Hepatic Remnant Lipoprotein Clearance by Heparan Sulfate Proteoglycans and Low-Density Lipoprotein Receptors Depend on Dietary Conditions in Mice

Erin M. Foley,* Philip L.S.M. Gordts,* Kristin I. Stanford,* Jon C. Gonzales, Roger Lawrence, Nicole Stoddard, Jeffrey D. Esko

Objective—Chylomicron and very low-density lipoprotein remnants are cleared from the circulation in the liver by heparan sulfate proteoglycan (HSPG) receptors (syndecan-1), the low-density lipoprotein receptor (LDLR), and LDLR-related protein-1 (LRP1), but the relative contribution of each class of receptors under different dietary conditions remains unclear.

Approach and Results—Triglyceride-rich lipoprotein clearance was measured in AlbCre*Ndst1^{f/f}, Ldlr^{−/−}, and AlbCre*Lrp1^{f/f} mice and mice containing combinations of these mutations. Triglyceride measurements in single and double mutant mice showed that HSPGs and LDLR dominate clearance under fasting conditions and postprandial conditions, but LRP1 contributes significantly when LDLR is absent. Mice lacking hepatic expression of all 3 receptors (AlbCre*Ndst1^{f/f} Lrp1^{−/−} Ldlr^{−/−}) displayed dramatic hyperlipidemia (870±270 mg triglyceride/dL; 1300±350 mg of total cholesterol/dL) and exhibited persistent elevated postprandial triglyceride levels because of reduced hepatic clearance. Analysis of the particles accumulating in mutants showed that HSPGs preferentially clear a subset of small triglyceride-rich lipoproteins (∼20–40 nm diameter), whereas LDLR and LRP1 clear larger particles (∼40–60 nm diameter). Finally, we show that HSPGs play a major role in clearance of triglyceride-rich lipoproteins in mice fed normal chow or under postprandial conditions but seem to play a less significant role on a high-fat diet.

Conclusions—These data show that HSPGs, LDLR, and LRP1 clear distinct subsets of particles, that HSPGs work independently of LDLR and LRP1, and that HSPGs, LDLR, and LRP1 are the 3 major hepatic triglyceride-rich lipoprotein clearance receptors in mice. (Arterioscler Thromb Vasc Biol. 2013;33:2065-2074.)

Key Words: cholesterol ■ heparan sulfate proteoglycans ■ lipoproteins ■ low-density lipoprotein receptor–related protein-1 ■ low-density lipoprotein receptor ■ syndecan-1 ■ triglycerides

Hypertriglyceridemia is characterized by the accumulation of triglyceride-rich lipoproteins (TRLs) in the blood. This condition affects 10% to 20% of the population in Western countries and increases the risk of atherosclerosis, coronary artery disease, and pancreatitis.1-4

TRLs consist of intestinal chylomicrons derived from dietary fats and very low-density lipoproteins (VLDLs) from the liver, as well as remnant particles resulting from the lipolytic processing of these lipoproteins in the peripheral circulation. Clearance of TRL remnants occurs in the liver via a multi-step process.5-7

The particles first pass through the fenestrated endothelium of the liver sinusoids and become sequestered in the perisinusoidal space of Disse, where they undergo further processing by lipoprotein lipase and hepatic lipase.6 Particles are then cleared by endocytic receptors on the surface of hepatocytes, leading to their lysosomal catabolism. Several receptors for remnant lipoproteins have been identified, including the low-density lipoprotein receptor (LDLR),8 members of the LDLR-related protein family (LRP1 and LRP5),9,10 syndecan-1,11 a type of heparan sulfate proteoglycan (HSPG), the very low-density lipoprotein receptor (VLDLR),12 scavenger receptor B1,13 and lipolysis-stimulated receptor.14 Understanding the relative contribution of these receptors to clearance, their coordinate regulation, and their role in maintaining plasma lipids under different dietary conditions is important because the information could help focus future drug development efforts to reduce hypertriglyceridemia.

In the present study, we sought to understand the relative contributions of HSPGs, LDLR, and LRP1 to clearance of remnant lipoproteins under various dietary conditions in mice. To this end, we interbred mice deficient in Ldlr, Lrp1, and the gene N-acetylglucosamine...
N-deacetylase-N-sulfotransferase-1 (Ndst1), which encodes an enzyme that affects the synthesis of the heparan sulfate chains on syndecan-1 and other hepatic proteoglycans, producing double mutants lacking various combinations of the 3 receptors, and the triple mutant lacking all 3 receptors. HSPGs and LDLR are the 2 dominant receptors mediating TRL clearance under fasting conditions and in the postprandial state, but LRP1 can substitute for LDLR in LDLR-deficient mice. No coordinate regulation of the 3 receptors was noted under these conditions, demonstrating their independence. HSPGs seem to bind and internalize a distinct subset of particles compared with LDLR and LRP1. Interestingly, when fed a high-fat Western diet, mice rely more on LDLR and LRP1 for remnant TRL clearance.

Materials and Methods

Materials and methods are available in the online-only Supplement.

Results

Inactivation of Ldlr, Lrp1, and Ndst1

We previously described mice in which the Cre transgene under the control of the albumin promoter was used to inactivate the heparan sulfate biosynthetic enzyme Ndst1 selectively in hepatocytes.15 Ndst1 affects the sulfation of all HSPGs including syndecan-1, the primary hepatic proteoglycan receptor.11 Like Sdc1−/− mice, AlbCre+Ndst1f/f mice display a 2-fold accumulation of fasting triglycerides and have delayed clearance of postprandial triglycerides because of loss of sulfation of the heparan sulfate chains located on syndecan-1 specifically in the liver.11 Syndecan-1 knockout mice were not used in this study because a conditional mutant has not been described. Liver-specific AlbCre+Ndst1f/f mice are designated by genotype or as HSPG receptor deficient throughout the rest of this study.

HSPG receptor–deficient mice were bred with Ldlr systemic knockout mice (Ldlr−/−) or mice bearing a floxed allele of Lrp1 (Lrp1f/f) to produce all combinations of double and triple receptor–deficient mice. Compound mutant mice were viable, fertile, and produced litters of normal size and with the expected Mendelian ratios of genotypes. To differentiate the contribution of the LDLR family of receptors and HSPGs, we chose to focus on 4 genotypes: Cre+Ndst1f/f (wild type), Cre+Ndst1f/f (HSPG receptor deficient), Cre+Lrp1f/fLdlr−/− (HSPG receptor only), and Cre+Ndst1f/fLrp1f/fLdlr−/− (triple receptor deficient).

To verify that the Cre transgene was sufficient to mediate recombination of 2 separate floxed genes (Ndst1f/f and Lrp1f/f), we analyzed several markers. First, we purified heparan sulfate from isolated hepatocytes and determined its disaccharide composition using mass spectrometry.16 Glucosamine N-sulfation was decreased from 43 N-sulfate groups/100 disaccharides in wild-type hepatocytes (sum of D0S0, D0S6, D2S0, and D2S6 in Table 1) to 19 sulfates/100 disaccharides in triple receptor–deficient hepatocytes. The reduction in sulfation was similar to that observed previously and in hepatocytes from Cre+Ndst1f/f mice (Table 1).15 Next, we showed by Western blotting that LRP1 expression was ablated in isolated hepatocytes from Cre+Lrp1f/fLdlr−/− and Cre+Ndst1f/fLrp1f/fLdlr−/− mice (Figure 1A). LRP1 and LDLR expression exhibited some variability in wild-type and HSPG receptor–deficient mice. However, when normalized to β-actin their levels of expression were not significantly different in any of the strains, indicating that altering HSPG receptor activity by inactivation of Ndst1 did not cause any compensatory changes in these receptors (Figure 1B and 1C). Similarly, hepatic syndecan-1 expression was not affected by inactivation of Ldlr, Lrp1, or Ndst1 and any combination of mutations in these genes (Figure 1D). Thus, the expression of syndecan-1 and LDLR family members does not seem to occur in a coordinated manner in hepatocytes under these conditions.

Hyperlipidemia in Compound Mutant Mice

To study the relative contribution of the 3 receptors to clearance of TRLs, we first analyzed plasma triglyceride and cholesterol in blood drawn from overnight-fasted animals raised on a normal chow diet (Figure 2; Table 2). HSPG receptor–deficient mice (Cre+Ndst1f/f) accumulated triglycerides compared with wild-type animals (Figure 2A; 69±26 versus 43±10 mg/dL, respectively; P<0.01), as shown previously.15 In contrast, mice lacking hepatic LRPI (Cre+Lrp1f/f) did not accumulate plasma triglycerides (Figure 2A). Double mutant mice deficient for both the HSPG receptors and the LRPI (Cre+Ndst1f/fLrp1f/f) also did not accumulate fasting triglycerides (66±20 mg/dL) beyond that observed in Cre+Ndst1f/f mice (P=0.7). Mice lacking LDLR (Ldlr−/−) accumulated triglycerides

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<th>Strain</th>
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<tr>
<td></td>
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<tr>
<td>Cre+Ndst1f/f (wild type)</td>
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<td>Cre+Ndst1f/fLrp1f/fLdlr−/− (triple receptor deficient)</td>
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Heparan sulfate purified from isolated hepatocytes was digested with heparin lyases I, II, and III. The resulting disaccharides were derivatized with isotopically labeled aniline and quantified by mass spectrometry (see Methods in the online-only Data Supplement). Disaccharides are designated using the code established in reference 17. Those containing the letter S contain a N-sulfate group. HSPG indicates heparan sulfate proteoglycan; LDLR, low-density lipoprotein receptor; LRP1, LDLR-related protein-1; and Ndst1, N-acetylglicosamine N-deacetylase-N-sulfotransferase-1.
4-fold over wild type (Figure 2B; 181±59 mg/dL [n=9]; P<0.0001). Inactivation of Ndst1 and Ldlr that is, the mutant expressing only LRP1 (Cre^+Ndst1^+/^−^/Ldlr^−^/−) resulted in further elevation in plasma triglyceride compared with either Ldlr^−^/− or Ndst1^−^/− (415±218 mg/dL [n=9]). Mice expressing only HSPG receptors (Cre^+Lrp1^+/^+/Ldlr^−^/− also exhibited elevated plasma triglycerides (548±140 mg/dL [n=9]) compared with Ldlr^−^/− and Lrp^−^/− mice (Figure 2B; P<0.0001). The triple receptor mutant (Cre^+Ndst1^+/^+/Lrp1^+/^+/Ldlr^−^/−) accumulated even greater levels of triglycerides (874±270 mg/dL [n=14]; P<0.001 compared with Cre^+Ndst1^+/^+/Ldlr^−^/− or Cre^+Lrp1^+/^+/Ldlr^−^/−). These findings suggest that LRP1 does not play a major role in TRL clearance when LDLR is present, consistent with previous studies.9,18

HSPGs are not thought to contribute to clearance of cholesterol-rich lipoprotein particles on the basis of the observation that HSPG receptor–deficient mice (Cre^+^/^+/Ndst1^−^/−) do not exhibit elevated plasma cholesterol levels compared with wild-type mice (Figure 2B; 67±9 mg/dL in wild type [n=12] versus 71±22 mg/dL in Cre^+^/^+/Ndst1^−^/− [n=13]; P=0.6).15 LRP1-deficient mice (Cre^+^/^+/Lrp1^−^/−) also did not exhibit elevated plasma cholesterol (71±15 mg/dL [n=7]) compared with the wild type (P=0.5), presumably because of compensation by LDLR. Mice lacking the HSPG receptors and LRP1 (Cre^+^/^+/Ndst1^−^/−/Lrp1^−^/−) also did not accumulate plasma cholesterol compared with wild type (52±7 mg/dL [n=12]; P<0.001). However, mice lacking HSPG receptors and LDLR (Cre^+^/^+/Ndst1^−^/−/Ldlr^−^/−) had ≈2-fold higher cholesterol (477±160 mg/dL [n=10]) than Ldlr^−^/− mice (291±63 mg/dL; P<0.01). Triple receptor–deficient mice (Cre^+^/^+/Ndst1^−^/−/Lrp1^−^/−/Ldlr^−^/−) exhibited higher fasting plasma cholesterol (1308±346 mg/dL [n=6]) than HSPG receptor–only mice (Cre^+^/^+/Lrp1^−^/−/Ldlr^−^/−; 779±149 mg/dL [n=9]; P<0.01). Thus, HSPGs can also mediate clearance of cholesterol-rich lipoproteins that LDLR family members normally clear.

HSPGs and LDL Receptor Family Members Mediate Clearance of TRLs of Unique Size

The accumulation of TRLs in single mutants deficient either in HSPGs or in LDLR suggested that these receptors can clear different subsets of TRLs. We recently showed that HSPG receptor–deficient mice accumulate particles enriched in apoAIV, whereas animals lacking LDLR and LRP1 do not.19 To study the composition of these particles in greater detail,
we analyzed samples by agarose electrophoresis, gel filtration, ultracentrifugation, and electron microscopy.

First, we analyzed TRLs (δ<1.006 g/mL) using nondenaturing agarose gel electrophoresis, which separates particles dependent on size and charge (Figure 3A). Wild-type TRLs migrated furthest, whereas the migration of TRLs that accumulated in HSPG receptor–deficient mice (Cre+Ndst1f/f) was retarded. The migration of TRLs from HSPG receptor–only animals (Cre+Lrp1f/fLdlr−/−) was even more delayed. Migration of TRLs from triple receptor mutant mice was intermediate between that observed in samples from the HSPG receptor–only and HSPG receptor–deficient mice. These findings confirm that particles of unique size and charge properties accumulate in each mutant and are consistent with an earlier study showing that mutants lacking LDLR accumulate large TRLs.8

To analyze the size of the accumulated particles, we fractionated whole plasma from fasted mice by gel filtration fast-phase liquid chromatography. HSPG receptor–deficient mice (Cre+Ndst1f/f) accumulated large TRLs consistent in size with chylomicron, VLDL, and their remnants. Similar sized triglyceride-rich particles were recovered from HSPG receptor–only mice (Cre+Lrp1f/fLdlr−/−), as well as particles resembling LDL or intermediate density lipoproteins in size. The accumulation of LDL/intermediate density lipoprotein–like particles was further accentuated in the triple receptor–deficient mutants. No significant change in HDL triglycerides

Table 2. Fasting Lipid Levels on a Chow Diet

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<th>Genotype</th>
<th>Triglyceride (mg/dL plasma)</th>
<th>Cholesterol (mg/dL plasma)</th>
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<tr>
<td>Cre−Ndst1f/f (wild type)</td>
<td>43±10</td>
<td>67±9</td>
</tr>
<tr>
<td>Cre+Ndst1f/f (HSPG receptor deficient)</td>
<td>69±26</td>
<td>71±22</td>
</tr>
<tr>
<td>Cre+Lrp1f/f (Lrp1 deficient)</td>
<td>33±5</td>
<td>71±15</td>
</tr>
<tr>
<td>Ldlr−/− (LDLR deficient)</td>
<td>181±59</td>
<td>291±63</td>
</tr>
<tr>
<td>Cre+Ndst1f/fLrp1f/f (LDLR only)</td>
<td>66±20</td>
<td>52±7</td>
</tr>
<tr>
<td>Cre−Ndst1f/fLdlr−/− (Lrp1 only)</td>
<td>415±218</td>
<td>477±160</td>
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<td>Cre+Lrp1f/fLdlr−/− (HSPG receptor only)</td>
<td>548±148</td>
<td>779±149</td>
</tr>
<tr>
<td>Cre+Ndst1f/fLrp1f/fLdlr−/− (triple receptor deficient)</td>
<td>874±270</td>
<td>1308±346</td>
</tr>
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</table>

All values are expressed as average±SE. HSPG indicates heparan sulfate proteoglycan; LDLR, low-density lipoprotein receptor; LRP1, LDLR-related protein-1; and Ndst1, N-acetylglucosamine N-deacetylase-N-sulfotransferase-1.
or cholesterol was noted in any of the mutants by this method (Figure 3B and 3C).

Higher resolution was achieved by evaluating the size of TRLs ($\delta < 1.006$ g/mL) by transmission electron microscopy (Figure 3D). Mice lacking HSPG receptors accumulated a range of particles, predominantly 20 to 50 nm in diameter. In contrast, the mutant lacking both LDLR family members (Cre$^{+}$Lrp$^{1/f}$Ldlr$^{-/-}$) accumulated somewhat larger TRLs (30–60 nm diameter). The TRLs from triple receptor–deficient mice essentially behaved like the particles derived from Cre$^{+}$Lrp$^{1/f}$Ldlr$^{-/-}$ mice. The altered distribution of particle size was significant between all groups except between triple receptor–deficient and Cre$^{+}$Lrp$^{1/f}$Ldlr$^{-/-}$ mice (Kruskal–Wallis test, $P < 0.001$).

When we compared the protein:triglyceride ratio in the TRLs, we observed that the ratio was smaller in Cre$^{+}$Ndst1$^{ff}$ mice compared with Cre$^{+}$Lrp$^{1/f}$Ldlr$^{-/-}$ and the triple receptor–deficient mice (0.38, 0.48, and 0.47, respectively). Thus, Cre$^{+}$Ndst1$^{ff}$ mice accumulate smaller TRL particles with lower protein content.

Finally, purified TRLs ($\delta < 1.006$ g/mL) were analyzed by gradient SDS-PAGE, and the individual apolipoproteins were visualized by silver staining. In all mutants, the TRLs contained apoB48, apoB100, apoE, and various apos (Figure 3E). In Cre$^{+}$Ndst1$^{ff}$ mice, both apoB48 and apoB100-containing lipoproteins accumulated. Combined inactivation of Ldlr and Lrp1 (Cre$^{+}$Lrp$^{1/f}$Ldlr$^{-/-}$ and Cre$^{+}$Ndst1$^{ff}$Lrp$^{1/f}$Ldlr$^{-/-}$) led to accumulation of apoB48-containing lipoproteins similar to the observation by Rohlmann et al.9 Particles containing apoE accumulated in Cre$^{+}$Ndst1$^{ff}$, Cre$^{+}$Lrp$^{1/f}$Ldlr$^{-/-}$, and triple receptor–deficient mice. The apoE accumulation was more profound in Cre$^{+}$Lrp$^{1/f}$Ldlr$^{-/-}$ and triple receptor–deficient mice compared with the Cre$^{+}$Ndst1$^{ff}$ mutant. We further characterized the apolipoprotein composition of accumulated TRLs.
particles by Western blot (Figure 3F). Both Cre\textsuperscript{+}\textit{Ndst1}\textsuperscript{f/f} and triple receptor–deficient mice accumulated apoAV-containing lipoproteins, whereas Cre\textsuperscript{+}\textit{Lrp1}\textsuperscript{f/f}\textit{Ldlr}\textsuperscript{−/−} mice did not. These findings are consistent with recent studies showing that HSPGs clear lipoprotein particles containing apoAV.

Together, these data suggest that HSPGs preferentially clear a subset of small TRL particles enriched in apoAV and apoE, whereas members of the LDLR family of receptors clear large particles, enriched in apoE, as reported previously.

**Postprandial Triglyceride Clearance and Liver Uptake Is Significantly Delayed in Compound Mutant Mice**

We next examined how receptor inactivation affected postprandial clearance. Fasted mice were given a bolus of corn oil by oral gavage, and blood was sampled at various time points post gavage to measure appearance and disappearance of triglycerides in the circulation. As shown in Figure 4A, HSPG receptor–deficient mice (Cre\textsuperscript{+}\textit{Ndst1}\textsuperscript{f/f}) had delayed clearance of postprandial triglycerides compared with the wild type (area under the curve=2244 versus 864, respectively). HSPG receptor–only mice (Cre\textsuperscript{+}\textit{Lrp1}\textsuperscript{f/f}\textit{Ldlr}\textsuperscript{−/−}) had an even greater delay in clearance (area under the curve=5406). Triple receptor–deficient mice had severely impaired clearance (area under the curve=11033), and triglyceride levels remained high even 8 hours post gavage.

To verify that this delay in postprandial clearance was because of altered liver clearance, we performed a \textsuperscript{1}H\textit{J}\text{retinol} excision study. In this experiment, the animals were gavaged with \textsuperscript{1}H\text{retinol} mixed with corn oil. In the intestine, \textsuperscript{1}H\text{retinol} is packaged into chylomicrons and these radioactive particles are subsequently cleared from the circulation in the liver in a time-dependent manner. At 8 hours post gavage counts remained high in plasma from the HSPG receptor–deficient mice and even greater in Cre\textsuperscript{+}\textit{Lrp1}\textsuperscript{f/f}\textit{Ldlr}\textsuperscript{−/−} and triple receptor–deficient mice (Figure 4B). In a separate experiment, counts remained significantly elevated in plasma from the triple receptor–deficient mice even 24-hour post gavage. Correspondingly, liver uptake of the radioactive TRLs was decreased compared with the wild type, with very low uptake occurring in livers from triple receptor–deficient mice (Figure 4C). There was no significant difference in uptake of radioactive counts into skeletal muscle or heart between the wild type and any of the mutants (Figure 4D and 4E), confirming that the observed persistence of postprandial triglycerides was because of defective clearance by hepatic receptors.

**High-Fat Diet Shifts the Relative Contribution of HSPG Receptors and LDL Receptor Family Members to TRL Clearance**

In a final set of experiments we examined how the various mutants would respond to a Western diet, high in fat and cholesterol content. Twelve-week-old mice were put on the Western diet and blood was taken for lipid analysis at 0, 1, 4, and 6 weeks after a short fasting period (Figure 5; Table 3). The Western diet induced little to no change in triglyceride levels of wild-type mice (65±3 mg/dL at t=0–82±10 mg/dL after 6 weeks on the Western diet; \(P>0.1\)). HSPG receptor–deficient mice exhibited a 2-fold increase in triglyceride levels after 6 weeks on the Western diet (82±7–172±30 mg/dL; \(P<0.05\)). This change was mild in comparison with the increase seen in mice lacking LDLR and LRPI. In the absence of these receptors, plasma triglycerides increased at all time points, from 497±39 at the start of the Western diet to 1900±135 mg/dL after 6 weeks. Inactivation of all 3 receptors increased plasma triglyceride to a similar level from 64±57 to 1850±170 mg/dL.

The Western diet also increased plasma cholesterol in Cre\textsuperscript{+}\textit{Lrp1}\textsuperscript{f/f}\textit{Ldlr}\textsuperscript{−/−} mice from 550±40 mg/dL at the start of the experiment to 2500±110 mg/dL after 6 weeks (\(P<0.001\)). The triple mutant was affected similarly (710±60 mg/dL increasing to 2500±220 mg/dL; \(P<0.001\)). The majority of the cholesterol was associated with the LDL fraction on the basis of gel filtration (Figure 5E). Plasma cholesterol levels increased slightly during high-fat feeding in both wild-type (72±4–130±14) and HSPG receptor–deficient mice (90±8–200±37; Figure 5b). Apolipoprotein analysis of purified TRLs (d<1.006 g/mL) by gradient SDS-PAGE and silver staining showed that the Cre\textsuperscript{+}\textit{Ndst1}\textsuperscript{f/f} mutant accumulated apoB\textsubscript{48} and apoE-containing lipoproteins (Figure 5C), whereas mutants lacking \textit{Ldlr} (Cre\textsuperscript{+}\textit{Lrp1}\textsuperscript{f/f}\textit{Ldlr}\textsuperscript{−/−} and Cre\textsuperscript{+}\textit{Ndst1}\textsuperscript{f/f}\textit{Lrp1}\textsuperscript{f/f}\textit{Ldlr}\textsuperscript{−/−}) accumulated apoB\textsubscript{100} and apoE-containing lipoproteins.

The Western diet did not induce changes in hepatic LDLR and LRPI expression in HSPG receptor–deficient mice (Figure 5E). Examination of other receptors showed that the Western diet did not affect hepatic scavenger receptor B1 expression in single, double, and triple mutants (Cre\textsuperscript{+}\textit{Ndst1}\textsuperscript{f/f}, 0.66±0.16 AU; [n=3]; Cre\textsuperscript{+}\textit{Ndst1}\textsuperscript{f/f}, 0.92±0.05 AU [n=3]; Cre\textsuperscript{+}\textit{Lrp1}\textsuperscript{f/f}\textit{Ldlr}\textsuperscript{−/−}, 0.60±0.16 AU [n=3]; and Cre\textsuperscript{+}\textit{Ndst1}\textsuperscript{f/f}\textit{Lrp1}\textsuperscript{f/f}\textit{Ldlr}\textsuperscript{−/−}, 0.53±0.16 AU [n=3]; Figure 5E). Lipolysis-stimulated receptor expression was also not altered (Cre\textsuperscript{+}\textit{Ndst1}\textsuperscript{f/f}, 0.49±0.19 AU [n=3]; Cre\textsuperscript{+}\textit{Ndst1}\textsuperscript{f/f}, 0.68±0.07 AU [n=3]; Cre\textsuperscript{+}\textit{Lrp1}\textsuperscript{f/f}\textit{Ldlr}\textsuperscript{−/−}, 0.46±0.13 AU [n=3]; and Cre\textsuperscript{+}\textit{Ndst1}\textsuperscript{f/f}\textit{Lrp1}\textsuperscript{f/f}\textit{Ldlr}\textsuperscript{−/−}, 0.34±0.11 AU [n=3]; Figure 5F). Together the data suggest that the LDLR family members play a dominant role in TRL clearance when mice consume a high-fat/high-cholesterol diet, whereas HSPGs play a more significant role when the animals are fed normal chow.

**Discussion**

A large body of work has established a role for HSPGs in hepatic clearance of TRL remnants. In cultured cells, HSPGs can interact directly with apolipoproteins and lipases and facilitate their internalization by endocytosis. Reduced plasma clearance rates and reduced hepatic uptake of labeled VLDL have been observed in mice after intravenous infusion with agents that neutralize heparan sulfate such as heparinase, heparin, suramin, or lactoferrin. ApoE variants have been described in which patients exhibit severe hyperlipoproteinemia, which has been associated with altered binding to heparan sulfate. Recently, we showed that Cre-mediated inactivation of \textit{Ndst1} or \textit{urocyclin 2-O-sulfotransferase (Hs2st)} in hepatocytes results in the accumulation of fasting triglycerides and delayed clearance of intestinally derived lipoproteins because of undersulfuration of heparan sulfate. In subsequent studies, we identified syndecan-1 as the primary HSPG receptor for TRLs in mouse and human hepatocytes.
Members of the LDL receptor family have also been demonstrated to act as receptors for remnant lipoprotein clearance. LDLR was originally characterized as a receptor for cholesterol-rich apoB100- and apoE-containing particles such as LDL. Mice, rabbits, and humans deficient for LDLR have only modest accumulation of triglycerides, suggesting a less dominant role for LDLR in TRL metabolism as compared with LDL metabolism.8,30,31 Hepatocytes also express LRP1, which was thought to participate in clearance of apoE-enriched apoB_{48}-bearing lipoproteins, including TRLs.32 However, hepatocyte-specific ablation of Lrp1 does not result in accumulation of fasting triglycerides or cholesterol (Figure 1; Table 2). A role for LRP1 becomes evident when LDLR is deficient (eg, in Cre^{Lrp1}^{+/f/f}Ldlr^{-/-} mice and when Lrp1 was inactivated by injection of Adenoviral-Cre).9 In other studies, in vivo inhibition of LDLR was achieved using tail vein injection of a blocking antibody.33 This treatment reduced plasma clearance of TRLs by ≈45%, whereas injection of the receptor-associated protein, an inhibitor of LRP1, reduced plasma clearance by 55%. When the LDLR blocking antibody and receptor-associated protein were administered together, plasma removal was only decreased by 60%, an incremental effect at best. In a different study, mice with systemic deletions of Vldlr and Ldlr were bred to mice in which Lrp1 was inactivated by an inducible Cre.34 VLDLR is not expressed in the liver and has been shown to mediate TRL clearance in tissues active in fatty acid metabolism (heart, adipose, and skeletal muscle). Notably, heparin blocked the association of DiI-labeled lipoproteins to hepatocytes from the deficient mice. These findings are consistent with our conclusions that HSPG receptors are a major contributor to clearance in the liver, and that the 2 receptor families do not fully compensate for one another.

Our genetic analysis of the relative contribution of HSPG receptors and LDLR and LRP1 to TRL clearance has several important implications. On the basis of fasting triglyceride data from double and triple mutant mice (Figure 2), we conclude that LDLR, LRP1, and HSPGs are the major TRL clearance receptors in mice, although the role of LRP1 only becomes apparent in the absence of LDLR. Postprandial clearance data also support this conclusion (Figure 4). In triple receptor–deficient mice, triglyceride levels remain elevated and liver uptake remains low even 8-hour post gavage, suggesting that other TRL receptors, such as lipolysis-stimulated receptor, LRP5, VLDLR, or scavenger receptor B1, do not compensate for the absence of these 3 major receptors. Nevertheless, we cannot exclude the possibility that these other receptors play a role in clearance under other conditions that were not addressed in our studies.10,13,35 Our findings also suggest that the different receptor subtypes clear distinct subsets of lipoprotein particles. On the basis of electron microscopy studies of particles that accumulate in the mutants, HSPG receptors apparently clear a subset of smaller TRLs, whereas members of the LDLR family of receptors clear larger particles (Figure 3). The idea that particle size partially determines the affinity of lipoprotein particles for their
receptors has been suggested and may be the result of apolipoprotein conformation or content. In this study, we provide additional evidence showing that HSPGs preferentially clear particles that are enriched in apoE and apoAV. On the basis of our findings, we suggest that the small particles preferentially cleared by HSPG receptors are enriched for apoE and apoAV. Whether these particles have other unique attributes is unknown. Conceivably, these particles might originate from lipolysis of a unique pool of VLDL or chylomicrons in the vasculature.

HSPGs can act as independent endocytic receptors for numerous ligands. Nevertheless, the idea that HSPGs act independently of other receptors in TRL clearance has

Table 3. Fasting Plasma Lipid Levels on a Western Diet

<table>
<thead>
<tr>
<th>Weeks on Diet</th>
<th>Cre-Ndst1&lt;sup&gt;ff&lt;/sup&gt; (Wild Type)</th>
<th>Cre-Ndst1&lt;sup&gt;ff&lt;/sup&gt; (HSPG Receptor Deficient)</th>
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<th>Cre-Ndst1&lt;sup&gt;ff&lt;/sup&gt;Lrp1&lt;sup&gt;ff&lt;/sup&gt;Ldi&lt;sup&gt;−/−&lt;/sup&gt; (Triple Receptor Deficient)</th>
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<td>Triglycerides, mg/dL</td>
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<td>133±14</td>
<td>1189±116</td>
<td>1317±144</td>
</tr>
<tr>
<td>6</td>
<td>82±10</td>
<td>172±30</td>
<td>1989±135</td>
<td>1847±173</td>
</tr>
<tr>
<td>Cholesterol, mg/dL</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>72±4</td>
<td>90±8</td>
<td>549±44</td>
<td>710±63</td>
</tr>
<tr>
<td>1</td>
<td>126±9</td>
<td>152±18</td>
<td>1588±56</td>
<td>1716±111</td>
</tr>
<tr>
<td>4</td>
<td>176±16</td>
<td>229±29</td>
<td>2167±89</td>
<td>2450±256</td>
</tr>
<tr>
<td>6</td>
<td>128±14</td>
<td>201±37</td>
<td>2496±106</td>
<td>2468±224</td>
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</table>

All values are expressed as average±SE. HSPG indicates heparan sulfate proteoglycan; LDLR, low-density lipoprotein receptor; LRP1, LDLR-related protein-1; and Ndst1, N-acetylgalcosamine N-deacetylase-N-sulfotransferase-1.
been questioned.6,40 Biochemical data have been presented suggesting that these 2 proteins act as coreceptors for lipoprotein particles or participate in a handoff process whereby HSPGs trap lipoprotein particles in the space of Disse and subsequently transfer them to LRP1 for endocytosis.6 Given our findings that the receptors clear different subsets of particles and that syndecan-1 expression is not affected by the loss of LDLR and LRP1 in hepatocytes (and vice versa), it seems likely that each receptor family acts independently.

The prominent role of syndecan-1 in clearance under fasting and postprandial conditions predicted that the proteoglycans would play an important role in clearing remnant lipoproteins in animals fed a Western diet. Surprisingly, triglyceride and cholesterol levels in HSPG receptor–deficient mice hardly changed when fed a Western diet. This is in sharp contrast to LDLR/LRP1-deficient mice in which high-fat feeding drastically increased both cholesterol and triglyceride levels. Furthermore, our data show that this discrepancy could not be explained by drastic changes in apolipoprotein composition of TRLs or changes in hepatic expression of LDLR, LRP1, scavenger receptor B1, and lipolysis-stimulated receptor in HSPG receptor–deficient mice on the Western diet. One interpretation of these findings is that hepatic HSPG receptors are active under basal (chow-fed) conditions and in response to acute dietary changes, that is, postprandial lipid loading, but do not recognize the particles that accumulate under chronic high-fat feeding. Conceivably, high levels of circulating LDL particles that accumulate under these conditions could decrease the availability of apoE and apoAV or shift particle size toward substrates poorly recognized by syndecan-1. We cannot exclude that changes in TRL lipid composition or the increase in blood lipids per se are contributing to this intriguing observation. Additional studies are clearly warranted to determine whether increasing the expression of apoE and apoAV pharmacologically stimulates HSPG-mediated clearance under high-fat diet conditions.3

The triple receptor–deficient mouse represents a unique hyperlipidemic model that can be used to study triglyceride and cholesterol metabolism under conditions where clearance is greatly delayed. In addition, the triple receptor mutant may prove to be a valuable tool for elaborating the mechanism of action of compounds that reduce or prevent atherosclerosis independent of hepatic TRL clearance.

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We thank Jennifer Patton and Joe Juliano for their assistance with fast-phase liquid chromatography and lipid analysis. We also thank Timo Merloo and Marilyn Farquhar for technical assistance with electron microscopy. We acknowledge Carlos Lameda Diaz for his kind assistance with glycosaminoglycan isolation. We are grateful to Joe Witztum for helpful discussions.

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Disclosures

None.

References

tors to hepatic clearance. The current findings are valuable, as they shed new light on the already complex etiology of hypertriglyceridemia. Interestingly, heparan sulfate proteoglycans play a major role when fed a balanced diet but are less crucial when consuming a high-density lipoprotein receptor and low-density lipoprotein receptor–related protein-1 and clear a distinct subset of small triglyceride-rich lipoproteins. We establish that heparan sulfate proteoglycans act independently of low-density lipoprotein receptor, and low-density lipoprotein receptor–related protein-1 are the 3 main receptors responsible for hepatic catabolism of triglyceride-rich lipoproteins. We identify that heparan sulfate 2-O-sulfotransferase is required for triglyceride-rich lipoprotein clearance.

Elevated plasma triglyceride concentrations contribute to increased risk of cardiovascular disease. This increase results from elevated triglyceride production in the liver and intestine or through decreased clearance from the circulation. In this study, we identified that heparan sulfate proteoglycans, low-density lipoprotein receptor, and low-density lipoprotein receptor–related protein–1 are the 3 main receptors responsible for hepatic catabolism of triglyceride-rich lipoproteins. We establish that heparan sulfate proteoglycans act independently of low-density lipoprotein receptor and low-density lipoprotein receptor–related protein–1 and clear a distinct subset of small triglyceride-rich particles. Interestingly, heparan sulfate proteoglycans play a major role when fed a balanced diet but are less crucial when consuming a high-fat diet. The data provide a better understanding of the genetic and environmental factors that influence the relative contribution of the receptors to hepatic clearance. The current findings are valuable, as they shed new light on the already complex etiology of hypertriglyceridemia.
Hepatic Remnant Lipoprotein Clearance by Heparan Sulfate Proteoglycans and Low-Density Lipoprotein Receptors Depend on Dietary Conditions in Mice
Erin M. Foley, Philip L.S.M. Gordts, Kristin I. Stanford, Jon C. Gonzales, Roger Lawrence, Nicole Stoddard and Jeffrey D. Esko

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Materials and Methods
Mice and animal husbandry - Lrp1 f/f, Ldlr−/−, and AlbCre* mice were purchased from The Jackson Laboratory. Ndst1 f/f AlbCre+ mice were generated and genotyped as described1. All mice were backcrossed more than 10 generations on a C57BL/6 background. Mice were housed and bred in vivaria approved by the Association for Assessment and Accreditation of Laboratory Animal Care located in the School of Medicine at the University of California, San Diego, following standards and procedures approved by the local Institutional Animal Care and Use Committee. Mice were weaned at 3 weeks, maintained on a 12-hour-light cycle, and fed ad libitum with water and standard rodent chow (Harlan Teklad) or a high fat/high cholesterol diet (TD.88137 from Harlan Teklad; 21% anhydrous milkfat (butterfat), 34% sucrose, and a total of 0.2% cholesterol).

Lipid analysis - Blood was drawn via the retroorbital sinus from mice fasted for 6-12 hr. Total cholesterol and triglyceride levels in plasma were determined using kits (Genzyme) against the Precipath L lipoprotein standard (Roche Diagnostics).

Ultracentrifugation studies – Plasma was pooled from several mice (70 µL per mouse, n = 3 mice per genotype). Lipoprotein fractions were separated by buoyant density ultracentrifugation according to established methods2. Briefly, 210 µL of pooled plasma was loaded into micro-ultracentrifuge tubes (Beckman). The samples were centrifuged 12 hours in a 42.2 Ti rotor at 38,000 rpm at 18°C. The top 50 µL containing VLDL and chylomicron remnants (δ < 1.006 g/ml) was removed and used for analysis. TRLs were analyzed by SDS-PAGE on 4-12% Bis-Tris gradient gels (NuPage, Invitrogen). Proteins were visualized by silver staining (Pierce) or after transfer to Immobilon-FL PVDF membrane (Millipore) as previously described3.

Heparan sulfate purification and disaccharide analysis - Hepatocytes were isolated and allowed to recover in culture overnight4. The cells were then treated overnight with Pronase (2 mg/ml; Roche Diagnostics) to degrade proteins, followed by purification of the glycopeptides by anion exchange chromatography using DEAE Sephacel (Amersham Biosciences). Columns were washed with low-salt buffer (0.15 M NaCl, 20mM sodium acetate; pH 6.0) and eluted with 1 M NaCl. Samples were desalted using PD-10 columns and then lyophilized. The glycosaminoglycans were digested with heparin lyases I, II, and III. The resulting disaccharides were derivatized with isotopically labeled aniline and quantified by mass spectrometry as described previously5. Molar percentages were calculated based on the relative area under each peak compared to standards.

Electron microscopy - Lipoprotein particles isolated by buoyant density ultracentrifugation (δ < 1.006 g/ml) were subjected to negative staining. Briefly, particles were diluted to a concentration of 0.5-1.5 mg/ml triglyceride in water, and sucrose was added to a final concentration of 0.1%. The particles were then allowed to adhere to a Formvar carbon-coated grid and stained with 1% uranyl acetate before visualization on a FEI Tecnai Spirit G2 BioTWIN transmission electron microscope equipped with a 4K Eagle digital camera.

Fast performance liquid chromatography (FPLC) - Pooled plasma samples were separated by gel filtration FPLC. Samples were loaded on a GE Superose 6 10/30 GL column (Cat# 175172-01) in 0.15 M sodium chloride containing 1 mM ethylenediaminetetraacetic acid and 0.02% sodium azide, pH 7.4. Fractions (0.5 mL)
were collected (0.5 mL/min). Total cholesterol and triglyceride levels were determined enzymatically using an automated reader (Cobas Mira; Roche Diagnostics) and kits: Cholesterol High-Performance Reagent (Roche Diagnostics) and Triglyceride-SL (Diagnostic Chemicals Ltd.).

**Western blots** – In Figure 1, freshly isolated hepatocytes were immediately solubilized in RIPA buffer plus protease inhibitors (Sigma). In Figure 5, freshly isolated livers were solubilized in RIPA buffer plus protease inhibitors (Sigma). For each sample, 20 µg protein was resolved on a 4-12% Bis-Tris NuPage gel (Invitrogen) and transferred to polyvinylidene fluoride membrane (Bio-Rad Laboratories). The membrane was blocked with Super-Block buffer (Pierce). Blots were incubated with antibodies against LDLR (AbCam), Lrp-1 (AbCam), Syndecan-1 (Pharmingen), SR-BI (Novus Biologicals), LSR (Sigma) or β-actin (Sigma) and appropriate HRP-conjugated secondary antibodies (Santa Cruz). Reactive bands were visualized by chemiluminescence.

**Agarose electrophoresis**– Lipoprotein samples (δ < 1.006 g/ml) were loaded onto a TITAN Gel (Helena Laboratories), electrophoresed, and stained per the manufacturer’s instructions.

**Postprandial clearance studies** - Mice were fasted for 12 hours. At 10 am, they were given a 200 µL bolus of corn oil (Sigma) by oral gavage. At the indicated time points, mice were sedated with isofluorane and bled via the tail vein. Triglyceride and cholesterol levels were measured as described above.

**[^3H]Retinol organ uptake experiments** - Clearance of chylomicrons derived from dietary triglyceride was measured by Vitamin A excursion essentially as described. Briefly, 27 µCi of [11,12-^3H]-retinol (Perkin Elmer; 44 Ci/mmol) in ethanol was mixed with 1 mL of corn oil (Sigma-Aldrich) and administered to overnight fasted mice by oral gavage (200 µL/mouse). Blood was obtained 8-hours post-gavage by cardiac puncture and counts remaining in the serum were assayed in duplicate by liquid scintillation counting. The mice were dissected, and organs were removed. Approximately 100-200 mg of tissue was solubilized in 1 mL of SOLVABLE (Perkin Elmer) at 55°C, and ^3H-counts in the tissue were assayed by liquid scintillation counting.

**Statistical analysis** - Statistical analyses were performed using Prism 4.0c (GraphPad Software) using the indicated tests. Significance was taken as *P* < 0.05. Data are expressed as average ± standard deviation.
