densitometric analysis of the autoradiographs was performed using a Versadoc imaging system (Bio-Rad).

**Genotyping**—Tail genomic DNA was used for genotyping. PCR conditions and primers for wild-type LSR were used as previously described (25). For the transgene, oligos used were forward primer, 5’-GCTTTTCTGGATCTGACTGT-3’, and reverse primer, 5’TTCCTCTCTCTCTCTCTCTCTT-3’, corresponding to intron 5 of LSR gene and the neomycin-cin cassette, respectively. PCR conditions for the neo gene were 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 90 s for 32 cycles. For both wild-type and mutant cases, PCR cycles were preceded by 5 min at 94 °C and ended by 7 min at 72 °C.

**Real-time RT-PCR**—Tissue or cell samples were stored in RNALater (Qiagen) following the manufacturer’s instructions. Lysates were prepared by homogenizing tissue in nucleic acid purification lysis solution (Applied Biosystems, 20–30 μg per 2 ml). Total RNA was purified using the 6100 Nucleic acid Prestation system (Applied Biosystems) following the manufacturer’s instructions.

cDNA was prepared from 250 ng of total RNA by RT-PCR and used for quantitation of mouse LSR, LDL-R, LRP1, and two reference genes (GAPDH and hypoxanthine guanine phosphoribosyltransferase) by real-time RT-PCR (see Table I for primer sequences used). LSR PCR primers were designed for the detection of all three forms of LSR, and primers for all genes were validated for identical efficiencies. cDNA equivalent to 15 ng of total RNA was added to SYBR Green JumpStart Taq ReadyMix (Sigma-Aldrich) containing 200 nM forward and reverse primers in a total of 25 μl, and real-time RT-PCR analysis was performed on an Applied 7500 system. Relative expression was then calculated as 2–ΔΔCt.

**Statistical Analysis**—All results are shown as the mean ± S.E. unless otherwise indicated. Statistical analysis was performed using Student’s t test comparing LSR+/+ to LSR−/− or paired t test as indicated; significance was considered as p < 0.05. Statistical analysis of real-time RT-PCR results were performed using the Relative Expression Software Tool-Multiple Condition Solver (REST-MCS®, Version 2). Areas under the curves of the postprandial lipemia data were calculated using the trapeze method.
RESULTS

The method used to produce LSR−/− mice was described previously (25). These mice were bred into C57Bl/6 background for >4 generations. Real-time RT-PCR was performed in liver mRNA isolated from LSR+/+ and their LSR−/− littermates to measure expression of LSR, LDL-R, and LRP1 relative to those of two reference genes, hypoxanthine guanine phosphoribosyltransferase (HPRT) and GAPDH. Results of Fig. 1A show that LSR mRNA, but not those of LDL-R or LRP1, was reduced in mice with a single functional LSR allele. Lower LSR gene expression led to decreased LSR protein in liver membrane preparations (Fig. 1B). The Sigma LSR affinity-purified antibody used revealed the β subunit (predicted molecular mass, 58.6 kDa) in liver total membranes (Fig. 1B, top panel), which was previously shown to be more abundant than the α and α' subunits (18). Western blots were also performed on enriched protein fractions solubilized from liver plasma membranes using the anti-LSR-C-term antibody (Fig. 1B, bottom panel). Densitometric analysis revealed that the average signal of the sum of the 2 bands that correspond to α (apparent molecular mass, 63 kDa) and β (apparent molecular mass, 58 kDa) subunits were 0.74 ± 0.04 (LSR−/−, n = 4) and 0.26 ± 0.04 arbitrary units (LSR+/+, n = 4, p < 0.001), respectively. Thus, in LSR−/− mice, both hepatic LSR mRNA and LSR protein were reduced by more than 50% relative to LSR+/+ with the same genetic background.

Western blots were performed on the same liver total membrane preparations used in Fig. 1B, top panel, to determine whether LDL-R and LRP1 protein levels were modified. Neither expression of hepatic LDL-R nor that of LRP1 was significantly decreased in LSR−/− animals (Fig. 1C).

We first measured changes in plasma TG occurring during the physiological post-prandial phase in LSR−/− and LSR+/+ mice on a normal chow diet. Blood was collected from 6-week-old mice at the beginning of the light cycle and then after 3 h with access only to water. The increase in plasma TG that occurred between these 2 time points was 2-fold and significantly (p < 0.04) greater in LSR−/− mice (Fig. 2A). Fasting LSR−/− and control mice were challenged with dietary lipid administered through gavage feeding, and plasma TG levels were recorded before and 1, 2, and 3 h after the lipid challenge. Fig. 2B shows that the lack of a single LSR allele caused a significant increase in postprandial TG levels most notably 2 h (p < 0.04) and 3 h (p < 0.03) after the meal. The area under the curve was 3.8-fold greater in LSR−/− as compared with LSR+/+ mice.

To rule out that these differences could result from changes in intestinal lipid absorption, two groups of mice were injected intravenously with a bolus of Intralipid followed by blood sampling at 2 and 30 min. Pilot studies showed that these time points provided a reproducible estimate of emulsion clearance. The % TG cleared within this time interval was calculated and is shown in Fig. 2C. A 2-fold reduction of TG clearance rate was recorded in LSR−/− mice relative to control LSR+/+ mice. We next determined if differences in TG clearance rate were due to reduced LpL or hepatic lipase activities. Activities of both enzymes in PHP were not different in LSR−/− and LSR+/+ animals (Fig. 2D). Finally, we crossed LSR−/− mice with LDLR−/− mice to obtain LDLR−/− LSR+/+ and LDLR−/− LSR−/− animals. These mice were challenged with dietary lipid by a single bolus gavage similar to the experiment in Fig. 2B, and plasma TG levels were determined over time. As shown in Fig. 2E, postprandial lipemia in LDLR−/− mice also lacking a
single LSR allele was significantly greater as compared with that in the control LDLR<sup>−/−</sup>-LSR<sup>−/−</sup>. The postprandial lipemia was even further elevated as compared with that measured in Fig. 2B. The area under the curve was increased 5-fold, and all time points after the gavage were significantly higher. Neither LpL nor hepatic lipase activities were found to be different in these LDLR<sup>−/−</sup>-LSR<sup>−/−</sup> animals as compared with the controls (data not shown). Thus, these data establish that LSR significantly contributes to the removal of dietary TG from the circulation.

To further explore the physiological role of LSR under conditions of dietary lipid challenge, 2 groups of 8-week old LDLR<sup>−/−</sup> and LSR<sup>+/+</sup> littermates were placed on a Western-type high fat, cholesterol-containing diet. As shown in Fig. 3A, before starting the diet plasma TG (top panel) and TC (bottom panel) were significantly (p < 0.05 and p < 0.04, respectively) increased in the LDLR<sup>−/−</sup> relative to controls. Western-type diets have been shown to lead in C57Bl/6J mice to a reduction of TG and increase in TC (33–35). The reduction of TG was significantly altered (p < 0.02, 12 weeks; p < 0.04, 30 weeks) and delayed in LDLR<sup>−/−</sup>, whereas the increase in TC was significantly amplified (p < 0.02, 12 weeks; p < 0.05, 30 weeks) relative to LSR<sup>+/+</sup> mice. The increase in TG reflected accumulation of the large TG-rich VLDL/chylomicron fraction, whereas the elevated TC levels reflected an increase in both the LDL and HDL fractions (Fig. 3B). The increase in LDL was relatively more pronounced than that of HDL, which appeared to be slightly larger and TG-enriched.

Lipid composition per milligram dry weight of liver, heart, adipose tissue, and skeletal muscle was not different between the two groups (Table 2), with the exception of a small but significant decrease in liver TG in LDLR<sup>−/−</sup> as compared with LDLR<sup>+/+</sup> animals on the Western-type diet. However, aorta TC and PL contents were significantly (TC, p < 0.02; PL, p < 0.002) increased in LDLR<sup>−/−</sup> mice relative to LDLR<sup>+/+</sup> mice on the same diet (Fig. 3C). Aorta TG was also increased in LDLR<sup>−/−</sup> mice but did not reach statistical significance. Consistent with these biochemical determinations was the detection of a greater amount of lipid deposits in Oil Red O and hematoxylin-stained aortas of LDLR<sup>−/−</sup> mice (Fig. 3D). These data are coherent with the detected increase in atherogenic TG-rich particles and LDL (Fig. 3B).

The Western-type diet also led to weight gain in both LDLR<sup>−/−</sup> and LSR<sup>+/+</sup> groups, but the differences were not statistically significant (Table 3). Interestingly, however, LSR<sup>+/+</sup> mice gained weight during the first 12 weeks of diet and remained stable, whereas LSR<sup>−/−</sup> mice gained weight during.
the first 12 weeks and continued to do so up to the 30-week final
time point. Paired t tests indicated that weight gain occurring
between 12 and 30 weeks was statistically significant (p < 0.05)
only in the LSR+/−/+ group and not in the LSR−/−/+ group. We
determined that this elevated body weight was directly corre-
lated to an increased fat mass (body weight versus fat mass, r = 0.987, p < 0.0001). Further analysis revealed that in LSR−/−/+,
but not in LSR+/−/+ mice, body weight at the end of the study was
significantly and positively correlated to both plasma TC and
TG values measured at the same time (Fig. 4), suggesting that
low hepatic LSR expression and, thus, diminished hepatic clear-
cance of remnants and LDL is directly associated with accumu-
lation of lipids in the peripheral tissues.

These in vivo data point toward LSR playing an important role
in the uptake of both TG-rich lipoproteins and LDL during
the postprandial phase and during times of dietary lipid chal-
lenge. We sought to determine in vitro the effect of LSR knock-
down by siRNA on lipoprotein endocytosis using the Hepa1–6
mouse hepatoma cell line. In these cells Western blots indi-
cated that both LSR and LDL-R were expressed (Fig. 5A).
However, LRP1 protein was not detected in immunoblots using
the same cell lysate preparations. Preliminary experiments indi-
cated that 79% knockdown in LSR mRNA expression was
achieved 24 h after transfection with 30 nM LSR siRNA as com-
pared with the negative control siRNA using GAPDH as the
reference gene (data not shown). LSR protein expression was
also clearly decreased, whereas the expression profiles of
LDL-R and LRP1 were not modified (Fig. 5A).

Lipoprotein uptake studies were performed on these cells using LDL and
VLDL labeled with DiI, a lipophilic fluorescent label that has
been used previously for measuring receptor-mediated uptake
of lipoproteins (36, 37). DiI-LDL was treated with CHD to

FIGURE 3. Plasma lipid levels, lipoprotein profile, and aorta lipid composition of LSR−/+/+ mice on Western-type diet. Eight-week-old LSR−/+/+ (n = 9) and
LSR−/−/+ (n = 12) mice were placed on a high fat, high cholesterol Western-type diet. A, at different time points (0 (before), 12 and 30 weeks on diet) blood
samples were obtained at 9 a.m. for analysis of plasma TG (top panel) and TC (bottom panel). B, plasma samples from 3 mice on the Western-type diet at t = 30
weeks were pooled and applied to a Superose 6B gel filtration column. Fractions were collected and analyzed for TG (top panel) and TC (bottom panel) to obtain
the lipoprotein profile, shown here as μg of TG or TC per fraction for LSR−/+/+ and LSR−/−/+ mice. Gel filtration analyses were run in duplicate using pooled
plasma samples from three different mice, each time with similar results; a representative profile is shown here. C, TG, TC, and PL contents were determined in
aortas isolated from LSR−/−/+ (n = 3) and LSR−/−/+ (n = 3) mice on the Western-type diet as described under “Experimental Procedures,” shown here as μg/mg
dry weight. DiI Oil Red O staining and hematoxylin counterstaining was performed on aorta cross-sections as described under “Experimental Procedures.”
Representative sections are shown here for aortas isolated from a control C57Bl/6J mice on a normal diet (left panel) and LSR−/+/+ (middle panel) and LSR−/−/+ littermates (right panel) after 30 weeks on the Western-type diet (L, lumen).
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inhibit its binding to the LDL receptor. After incubation of cells with Dil-CHD-LDL or Dil-VLDL in the presence of oleate to activate LSR, the intensity of cell labeling with Dil was decreased in the cells treated with LSR siRNA as compared with that of the controls (Fig. 5B). Image analysis on a fixed number of cells revealed that the fluorescence intensity in cells that had been transfected with LSR siRNA was significantly lower as compared with those transfected with the negative control siRNA (Fig. 5C). Therefore, specific targeted knockdown of LSR expression using siRNA led to decreased uptake of both Dil-CHD-LDL and Dil-VLDL in liver cells. These in vitro data taken together with the in vivo animal studies using the LSR−/+ mouse led us to conclude that the LSR gene product contributes significantly to receptor-mediated clearance of TG-rich particles and LDL in the liver.

DISCUSSION

We have, thus, shown that inactivation of a single allele of the candidate LSR gene in mice caused impaired clearance of dietary lipid and moderate increases in plasma TC and TG levels under physiological conditions. The ability to clear lipids after either a single dietary lipid challenge or intravenous bolus injection of Intralipid was reduced 2-fold in LSR−/+ mice. This was not due to the LDL-R as 1) hepatic LDL-R expression was not modified in LSR−/+ mice, and 2) the delay in clearance of dietary lipid was observed even when a single allele of LSR was inactivated in LDLR−/− mice. These in vivo data are consistent with our observation that postprandial, but not fasting TG levels, are significantly and inversely correlated with the number of LSR receptors expressed on hepatocyte plasma membranes (17) and demonstrate the contribution of the LSR gene product to the clearance of TG-containing particles during the postprandial phase.

Combined inactivation of a single LSR allele and the absence of the LDL-R led to postprandial triglyceridemia that was higher (5-fold) than if only a single LSR allele is inactivated and LDL-R is active (3.8-fold), suggesting a cooperativity between the two receptors in the removal of TG-rich lipoproteins. These data further support the idea that the LDL-R itself contributes to the clearance of TG-rich particles (4, 5) but cannot be the rate-limiting step. Indeed, chylomicron remnant clearance is normal in LDL-R-deficient subjects or Watanabe heritable hyperlipidemic rabbits (8, 9), indicating that although the LDL-R pathway participates, it is not the critical pathway for the removal of these lipoproteins.

Recently, selective inactivation of LRP1 in adipose tissue in mice was shown to lead to delayed clearance of postprandial lipids accompanied by decreased adiposity despite high lipase LpL activity (38), thereby preventing weight gain and insulin resistance. LRP1-mediated endocy-

TABLE 2

| Tissue lipid content in LSR+/+ and LSR−/− mice on Western-type diet | LSR+/+ | LSR−/− |
|---|---|---|
| | µg/mg dry weight, mean ± SE | µg/mg dry weight, mean ± SE |
| Liver | | |
| TG | 108.5 ± 4.95 (n = 8) | 95.1 ± 4.05 (n = 11) (p < 0.05) |
| TC | 13.9 ± 1.61 (n = 8) | 10.4 ± 1.38 (n = 11) |
| PL | 43.1 ± 5.57 (n = 8) | 44.2 ± 5.17 (n = 11) |
| Heart | | |
| TG | 39.4 ± 3.01 (n = 7) | 37.9 ± 2.20 (n = 12) |
| TC | 9.7 ± 0.66 (n = 7) | 9.2 ± 0.61 (n = 12) |
| PL | 40.1 ± 2.87 (n = 7) | 45.9 ± 3.19 (n = 12) |
| Adipose tissue | | |
| TG | 20.8 ± 2.09 (n = 6) | 17.7 ± 1.80 (n = 12) |
| PL | 2.0 ± 0.12 (n = 6) | 1.7 ± 0.29 (n = 12) |
| Skeletal muscle | | |
| TG | 164.2 ± 16.43 (n = 7) | 142.1 ± 21.01 (n = 12) |
| TC | 5.0 ± 0.69 (n = 7) | 4.8 ± 0.58 (n = 12) |
| PL | 7.6 ± 0.61 (n = 7) | 8.8 ± 0.83 (n = 12) |

**TABLE 3**

| Body weights of LSR+/+ and LSR−/− on Western-type diet | LSR+/+ | LSR−/− |
|---|---|---|
| | | |
| Weeks on diet | Body weight | Body weight |
| | n = 9 | n = 12 |
| 0 | 23.8 ± 0.8 | 24.0 ± 0.6 |
| 12 | 33.0 ± 1.5 | 34.8 ± 1.4 |
| 30 | 32.9 ± 2.8 | 38.4 ± 2.4* |

* Student’s paired t test between values for 12 and 30 wks, p < 0.05.

**FIGURE 4.** Correlation between body weight and plasma TG or TC levels in LSR−/− mice on Western-type diet. Correlation analysis was performed on the body weight and plasma TG (A and B) and TC (C and D) levels in LSR−/− (A and C) and LSR+/+ (B and D) mice after 30 weeks on a Western-type diet. Correlation coefficients (r) and p values for r statistical significance are indicated in each graph.
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We previously demonstrated that FFAs inhibit LDL binding to the LDL-R (39). In the space of Disse, cholesterol-rich particles that do not generate FFAs are endocytosed primarily through the LDL-R. We propose that under conditions in which lipolytic products are being generated, LSR represents an alternate pathway for LDL, with the caveat that LDL be present at sufficiently high concentrations to compete with the higher affinity TG-rich particles for endocytic uptake through LSR. This situation would be predicted to occur in cases where the LDL-R is defective or inactive and, thus, provides a straightforward explanation for the large amounts of LDL removed daily by the liver in subjects lacking the LDL-R (6). This is further reinforced by in vitro data showing that CHD treatment of LDL used to inhibit its binding to LDL-R and for measuring in vivo LDL-R independent clearance (40) did not alter LDL binding to LSR (15, 18). In addition, Dil-CHD-LDL uptake in Hepa1–6 cells in the presence of oleate was significantly reduced after siRNA-mediated knockdown of LSR expression. The finding that LSR is an alternate pathway for LDL uptake also provides a novel strategy to reduce plasma cholesterol in subjects with familial hypercholesterolemia. Pharmacological agents that would mimic FFA-activating effects of LSR are likely to decrease LDL levels in familial hypercholesterolemia patients.

Our conclusion that LSR contributes to the removal of apoB-containing lipoproteins is not in contradiction but, rather, is complementary to the recent findings that reduced liver heparan sulfate sulfation impairs apoB-containing lipoprotein clearance (14). Upon entry into the space of Disse, TG-rich lipoproteins bind through apoE to HSPGs and undergo further lipolysis. It is our current view that this leads to production of FFAs that activates LSR and inhibits the LDL-R (39). We demonstrate here that dietary lipid clearance is impaired in LSR/−/− mice despite normal post-heparin lipolytic activities. Therefore, although processing in the space of Disse is necessary for normal clearance, LSR acts downstream of the lipolytic process as a hepatic receptor for the removal of TG-rich particles from the circulation. Hypercholesterolemia was aggravated in LDLR/−/− mice in which sulfation of heparan sulfate was

tosis of TG-rich particles was proposed to account for a large part of dietary TG clearance in the adipose tissue. However, unlike the liver, adipose tissue capillary endothelium is nonfenestrated and may render entry of large, bulky chylomicrons and remnants difficult. The data, however, unambiguously show that LRP1 is necessary for proper processing of functional LpL, as in its absence, the enzyme is present but fails to deliver lipid to the underlying adipose tissue (38). If this is indeed the function of LRP1 in adipose tissue, a similar mechanism of action may exist in the liver and explain the hyperlipidemic effect in hepatic LRP1 and LDL-R inactive mice (11).

We did not find any detectable change in protein expression of LDL-R or LRP1 in the skeletal muscle, adipose tissue, or brain of LSR/−/− mice (data not shown). Interestingly, we did notice a tendency toward hepatic LRP1 protein expression to actually increase in livers from LSR/−/− mice. This could not explain the increased postprandial lipemia and decreased lipid clearance that was observed in these animals. It is possible that LRP1 may serve as a backup pathway, as it does for the LDL-R. LSR, by virtue of its activation of its lipolytic product, FFAs, is dependent on the processing of lipoproteins by lipases in the space of Disse. Therefore, if LRP1 has a similar function in the liver as in the adipose tissue, we could speculate that the regulation of LRP1 and LSR expression and activities may be related in the liver in such a way to ensure lipoprotein processing and uptake by the hepatocyte.

Dietary lipid challenge in the form of a Western-type diet magnified the hypercholesterolemia in LSR−/− mice and led to a significant change in the lipoprotein profile. Greater accumulation of TC and PL in the aortas of these mice was consistent with the accumulation of atherogenic TG-rich particles as well as LDL. Real-time RT-PCR analysis at the end of the dietary study revealed that only LSR expression was significantly reduced. LDL-R mRNA was only slightly increased, and there was no change in LRP1 mRNA expression (data not shown). Therefore, LSR contributes to the clearance of not only chylomicron remnants but LDL as well under conditions of high dietary lipid load.

FIGURE 5. Dil-CHD-LDL and Dil-VLDL uptake in Hepa1–6 cells transfected with siRNA directed against LSR. A, Western blot of cell lysates recovered from Hepa1–6 h after transfection with 30 nm control siRNA or siRNA directed against LSR, as described under “Experimental Procedures.” Immunoblots were performed to detect LSR, LDL-R, and LRP1. B, lipoprotein uptake studies were performed as described under “Experimental Procedures” using Dil-CHD-LDL or Dil-VLDL. Representative pictures are shown for Dil-CHD-LDL or Dil-VLDL uptake in cells transfected with control or LSR siRNAs (number of cells per frame is 45 and 34 for Dil-CHD-LDL control and LSR siRNA, respectively, and 66 and 58 for Dil-VLDL control and LSR siRNA, respectively). C, image analysis was also performed on photographs obtained in Fig. 4B using the same number of cells per field (n = 4 different fields) for control and LSR siRNA-transfected Hepa1–6. Results are presented as the mean ± S.D. All experiments were performed in duplicate.

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reduced (14), suggesting that either HSPGs themselves and/or HSPG-dependent processes are involved in the LDL-R independent uptake of LDL. This would be consistent with LSR representing an HSPG-dependent pathway, relying on functional HSPGs for lipolytic processing of particles and production of FFAs for its activation as hepatic receptor for both remnants and LDL.

We have published a series of evidence demonstrating that the activation of LSR by free fatty acids leads to the binding, uptake, and degradation of apoB- or apoE-containing lipoprotein fibroblasts and rodent hepatocytes (15,17–19). We have also shown that transient transfection of the α and β subunits of the LSR gene lead to the reconstitution of LSR activity as a receptor for lipoproteins (18). We now have data demonstrating decreased uptake of Dil-CHD-LDL and Dil-VLDL after targeted knockdown of LSR in a mouse liver cell line. Under these conditions, neither the LDL-R nor LRP1 expression was modified. Furthermore, the cell studies were performed in the presence of oleate, which activates LSR but also inhibits LDL-R activity. These in vitro data are consistent with the findings of decreased lipid clearance in LSR<sup>-/-</sup> mice and the accumulation of TG-rich lipoproteins and LDL in the circulation of these animals when challenged with a lipid load in the form of a Western-type diet. Taken together, these results clearly show that the LSR gene product has a direct effect on lipoprotein clearance.

Detailed analysis of the lipoprotein profile of LSR<sup>-/-</sup> mice challenged with dietary lipid suggests that TG-enriched HDL also accumulates in the plasma. We, therefore, cannot entirely rule out the possibility that LSR also contributes to part of HDL clearance. LSR displays very low affinity for apoE-free HDL (15) nor do liver HSPGs themselves seem to be involved in HDL metabolism (14). A recent study identified a glycosylphosphatidylinositol-anchored high density lipoprotein-binding protein 1 (GPIHBP1) expressed in heart and adipose tissue and involved in lipolytic processing of chylomicrons (41). It is possible that a similar situation may exist in the liver involving the processing of HDL through the hepatic lipase system. Further studies are needed to clarify the role of LSR in HDL metabolism.

In LSR<sup>-/-</sup> animals on a high fat Western-type diet, weight gain was directly proportional to the degree of hyperlipidemia, suggesting that partial LSR inactivation decreases liver clearance of lipoproteins, thereby favoring accumulation of lipids in adipose tissue and, as a consequence, increase in body mass. Continued monitoring of LSR<sup>-/-</sup> mice showed significantly increased body weight as fat mass in older animals maintained even on a normal diet. This is in line with the study of adipose tissue specific LRP1 inactivation that provides clear evidence that preserving a proper balance of lipid deposition between peripheral tissues and the liver is critical for maintaining energy homeostasis (38). Our studies further indicate that LSR activity and expression are decreased in different obese mouse models, including the ob/ob mice. If placed under replacement leptin therapy to induce weight loss, a concomitant increase in LSR expression and normalization of elevated postprandial lipemia was observed. Therefore, LSR represents a new molecular mechanism contributing to the regulation of lipid partitioning between the liver and the peripheral tissues.

The contribution of the LSR gene product to the removal of atherogenic TG-rich particles and LDL has now been clearly demonstrated in vivo in animals with only one single functional LSR allele. LSR affinity for β-VLDL from apoE2/2 patients with type III hyperlipidemia is reduced relative to VLDL from apoE3/3 subject (19). This fact combined with the observation of a direct correlation between weight gain and hyperlipidemia observed in LSR<sup>-/-</sup> mice on a Western-type diet points to a coherence of the LSR model with the type III hyperlipidemia 2-hit hypothesis, which associates apoE2/2 as a susceptibility factor with a second hit, e.g. obesity, to cause hyperlipidemia (2, 42). In view of the embryonic lethality of complete LSR inactivation, it is unlikely that human subjects with homozygous LSR defects will ever be identified. However, it is possible that human subjects with genetic polymorphisms of the LSR gene leading to impaired receptor function causing mixed hyperlipidemia will be identified in the future. Further studies of LSR will bring insight into the molecular mechanisms linking hyperlipidemia to obesity and atherosclerosis and allow the development of new strategies for the prevention or treatment of these lipid-related disorders.

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