The hepatic uptake of VLDL in $lrp^{-}ldlr^{-/-}vldlr^{-/-}$ mice is regulated by LPL activity and involves proteoglycans and SR-BI

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Abstract LPL activity plays an important role in preceding the VLDL remnant clearance via the three major apolipoprotein E (apoE)-recognizing receptors: the LDL receptor (LDLr), LDL receptor-related protein (LRP), and VLDL receptor (VLDLr). The aim of this study was to determine whether LPL activity is also important for VLDL remnant clearance irrespective of these receptors and to determine the mechanisms involved in the hepatic remnant uptake. Administration of an adenovirus expressing LPL (Ad*LPL*) into $lrp^{-}ldlr^{-/-}vldlr^{-/-}$ mice reduced both VLDL-triglyceride (TG) and VLDL-total cholesterol (TC) levels. Conversely, inhibition of LPL by Ad*APOC1* increased plasma VLDL-TG and VLDL-TC levels. Metabolic studies with radiolabeled VLDL-like emulsion particles showed that the clearance and hepatic association of their remnants positively correlated with LPL activity. This hepatic association was independent of the bridging function of LPL and HL, since heparin did not reduce the liver association. In vitro studies demonstrated that VLDL-like emulsion particles avidly bound to the cell surface of primary hepatocytes from $lrp^{-}ldlr^{-/-}vldlr^{-/-}$ mice, followed by slow internalization, and involved heparin-releaseable cell surface proteins as well as scavenger receptor class B type I (SR-BI). Collectively, we conclude that hepatic VLDL remnant uptake in the absence of the three classical apoE-recognizing receptors is regulated by LPL activity and involves heparan sulfate proteoglycans and SR-BI.—Hu, L., C. C. van der Hoogt, S. M. S. Espirito Santo, R. Out, K. E. Kypreos, B. J. M. van Vlijmen, T. J. C. Van Berkel, J. A. Romijn, L. M. Havekes, K. Willems van Dijk, and P. C. N. Rensen. **The hepatic uptake of VLDL in $lrp^{-}ldlr^{-/-}vldlr^{-/-}$ mice is regulated by LPL activity and involves proteoglycans and SR-BI. *J. Lipid Res.* 2008.** 49: **1553–1561.**

Supplementary key words lipoprotein lipase • low density lipoprotein receptor • very low density lipoprotein receptor • low density lipoprotein receptor-related protein • triglyceride-rich emulsion particles • transgenic mice • adenovirus-mediated gene transfer • apolipoprotein E

LPL is the key enzyme responsible for the hydrolysis of triglycerides (TGs) in TG-rich lipoproteins such as chylomicrons and VLDL (1, 2). During lipolysis, the lipoproteins are reduced in size and enriched with apolipoprotein E (apoE). Subsequently, their core remnants are taken up mainly by the liver via apoE-recognizing receptors [i.e., the LDL receptor (LDLr) and the LDLr-related protein (LRP)] (2). Therefore, mice deficient for the LDLr and hepatic LRP show marked accumulation of TG-rich lipoprotein remnants (3). Although core remnants may be directly internalized via the LDLr, the binding and internalization via the LRP is thought to involve previous binding of core remnants to heparan sulfate proteoglycans (HSPGs) in the space of Disse via heparin binding proteins such as apoE (4, 5).

The third major apoE-recognizing receptor, the VLDL receptor (VLDLr), is expressed abundantly in tissues active in fatty acid metabolism [i.e., heart, skeletal muscle,

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and white adipose tissue (WAT)] and functions as a peripheral lipoprotein remnant receptor. Like the LDLr and LRP, the VLDLr binds TG-rich lipoproteins via apoE, and this binding is modulated by LPL (6, 7). VLDLr-deficient mice have normal plasma lipoprotein levels when fed a chow diet (8). However, when TG metabolism is stressed by feeding a high-fat diet or by cross-breeding on an *ob/ob* or $ldlr^{-/-}$ background, the VLDLr deficiency results in moderate accumulation of plasma TG-rich lipoproteins (9, 10). Recently, we demonstrated that the VLDLr plays a major role in postprandial lipoprotein metabolism by facilitating LPL-mediated TG hydrolysis (11).

Additional deletion of the VLDLr from LDLr and hepatic LRP double-deficient mice aggravates their phenotype upon stressing TG metabolism, either by high-fat feeding or by giving an intragastric olive oil bolus (12). On a chow diet, the steady-state fasted plasma TG and total cholesterol (TC) levels are increased by 8- to 9-fold in $lrp^{-}ldlr^{-/-}vldlr^{-/-}$ mice compared with wild-type mice (12). However, because $lrp^{-}ldlr^{-/-}vldlr^{-/-}$ mice have continuous lipid input into their circulation via the production of VLDL, similar to wild-type mice, the remnants in these mice must be cleared from plasma to maintain the steady-state lipid levels. This indicates that, although less efficient than via the classical remnant receptors, additional pathway(s) are present for the clearance of lipoprotein remnants in vivo.

Two of these nonclassical pathways may involve HSPGs and the scavenger receptor class B type I (SR-BI). HSPGs have been reported to directly internalize apoE-enriched TG-rich particles, both in vitro (13) and in vivo (14). In fact, under normal physiological conditions, hepatic HSPGs are critically important for the clearance of remnant lipoproteins, since targeted inactivation of the biosynthetic gene *Ndst1* in hepatocytes, resulting in 50% reduction in the sulfation of liver heparan sulfate, resulted in the accumulation of TG-rich lipoproteins in wild-type as well as $ldlr^{-/-}$ mice (15). In addition, recent studies using $srb1^{-/-}$ mice have shown that SR-BI can function as an internalizing receptor for chylomicrons (16, 17) and VLDL (18).

Sehayek et al. (19) showed that the lipolytic activity of LPL (i.e., hydrolysis of TG within the lipoprotein core) is required for apoE-dependent uptake of lipoprotein remnants via the LDLr and possibly the LRP in vitro. However, it remains unclear whether modulation of LPL activity also affects the catabolism of TG-rich particles in the absence of the three major apoE-recognizing receptors in vivo, via HSPGs and/or SR-BI. Therefore, the aim of this study was to determine the role of LPL activity in hepatic VLDL metabolism in mice that lack the LDLr, hepatic LRP, and the VLDLr as well as the mechanisms involved in the hepatic uptake. Therefore, we either increased LPL activity by adenovirus-mediated overexpression (20) or decreased LPL activity by adenovirus-mediated expression of the LPL inhibitor apoC-I (21). In these studies, we demonstrate that the receptor-independent hepatic uptake of VLDL core remnants in vivo is regulated by LPL activity and involves HSPGs and SR-BI.

MATERIALS AND METHODS

Animals

Male MX1Cre$^{+}lrp^{loxP/loxP}ldlr^{-/-}vldlr^{-/-}$ mice (12), 4–6 months of age, were used in the experiments. Mice were obtained from our breeding colony at the Institutional Animal Facility, housed under standard conditions in conventional cages, and fed regular chow ad libitum. LRP deficiency was induced by intraperitoneal injection of polyinosinic:polycytidylic ribonucleic acid (pI:pC; Sigma, St. Louis, MO), which results in the complete absence of LRP protein in liver membrane extracts (12). These mice will be referred to as $lrp^{-}ldlr^{-/-}vldlr^{-/-}$. Experiments were performed after 4 h of fasting at 12:00 PM with food withdrawn at 8:00 AM, unless indicated otherwise. The Institutional Ethical Committee on Animal Care and Experimentation approved all experiments.

Adenoviruses and administration to mice

The generation of an adenovirus expressing human apoC-I (Ad*APOC1*) has been described in full detail (21). An adenovirus expressing human LPL (Ad*LPL*) (16) was a kind gift of Dr. Silvia Santamarina-Fojo. An adenovirus expressing β-galactosidase (Ad*LacZ*) was used as a control. Viruses were grown and purified by standard procedures, and typical titers of 1×10^{10} to 1×10^{11} plaque-forming units (pfu)/ml were obtained. Viruses were stored in aliquots at −80°C until use. Basal serum lipid levels were measured at 4 weeks after pI:pC injection, at least 3 days before adenovirus injection into mice. At day 0, mice were injected into the tail vein with Ad*APOC1*, Ad*LPL*, or Ad*LacZ* (1×10^{9} pfu/mouse), diluted with PBS to a total volume of 200 μl. To prevent sequestration of low doses of virus by Kupffer cells, mice were preinjected with 0.5×10^{9} pfu Ad*LacZ* at 3 h before injection of the adenoviruses of interest (22).

Plasma lipid and lipoprotein analysis

In all experiments, blood was collected from the tail vein into chilled paraoxon (Sigma)-coated capillary tubes to prevent ongoing in vitro lipolysis (23). The tubes were placed on ice and centrifuged at 4°C, and the obtained plasma was assayed for TC and TG using the commercially available enzymatic kits 236691 and 11488872 (Roche Molecular Biochemicals, Indianapolis, IN), respectively. For determination of the distribution of lipids over plasma lipoproteins by fast-performance liquid chromatography (FPLC), 50 μl of pooled plasma per group was injected onto a Superose 6 column (Äkta System; Amersham Pharmacia Biotech, Piscataway, NJ), and eluted at a flow rate of 50 μl/min with PBS and 1 mM EDTA, pH 7.4. Fractions of 50 μl were collected and assayed for TC and TG as described above.

Postheparin plasma LPL levels

Blood was collected at 10 min after tail vein injection of heparin diluted in PBS (0.1 U/g body weight; Leo Pharmaceutical Products B.V., Weesp, The Netherlands). Plasma was isolated, snap-frozen, and stored at −80°C until analysis for total LPL activity as described previously (24).

Preparation of VLDL-like emulsion particles

TG-rich VLDL-like emulsion particles (80 nm) were prepared as described (25). Emulsions were obtained by adding the following labels to 100 mg of emulsion lipids prior to sonication: *1*) 200 μCi of glycerol tri[^{3}H]oleate (triolein) (TO) and 20 μCi of [^{14}C]cholesteryl oleate (CO); *2*) 500 μg of 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanide perchlorate (DiI) (Molecular Probes, Leiden, The Netherlands); or *3*) 80 μCi of [^{3}H]cholesteryl oleoyl ether (COEth).

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In vivo kinetics of VLDL-like emulsion particles

The in vivo kinetics of [^{3}H]TO and [^{14}C]CO double-labeled emulsion particles were assessed at 5 days after virus administration. Then, mice were anesthetized with acepromazine-midazolam-fentanyl (1:2:5, v/v/v), the abdominal cavities were opened, and the radiolabeled emulsion particles (1.0 mg of TG in 200 μl of PBS) were injected into the vena cava inferior at 8:00 AM. In addition, the effect of heparin on the kinetics of emulsion particles was evaluated. For this, mice were anesthetized and the radiolabeled emulsion particles (1.0 mg of TG in 200 μl of PBS) were administered into the tail vein at 10 min after intravenous injection of heparin (500 U/kg) or PBS at 8:00 AM. At the indicated time points after injection, blood was taken from the vena cava inferior or tail vein to determine the serum decay of [^{3}H]TO and [^{14}C]CO by scintillation counting (Packard Instruments, Downers Grove, IL). At 30 min after injection, mice were euthanized and tissues were collected, including liver, heart, skeletal muscle, and WAT. Since the various WAT compartments can have different LPL activity levels (26), we analyzed perirenal, intestinal, and gonadal WAT. Tissues were weighed and dissolved overnight in Solvable (Packard Bioscience, Meriden, CT), after which ^{3}H and ^{14}C activities were determined in Ultima Gold (Packard Bioscience). Radioactivity values were corrected for the serum radioactivity (liver, 84.7 μl/g; heart, 68.1 μl/g; WAT, 16.1 μl/g; skeletal muscle, 13.7; μl/g) present at the time of sampling (25). The total plasma volumes of the mice were calculated from the equation V (ml) = 0.04706 × body weight (g), as determined from ^{125}I-BSA clearance studies as described previously (27).

Visualization of uptake of fluorescently labeled VLDL-like emulsion particles by isolated mouse hepatocytes

Mouse hepatocytes were isolated from anesthetized wild-type or $lrp^- ldlr^{-/-} vldlr^{-/-}$ mice and subjected to Percoll® gradient centrifugation to discard nonviable cells (28). The cells (viability >99% as judged from 0.2% trypan blue exclusion) were attached to collagen S-coated (3.87 μg/cm^2) 2.5 cm glass cover slips in 9.6 cm^2 six-well dishes (0.8 × 10^6 cells/well) by culturing in DMEM + 10% fetal calf serum (3–4 h at 37°C). The cover slips were washed to remove unbound cells and incubated (2 h at 4°C) with DiI-labeled VLDL-like emulsion particles (100 μg TG/ml). The cover slips were washed twice with DMEM + 2% BSA to remove unbound particles and transferred to a Zeiss IM-35 inverted microscope (Oberkochen, Germany) with a Zeiss plan apochromatic 63×/1.4 numerical aperture oil objective and fitted with a temperature-controlled incubation chamber, which was equipped with a Bio-Rad 600 MRC confocal laser scanning microscopy system. The cells were further incubated (30 min at 37°C) in DMEM + 2% BSA, after which the (intra)cellular localization of DiI was visualized (λ_{ex} 543 nm).

Association of radiolabeled HDL- and VLDL-like emulsion particles with isolated mouse hepatocytes

Mouse hepatocytes were isolated from anesthetized wild-type or $lrp^- ldlr^{-/-} vldlr^{-/-}$ mice (28). Cells (1–2 mg protein/ml) were incubated (3 h at 37°C) in DMEM + 2% BSA with [^{3}H]COEth-labeled HDL (20 μg protein/ml) or emulsion particles (100 μg TG/ml) in the absence and presence of oxidized LDL (oxLDL; 100 μg protein/ml) or heparin (1,000 U/ml) under gentle shaking in a circulating lab shaker (Adolf Kühner AG, Basel, Switzerland) at 150 rpm. After incubation, cells were pelleted by centrifugation (1 min at 50 *g*), and unbound radiolabeled ligands were removed by washing twice with ice-cold 50 mM Tris-HCl, 150 mM NaCl, 5 mM $CaCl_2$ (Tris-buffered saline), pH 7.4, containing 0.2% BSA, and once with Tris-buffered saline without BSA. The cell pellet was lysed in 0.1 M NaOH, and the cell-associated radioactivity and protein content were measured. [^{3}H]COEth association was calculated as dpm/mg cell protein.

Statistical analysis

All data are presented as means ± SD. Data were analyzed using the Mann-Whitney U-test unless indicated otherwise. $P <$ 0.05 was regarded as statistically significant.

RESULTS

LPL activity modulates VLDL-cholesterol levels in $lrp^- ldlr^{-/-} vldlr^{-/-}$ mice

To study the impact of LPL activity on the clearance of VLDL-associated TG and cholesterol in the absence of the apoE-recognizing receptors, we used mice deficient for the LDLr, hepatic LRP, and VLDLr, as described previously (12). Upon deletion of hepatic LRP from MX1Cre$^+$$lrp^{lox/lox}$ $ldlr^{-/-} vldlr^{-/-}$ mice, their plasma lipid levels were determined and the mice were assigned to three groups, matched for TC and TG plasma levels (**Table 1**). The mice received Ad*LPL* to increase LPL levels (25), Ad*APOC1* to inhibit LPL activity (26), or Ad*LacZ* as a control group. The effect of these interventions on plasma lipid levels was assessed at 5 days after injection.

Ad*LPL* administration resulted in a 3.0-fold increase in postheparin LPL plasma activity (33.7 ± 5.4 vs. 11.4 ± 1.6 μmol FFA generated/h/ml; $P < 0.05$), with a concomitant 6.8-fold reduction in plasma TG levels (1.1 ± 0.1 vs. 7.5 ± 0.9 mM; $P < 0.01$) and 1.2-fold reduction in plasma TC levels (15.7 ± 3.9 vs. 19.0 ± 1.9 mM; $P < 0.05$) compared with mice administered Ad*LacZ* (Table 1). From FPLC fractionation of pooled plasma, it was apparent that the decrease in plasma TG and TC was confined to the VLDL fractions (**Fig. 1**).

Ad*APOC1* administration did not result in altered total postheparin plasma LPL levels (11.5 ± 7.6 vs. 11.4 ± 1.6 μmol FFA/h/ml) (Table 1). This is in accordance with

TABLE 1. Effect of adenovirus administration on plasma lipid levels and postheparin LPL plasma activity in $lrp^- ldlr^{-/-} vldlr^{-/-}$ mice

Adenovirus	LPL Activity μmol FFA generated/h/ml	TG	TC
		mM	
Before adenovirus			
Ad*LPL*	n.d.	3.9 ± 0.7	19.5 ± 3.0
Ad*LacZ*	n.d.	4.0 ± 0.8	19.4 ± 3.3
Ad*APOC1*	n.d.	3.8 ± 0.6	19.5 ± 8.1
After adenovirus			
Ad*LPL*	33.7 ± 5.4[a]	1.1 ± 0.1[b]	15.7 ± 3.9[a]
Ad*LacZ*	11.4 ± 1.6	7.5 ± 0.9	19.0 ± 1.9
Ad*APOC1*	11.5 ± 7.6	37.6 ± 10.1[b]	30.1 ± 6.7[b]

Plasma was obtained from fasted $lrp^- ldlr^{-/-} vldlr^{-/-}$ mice before and after administration of Ad*LacZ*, Ad*LPL*, or Ad*APOC1* and assayed for triglycerides (TGs) and total cholesterol (TC). After the second blood withdrawal, heparin was injected and postheparin plasma was obtained and assayed for LPL activity. Values are expressed as means ± SD. n.d., not determined. Statistical differences were assessed compared with Ad*LacZ*.

[a] $P < 0.05$.
[b] $P < 0.01$.

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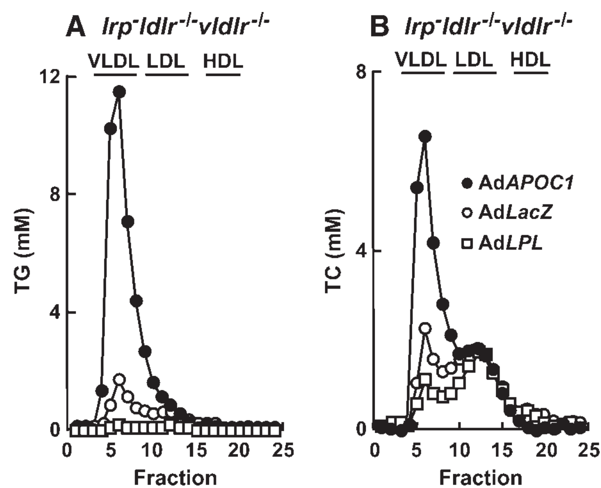

Fig. 1. Effect of modulation of LPL activity by LPL and apolipoprotein C- I (apoC-I) on plasma triglyceride (TG) and total cholesterol (TC) distribution. $lrp^-ldlr^{-/-}vldlr^{-/-}$ mice were injected with Ad*LacZ* (open circles), Ad*LPL* (open squares), or Ad*APOC1* (closed circles) (1×10^9 plaque-forming units). Four days after injection, 4 h fasted plasma samples were drawn, pooled, and subjected to fast-performance liquid chromatography fractionation. Fractions were analyzed for TG (A) and TC (B).

previous findings that human *APOC1* transgenic mice do not show a change in plasma total LPL levels compared with wild-type mice (24) but, rather, apoC-I acts by modulating local LPL activity. Administration of Ad*APOC1* resulted in 5.0-fold increased plasma TG levels (37.6 ± 10.1 vs. 7.5 ± 0.9 mM; $P < 0.01$) in addition to 1.6-fold increased plasma TC levels (30.1 ± 6.7 vs. 19.0 ± 1.9 mM; $P < 0.01$) compared with Ad*LacZ* control mice (Table 1). These increased plasma TG and TC levels were due to increased VLDL levels, as was shown after FPLC fractionation of pooled plasma (Fig. 1).

Taken together, these results suggest that LPL activity not only regulates the clearance of VLDL-TG but also determines the clearance of VLDL-cholesterol in the absence of the LRP, LDLr, and VLDLr in vivo.

LPL activity modulates the liver association of VLDL-like emulsion core remnants in $lrp^-ldlr^{-/-}vldlr^{-/-}$ mice

To provide direct in vivo evidence that LPL activity determines the clearance of VLDL-cholesterol, [^{3}H]TO and [^{14}C]CO double-labeled TG-rich VLDL-like emulsion particles were injected into $lrp^-ldlr^{-/-}vldlr^{-/-}$ mice at 5 days after Ad*LPL*, Ad*APOC1*, or Ad*LacZ* administration (**Fig. 2**).

The clearance of [^{3}H]TO was substantially accelerated in Ad*LPL*-treated mice compared with control mice, as evidenced by a 2.5-fold decreased serum half-life of ^{3}H activity ($t_{1/2}$ = 18 ± 7 vs. 45 ± 11 min) (Fig. 2A). On the other hand, mice that were treated with Ad*APOC1* showed a 1.7-fold increased half-life ($t_{1/2}$ = 77 ± 13 min). Thus, the LPL activity was positively correlated with the serum clearance of ^{3}H activity ($P < 0.05$). Increased LPL activity was accompanied by a significantly increased uptake of TO-derived ^{3}H activity in LPL-expressing organs such as heart ($P < 0.01$), muscle ($P < 0.05$), and WAT ($P < 0.05$ for perirenal WAT) and also in the liver ($P < 0.01$) (Fig. 2B).

Although serum [^{14}C]CO decay was slower compared with the [^{3}H]TO decay, the [^{14}C]CO serum half-life was also dependent on LPL activity. This was evidenced by a 1.4-fold decreased half-life of ^{14}C label in Ad*LPL*-treated mice ($t_{1/2}$ = 71 ± 24 min) and a 3.7-fold increased half-life in Ad*APOC1*-treated animals ($t_{1/2}$ = 364 ± 241 min) compared with controls ($t_{1/2}$ = 99 ± 8 min) ($P < 0.05$) (Fig. 2C). In addition, the association of ^{14}C activity with the liver was 7.0-fold increased in Ad*LPL* animals, compared with control mice, and decreased to zero by Ad*APOC1* ($P < 0.01$) (Fig. 2D). Further analysis of the distribution of

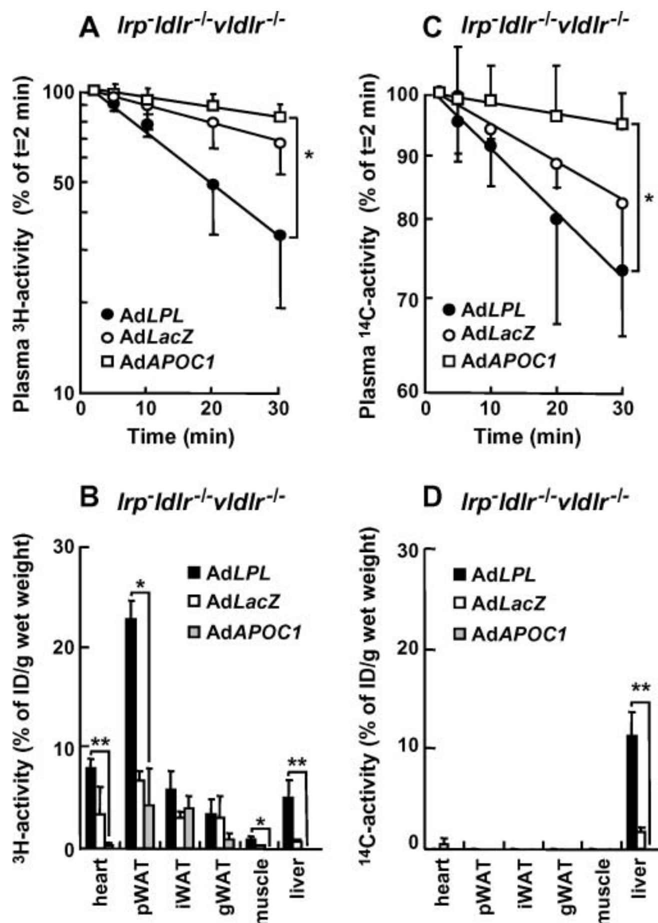

Fig. 2. Effect of modulation of LPL activity by LPL and apoC-I on the serum decay and organ distribution of VLDL-like emulsion particles. $lrp^-ldlr^{-/-}vldlr^{-/-}$ mice were injected with Ad*LacZ*, Ad*LPL*, or Ad*APOC1* (1×10^9 plaque-forming units). Five days after injection, mice were anesthetized and injected with [^{3}H]triolein (TO) and [^{14}C]cholesteryl oleate (CO) double-labeled emulsion particles (1 mg of TG). Serum samples were collected at the indicated times and measured for ^{3}H activity (A) and ^{14}C activity (C). At 30 min, the animals were euthanized and tissues were collected. Tissues were dissolved in Solvable (overnight at 60°C) and measured for ^{3}H activity (B) and ^{14}C activity (D) corrected for serum radioactivity. Asterisks indicate statistically significant differences (* $P < 0.05$, ** $P < 0.01$) as analyzed by one-way ANOVA (A, C) or Mann-Whitney U-test (B, D). pWAT, perirenal white adipose tissue; iWAT, intestinal WAT; gWAT, gonadal WAT. Data are presented as means ± SD.

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^{14}C activity over cholesterol and cholesteryl esters in the liver revealed that the ^{14}C activity was almost exclusively recovered in the cholesteryl esters (data not shown), which indicates that particles are indeed associated with the liver; however, they have not entered the lysosomal degradation pathway yet. Taken together, these results show that LPL activity positively correlates with liver association of particle core remnants.

Heparin accelerates the lipolysis of VLDL-like emulsion particles followed by rapid liver association of their core remnants in wild-type and $lrp^{-}ldlr^{-/-}vldlr^{-/-}$ mice

To gain insight into the potential pathways by which VLDL-like emulsion particles associate with the liver in the absence of the LDLr, hepatic LRP, and VLDLr, we next determined the kinetics of [^{3}H]TO and [^{14}C]CO double-labeled TG-rich VLDL-like emulsion particles in wild-type mice and $lrp^{-}ldlr^{-/-}vldlr^{-/-}$ mice following an intravenous injection of heparin (**Fig. 3**). Heparin results in the release of LPL from the vascular endothelium, resulting in a higher systemic plasma TG hydrolase activity, and prevents the association of both LPL and HL with the liver.

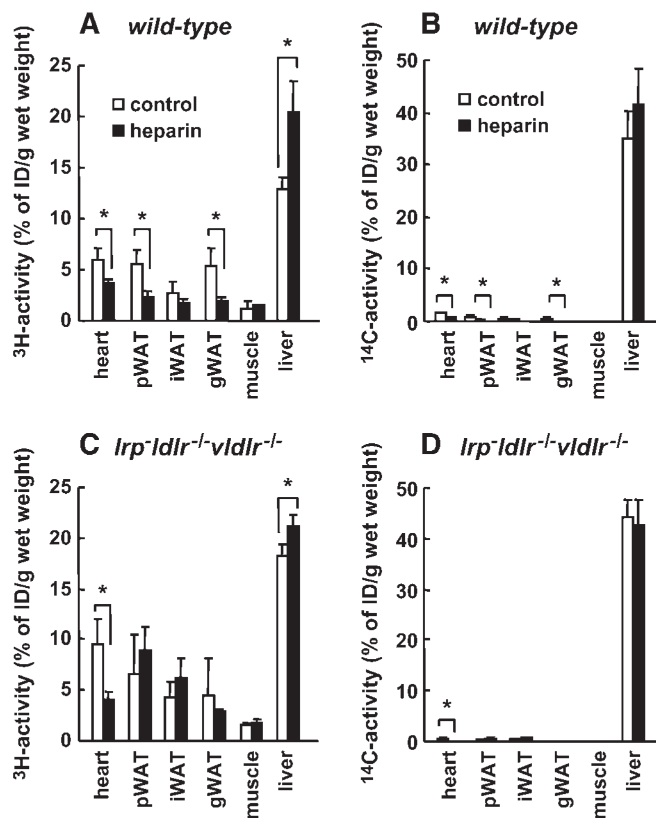

Fig. 3. Effect of heparin on the organ distribution of VLDL-like emulsion particles. Wild-type mice (A, B) and $lrp^{-}ldlr^{-/-}vldlr^{-/-}$ mice (C, D) were anesthetized and injected with 500 U/kg heparin (closed bars) or vehicle (open bars). After 10 min, mice were injected with [^{3}H]TO and [^{14}C]CO double-labeled emulsion particles (1 mg of TG). At 30 min, the animals were euthanized and tissues were collected. Tissues were dissolved in Solvable (overnight at 60°C) and measured for ^{3}H activity (A, C) and ^{14}C activity (B, D) corrected for serum radioactivity. Asterisks indicate statistically significant differences (* $P < 0.05$). Data are presented as means ± SD.

In wild-type mice, heparin accelerated the serum clearance of both [^{3}H]TO ($t_{1/2}$ = 1.3 ± 0.1 vs. 8.7 ± 1.6 min) and [^{14}C]CO ($t_{1/2}$ = 2.9 ± 0.1 vs. 12.0 ± 2.4 min) (data not shown). This was accompanied by a decreased uptake of [^{3}H]TO-derived radiolabel by peripheral tissues and an increased uptake by the liver (Fig. 3A). [^{14}C]CO-derived radiolabel mainly associated with the liver, both in the absence (35.0 ± 5.1% of injected dose/g) and presence (41.3 ± 7.0% injected dose/g) of heparin (Fig. 3B).

In $lrp^{-}ldlr^{-/-}vldlr^{-/-}$ mice, heparin also accelerated the serum clearance of both [^{3}H]TO ($t_{1/2}$ = 1.7 ± 0.3 vs. 2.9 ± 0.7 min) and [^{14}C]CO ($t_{1/2}$ = 2.8 ± 0.8 vs. 6.4 ± 2.0 min) (data not shown). The serum decay of the VLDL-like emulsion particles in $lrp^{-}ldlr^{-/-}vldlr^{-/-}$ mice was faster than that observed in the previous experiment. This was probably due to the lower TG levels in this specific batch of mice at the time of the experiment (2.2 ± 1.0 vs. 4.0 ± 0.8 mM) and the fact that infection of mice with a recombinant adenovirus per se did affect TG levels to some extent in the previous experiment (7.5 ± 0.9 vs. 4.0 ± 0.8 mM). Similar to wild-type mice, the increased lipolysis after heparin injection was accompanied by an increased association of [^{3}H]TO-derived radiolabel with the liver (Fig. 3C). Again similar to wild-type mice, heparin did not affect the liver association of the core remnants in $lrp^{-}ldlr^{-/-}vldlr^{-/-}$ mice, as judged from an effective liver association of [^{14}C]CO both in the absence (44.3 ± 3.5% of injected dose/g) and presence (42.6 ± 5.0% injected dose/g) of heparin (Fig. 3D). Since heparin releases LPL and HL from endothelial surfaces and the liver, the effective liver association of particle core remnants in $lrp^{-}ldlr^{-/-}vldlr^{-/-}$ mice apparently does not depend on the bridging function of LPL and HL.

Association of VLDL-like emulsion particles with hepatocytes from $lrp^{-}ldlr^{-/-}vldlr^{-/-}$ mice is followed by slow internalization

We have previously shown that the hepatic binding and uptake of TG-rich lipoproteins and VLDL-like emulsion particles is mainly exerted by hepatocytes (29). To establish whether binding of VLDL-like emulsion particles to hepatocytes that lack the LDLr, LRP, and VLDLr can still lead to internalization, we incubated freshly isolated hepatocytes with DiI-labeled emulsion particles (**Fig. 4**). These emulsion particles avidly bound to the cell surface upon incubation at 4°C. LDLr- and LRP-independent internalization of cell-bound emulsion particles was observed on further incubation at 37°C, as evidenced by the detection of fluorescence in compartments below the cell surface after 30 min of incubation (Fig. 4A). However, the rate of internalization was slower compared with that of wild-type hepatocytes, which internalized the majority of cell-associated particles within the same time period (Fig. 4B).

Association of VLDL-like emulsion particles with hepatocytes from $lrp^{-}ldlr^{-/-}vldlr^{-/-}$ mice involves HSPG-bound ligands and SR-BI

Since both cell surface HSPGs (15) and SR-BI (18) have recently been implicated in the hepatic uptake of VLDL, we examined their contribution to the association

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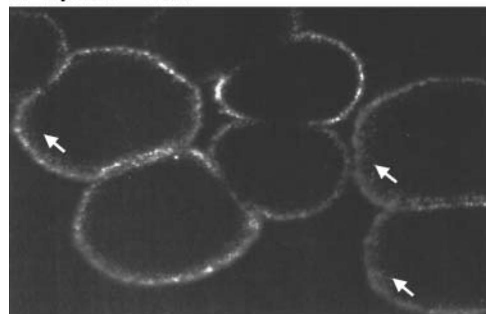

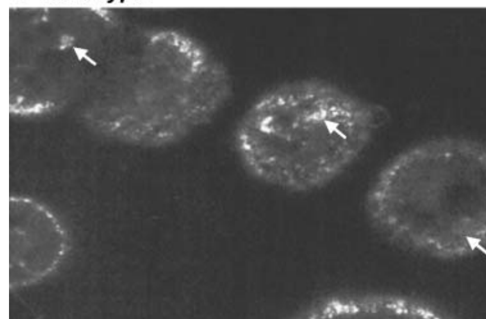

Fig. 4. Effect of LRP and (V)LDL receptor deficiency on the uptake of VLDL-like emulsion particles by hepatocytes. Freshly isolated hepatocytes from *lrp⁻ ldlr⁻/⁻ vldlr⁻/⁻* mice (A) and wild-type mice (B) were incubated (2 h at 4°C) in DMEM + 2% BSA with 50 nm sized 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanide perchlorate (DiI)-labeled emulsion particles (100 μg TG/ml). The cells were washed to remove unbound particles and further incubated at 37°C. After 30 min, localization of DiI was determined by confocal laser-scanning microscopy. Intracellular fluorescently labeled compartments are indicated by arrows. Under the applied conditions, autofluorescence was negligible.

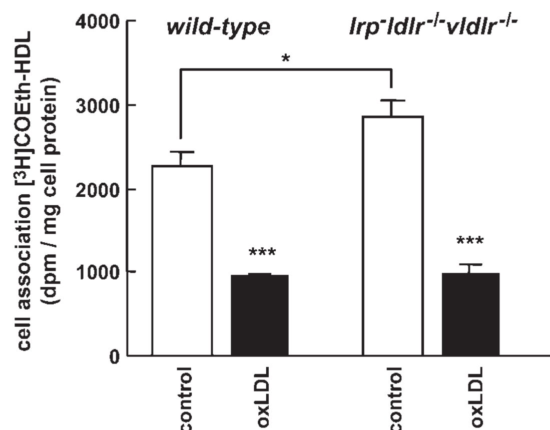

Fig. 5. Effect of LRP and (V)LDL receptor deficiency on the association of HDL with hepatocytes. Freshly isolated hepatocytes from wild-type mice (left) and *lrp⁻ ldlr⁻/⁻ vldlr⁻/⁻* mice (right) were incubated (3 h at 37°C) in DMEM + 2% BSA with [^{3}H]cholesteryl oleoyl ether (COEth)-labeled HDL (20 μg protein/ml) in the absence or presence of oxidized LDL (oxLDL; 100 μg protein/ml). The cells were washed to remove unbound particles, and the cell association of [^{3}H]COEth was measured as dpm/mg cell protein. Values are means ± SD (n = 3). Asterisks indicate statistically significant differences (* $P < 0.05$, *** $P < 0.001$).

of [^{3}H]COEth-labeled VLDL-like emulsion particles with freshly isolated hepatocytes from wild-type mice versus *lrp⁻ ldlr⁻/⁻ vldlr⁻/⁻* mice after incubation for 3 h at 37°C. Deficiency for the LRP, LDLr, and VLDLr did upregulate SR-BI expression to some extent, since the association of [^{3}H]COEth-HDL with hepatocytes from *lrp⁻ ldlr⁻/⁻ vldlr⁻/⁻* mice was higher than with hepatocytes from wild-type mice (+26%; $P < 0.05$) (**Fig. 5**). The established SR-BI inhibitor oxLDL (30) inhibited the association of HDL with both cell types (∼−60%; $P < 0.001$).

Remarkably, the cell association of [^{3}H]COEth emulsion particles was unaffected by deficiency for the LRP, LDLr, and VLDLr (**Fig. 6**), confirming the contribution of pathways other than these classical apoE receptors to the association of emulsion particles with hepatocytes. Heparin, at a concentration of 1,000 U/ml that releases all heparin binding proteins from cell surface HSPGs, including apolipoproteins from cell receptors (13), strongly inhibited the cell association (>95%). Also, oxLDL effectively inhibited the association of [^{3}H]COEth emulsion particles with wild-type (−60%) and *lrp⁻ ldlr⁻/⁻ vldlr⁻/⁻* (−78%) hepatocytes to a similar extent as [^{3}H]COEth-HDL (∼60%), indicating the involvement of SR-BI in the cell association of emulsion particles. To evaluate the effect of the bridging function of LPL and apoE, we also determined the cell association of emulsion particles after previous enrichment with heat-inactivated LPL and apoE. Both LPL and apoE reduced the association of emulsion particles with wild-type and *lrp⁻ ldlr⁻/⁻ vldlr⁻/⁻* hepatocytes. However, both heparin and oxLDL still inhibited the association of these LPL- and apoE-containing emulsion particles with both types of hepatocytes (Fig. 6).

DISCUSSION

The aim of the current study was to investigate the role of LPL activity in the classical apoE receptor-independent clearance of lipoprotein remnants by modulating LPL activity in *lrp⁻ ldlr⁻/⁻ vldlr⁻/⁻* mice and to determine the mechanisms involved in their hepatic uptake. We demonstrate that the hepatic uptake of VLDL core remnants independent of these three main apoE-recognizing receptors is positively regulated by LPL activity and involves HSPGs and SR-BI.

How could modulation of LPL activity affect the hepatic uptake of lipoprotein remnants in the absence of the LDLr, hepatic LRP, and VLDLr? In vitro studies have shown that LPL via its bridging function can mediate the binding and uptake of lipoproteins and their remnants via HSPGs (31–34), LRP (35), and the LDLr (34, 36). A function of catalytically inactive LPL in the hepatic lipoprotein uptake has also been demonstrated in vivo (37, 38). Likewise, HL has been demonstrated to participate in the cell binding and uptake of remnant lipoproteins in vitro (39, 40) and to mediate the clearance of remnant lipoproteins from plasma by its nonlipolytic bridging function in vivo (41). However, heparin, which effectively releases LPL and HL from cellular binding sites and results in increased lipolysis by enhancing

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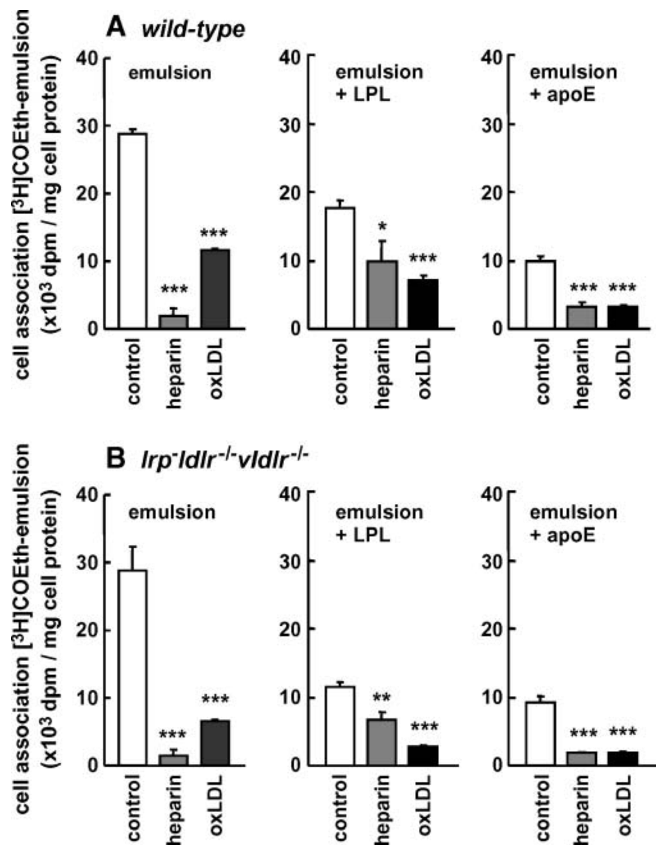

Fig. 6. Effect of LRP and (V)LDL receptor deficiency on the association of VLDL-like emulsion particles with hepatocytes. Freshly isolated hepatocytes from wild-type mice (A) and $lrp^- ldlr^{-/-}$ $vldlr^{-/-}$ mice (B) were incubated (3 h at 37°C) in DMEM + 2% BSA with 50 nm sized $[^3H]$COEth-labeled emulsion particles (100 μg TG/ml), without or with previous enrichment (30 min at 37°C) with heat-inactivated LPL (1 μg/ml) or human apoE (6 μg/ml) in the absence or presence of heparin (1,000 U/ml) or oxLDL (100 μg protein/ml). The cells were washed to remove unbound particles, and the association of $[^3H]$COEth was measured as dpm/mg cell protein. Values are means ± SD (n = 3). Asterisks indicate statistically significant differences (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

the interaction of the catalytically active lipases with lipoproteins, did not abrogate the rapid binding of VLDL-like emulsion-associated $[^{14}C]$CO activity with the liver in either wild-type and $lrp^- ldlr^{-/-} vldlr^{-/-}$ mice. Furthermore, the in vitro studies with isolated hepatocytes showed that enrichment of VLDL-like emulsion particles with catalytically inactive LPL decreased rather than increased the cell association of the particles. These data indicate that the rapid initial hepatic association of the particle core remnants in $lrp^- ldlr^{-/-} vldlr^{-/-}$ mice is likely to be independent of the bridging function of LPL and HL. This conclusion is corroborated by our observation that Ad*APOC1* reduced the hepatic association of the particle core remnants without affecting either postheparin plasma levels of LPL activity (11.5 ± 7.6 vs. 11.4 ± 1.6 μmol FFA/h/ml) or LPL protein (295 ± 40 vs. 254 ± 53 ng/ml) compared with Ad*LacZ*. Taken together, the positive correlation between plasma LPL activity and hepatic VLDL remnant uptake in lrp^- $ldlr^{-/-} vldlr^{-/-}$ mice does not seem to be caused by the bridging functions of LPL and HL. Most likely, it is a consequence of the catalytic function of LPL, which accelerates the generation of VLDL core remnants that subsequently become associated with the liver via mechanisms unrelated to the classical apoE receptors and lipases.

So what mechanisms do underlie the binding and uptake of VLDL remnants by the liver through apparently nonclassical pathways? Although it has been suggested that LRP5 (42), apoB-48 receptor (43), and LR11 (44) may play a role in the metabolism of apoB-containing lipoproteins, the most likely candidates appear to be HSPGs (15) and SR-BI (16, 18).

It has previously been shown that lactoferrin inhibits the binding of chylomicron remnants and β-VLDL to HSPGs in vitro (45), since lactoferrin effectively binds to HSPGs, probably due to its structural homology with the heparin binding site of apoE and its overall positive charge (46). In addition, lactoferrin reduces the hepatic association of the currently applied VLDL-like emulsion particles by 90% in vivo (25), indicating that hepatic association of these particles is dependent on HSPG. Protamine, like lactoferrin, binds to HSPGs by electrostatic interaction, thereby reducing remnant binding (47). We have shown that protamine administration to mice that lack both the LDLr and hepatic LRP completely inhibited the liver association of the emulsion particles (P.C.N. Rensen, unpublished observations). These observations thus underscore the involvement of HSPGs in the hepatic association of VLDL-like emulsion particle remnants. Indeed, treatment of hepatocytes from wild-type and $lrp^- ldlr^{-/-} vldlr^{-/-}$ mice with heparin at a concentration that releases a number of ligands from cell surface HSPGs, including apolipoproteins from cell receptors (48, 49), strongly inhibited the cell association (>95%). This indicates the involvement of HSPG-bound ligands in the binding and possibly also the uptake of VLDL-like emulsion particles by hepatocytes.

Our observation that heparin did not reduce the liver association of the VLDL-like particle core remnants in wild-type and $lrp^- ldlr^{-/-} vldlr^{-/-}$ mice is in accordance with previous observations in $ldlr^{-/-}$ mice (50). The seeming discrepancy that heparin blocks the association of emulsion particles with hepatocytes in vitro, but not with the liver in vivo, is probably related to the additional effect of heparin in vivo (i.e., accelerated remnant formation through increasing plasma LPL activity) as well as to the difference between the doses of heparin used in the in vitro and in vivo studies. Al-Haideri et al. (13) have shown that a high concentration of 1,000 U/ml releases all heparin binding proteins from cells, thereby blocking the association of similar emulsion particles to fibroblasts, whereas a low concentration of 10 U/ml only displaces a number of ligands from HSPGs and had no effect on the uptake of emulsion particles by fibroblasts. Accordingly, we observed that 1,000 U/ml heparin blocked the association of emulsion particles with hepatocytes in vitro, and the generally applied dosage of 500 U/kg heparin (which corresponds to 10–15 U/ml plasma) did not reduce the liver binding of emulsion particles in vivo. Taken together with the fact that heparin at a concentration of 100 U/ml only mar-

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ginally affects the binding of newly secreted apoE to cells (5) and does not release apoE from immobilized HSPGs (51), it is well possible that under the in vivo conditions hepatocytes still contain sufficient apoE on the cell surface to allow sequestration of remnants on the hepatocyte surface and to facilitate remnant uptake via its secretion-capture role (5). In addition, it has been shown that the VLDL-like emulsion particles that we used in this study rapidly acquire apoE from plasma (25, 52), which would allow plasma-derived apoE to also play a role in the subsequent uptake of emulsion particles by hepatocytes.

SR-BI was recently identified as a receptor that internalizes VLDL holoparticles (18). Our in vitro data showed that SR-BI expression by hepatocytes was functionally upregulated to some extent by the deficiency for the LDLr, LRP, and VLDLr. The established SR-BI inhibitor oxLDL (30) markedly reduced the association of [^{3}H]COEth-labeled VLDL-like emulsion particles with hepatocytes from both wild-type and *lrp*$^-$ *ldlr*$^{-/-}$ *vldlr*$^{-/-}$ mice to a similar extent as that of [^{3}H]COEth-labeled HDL, which indeed indicates the involvement of SR-BI in the binding and uptake of VLDL-like emulsion particles by hepatocytes. SR-BI recognizes an array of ligands, including apolipoproteins and negatively charged phospholipids. Since the phospholipid surface of the emulsion particles has a negligible charge, as is evident from the low electrophoretic mobility on agarose gels compared with (oxidized) lipoproteins (25), it is unlikely that SR-BI directly binds to the emulsion-associated phospholipids. Instead, the association of VLDL core remnants with hepatocytes through SR-BI most likely involves apoE. Mice deficient for the LDLr have elevated plasma apoE levels compared with wild-type mice, which are maintained upon the deletion of LRP (3, 53) and the VLDLr (12), and apoE is a well-established ligand for SR-BI (54–56).

In conclusion, we have demonstrated that in the absence of the three major apoE-recognizing receptors, the uptake of VLDL remnants by the liver is regulated by the catalytic function of LPL in plasma and involves both hepatic HSPGs and SR-BI.

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