The VLDL receptor plays a major role in chylomicron metabolism by enhancing LPL-mediated triglyceride hydrolysis

Jeltje R. Goudriaan,* Sonia M. S. Espirito Santo,* Peter J. Voshol,1,*† Bas Teusink,* Ko Willems van Dijk,3,*** Bart J. M. van Vlijmen,* Johannes A. Romijn,†
Louis M. Havekes,*8 and Patrick C. N. Rensen*§

Institute for Applied Scientific Research Prevention and Health,* Gaukbury Laboratory, 2301 CE Leiden, The Netherlands; and Department of Endocrinology and Diabetes,† Department of Cardiology and General Internal Medicine,§ and Molecular Genetics and Cell Biology-Department of Human Genetics,** Leiden University Medical Center, 2300 RC Leiden, The Netherlands

Abstract  The VLDL receptor (VLDLr) is involved in tissue delivery of VLDL-triglyceride (TG)-derived FFA by facilitating the expression of lipoprotein lipase (LPL). However, vldlr−/− mice do not show altered plasma lipoprotein levels, despite reduced LPL expression. Because LPL activity is crucial in postprandial lipid metabolism, we investigated whether the VLDLr plays a role in chylomicron clearance. Fed plasma TG levels of vldlr−/− mice were 2.5-fold increased compared with those of vldlr+/+ littermates (1.20 ± 0.37 mM vs. 0.47 ± 0.18 mM; P < 0.001). Strikingly, an intragastric fat load led to a 9-fold increased postprandial TG response in vldlr−/− compared with vldlr+/+ mice (226 ± 188 mM/h vs. 25 ± 11 mM/h; P < 0.05). Accordingly, the plasma clearance of [3H]TG-labeled protein-free chylomicron-mimicking emulsion particles was delayed in vldlr−/− compared with vldlr+/+ mice (half-life of 12.0 ± 2.6 min vs. 5.5 ± 0.9 min; P < 0.05), with a 60% decreased uptake of labeled into adipose tissue (P < 0.05). VLDLr deficiency did not affect the plasma half-life and adipose tissue uptake of albumin-complexed [14C]FFA, indicating that the VLDLr facilitates postprandial LPL-mediated TG hydrolysis rather than mediating FFA uptake. We conclude that the VLDLr plays a major role in the metabolism of postprandial lipoproteins by enhancing LPL-mediated TG hydrolysis. — Goudriaan, J. R., S. M. S. Espirito Santo, P. J. Voshol, B. Teusink, K. W. van Dijk, B. J. M. van Vlijmen, J. A. Romijn, L. M. Havekes, and P. C. N. Rensen. The VLDL receptor plays a major role in chylomicron metabolism by enhancing LPL-mediated triglyceride hydrolysis. J. Lipid Res. 2004. 45: 1475–1481.

Supplementary key words adipose tissue • free fatty acids • lipoprotein lipase • postprandial lipid metabolism • very low density lipoprotein-like emulsion • transgenic mice

Abbreviations: apoE, apolipoprotein E; HL, hepatic lipase; LDLr, LDL receptor; LPL, lipoprotein lipase; TG, triglyceride; VLDLr, VLDL receptor.

1 To whom correspondence should be addressed.

E-mail: pj.voshol@pg.tno.nl

Published, JLR Papers in Press, May 16, 2004.
DOI 10.1194/jlr.M400009-JLR200

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ing the VLDLr by gene targeting. Although the initial in vitro data suggested that the VLDLr may play a prominent role in TG metabolism (4, 5), these vldlr−/− mice exhibited no differences in plasma lipoproteins on a normal chow diet (9). However, stressing the FA metabolism by prolonged fasting (10), a high-fat diet (10), or cross-breeding on a hyperlipidemic ob/ob (10) or ldlr−/− (11) background, VLDLr deficiency led to a hypertriglyceridemic phenotype in mice. In addition, under conditions of dietary stress, we showed that despite the development of hypertriglyceridemia, vldlr−/− mice were protected from diet-induced obesity (10). Recently, Yagyu et al. (12) showed that the VLDLr can play a role in the delivery of VLDL-TG-derived FFAs to adipose tissue, because VLDLr deficiency resulted in defective VLDL catabolism in vivo associated with reduced activity of LPL, albeit under prolonged fasting conditions. Compared with the fasting state, LPL activity in adipose tissue is increased postprandially (13), and the flux of TG-derived FFA to adipose tissue is greatly enhanced during the postprandial state (14). Therefore, we hypothesize that the VLDLr plays a crucial role in postprandial lipoprotein metabolism.

Thus, the aim of the present study was to evaluate the role of the VLDLr in chylomicron metabolism. We show that VLDLr deficiency in mice severely impairs chylomicron catabolism. Furthermore, postprandial TG-derived FFA uptake in adipose tissue is severely impaired, indicating that the VLDLr is required for optimal LPL-mediated chylomicron processing in vivo. These findings can fully explain our earlier observation that vldlr−/− mice are protected against diet-induced and genetically induced obesity (10). From the present study, we conclude that the VLDLr plays a major role in postprandial lipoprotein metabolism by facilitating LPL-mediated TG hydrolysis.

MATERIALS AND METHODS

Animals

Vldlr+/− mice originated from the Jackson Laboratories and were bred in our animal facility in Leiden. Nontransgenic littermates (vldlr+/+) were used as controls. All mice used in this study were housed under standard conditions with free access to water and food and a normal 12 h light/dark cycle (lights on at 7:00 AM and off at 7:00 PM). Principles of laboratory animal care were followed, and the animal ethics committee of our institute approved all animal experiments.

Plasma lipid analysis

To determine postprandial and fasting lipid levels, tail vein blood was collected from male vldlr−/− and vldlr+/+ mice at 7:00 AM just after the end of the dark cycle (fed state) and at 11:00 AM (after a 4 h fasting period) into chilled pararaffon-coated capillary tubes to prevent ongoing lipolysis (15). These tubes were placed on ice and immediately centrifuged at 4°C. Plasma levels of total cholesterol, TG (corrected for free glycerol), and FFA were determined enzymatically using commercially available kits and standards (236691, Boehringer, Mannheim, Germany; 337-B GPO-Trinder kit, Sigma, St. Louis, MO; Nefa-C kit, Wako Chemicals GmbH, Neuss, Germany).

LPL and hepatic lipase enzyme activity

Female vldlr−/− and vldlr+/+ mice were fasted for 4 h, and postheparin plasma was obtained 10 min after a tail vein injection of heparin (0.1 U/g body weight) (Leo Pharma BV, Weesp, The Netherlands). The LPL activity was determined in postheparin plasma as described (16).

Intragastric fat load

To investigate the handling of postprandial TG, vldlr−/− and vldlr+/+ mice were given an intragastric 200 μl olive oil bolus after an overnight fast. Blood samples were drawn at the indicated time points after administration. TG concentrations were determined in plasma as described above and are presented as relative increase from time 0.

Preparation of emulsion particles

Protein-free chylomicron-mimicking TG-rich emulsion particles were prepared essentially as described by Rensen et al. (17) from 100 mg of total lipid at a weight ratio of triolein (Sigma)-egg yolk phosphatidylcholine (Lipoid, Ludwigshafen, Germany)-lyso phosphatidylcholine (Sigma)-cholesteryl oleate (Sigma)-cholesterol (Sigma) of 70:22.7:2.3:3.0:2.0 in the presence of 200 μCi of glycerol tril[9,10(n)H]oleate ([3H]TG) (Amersham, Little Chalfont, UK). Lipids were hydrated in 10 ml of 2.4 M NaCl, 10 mM HEPES, and 1 mM EDTA, pH 7.4, and sonicated for 30 min at 10 μm output using a Soniprep 150 (MSE Scientific Instruments) equipped with a water bath for temperature (54°C) maintenance. Subsequently, the emulsion was separated into fractions with different average sizes by density gradient ultracentrifugation (17).

In vivo lipolysis: bolus experiment

Fed female vldlr−/− and vldlr+/+ mice were anesthetized (0.5 mg/kg Domitor, Pfizer Animal Health, Capelle a/d IJssel, The Netherlands; 0.05 mg/kg Pentanyl Bipharma, Pharma Hameln, Hameln, Germany; and 5 mg/kg midazolam, Roche, Mijdrecht, The Netherlands), and the abdomens were opened. [3H]TG-labeled emulsion particles were injected via the vena cava inferior at t = 0 as a large bolus (1.0 mg of TG), resulting in plasma TG levels well above the endogenous TG concentration. At the time points indicated after injection, small blood samples (30 μl) were taken from the vena cava inferior and serum 3H-radioactivity was measured. At 15 min, the mice were sacrificed, and their organs excised and weighed. Radioactivity was counted after the tissues were dissolved in Solvable (Packard Bioscience, Groningen, The Netherlands), and corrections were made for the serum radioactivity in the tissues as described by Rensen et al. (17).

In vivo lipolysis: infusion experiment

After 2 weeks on a high-fat diet (46.2% of the calories as fat; Hope Farms, Woerden, The Netherlands), fed male vldlr−/− and vldlr+/+ mice were anesthetized and an infusion needle, connected to a Harvard microdialysis low-flow 11 minipump (Holliston, MA), was inserted into the tail vein. [3H]TG-labeled emulsion particles (1.0 mg TG/ml) and a trace amount of [14C]palmitic acid (Amersham) complexed to BSA (2%) in the presence of citrate (3 μg/ml) were infused at a rate of 0.2 ml/h for 2 h to achieve steady-state TG levels. After 1.5 and 2 h, a 150 μl blood sample was taken by tail bleeding. Subsequently, the mice were killed and their organs quickly removed and frozen in liquid nitrogen. Plasma levels of TG and FFA were determined as described above. Lipids were extracted from plasma according to Bligh and Dyer (18). The lipid fraction was dried under nitrogen, dissolved into chloroform-methanol (5:1, v/v), and subjected to TLC (LK5D gel 150; Whatman) using hexane-diethyl-ether-acetic acid (85:16:1, v/v/v) as mobile phase. Lipid spots
were visualized by I$_2$ vapor and scraped off, lipids were dissolved in hexane, and radioactivity was measured. Tissues were dissolved in 3 M KOH in 50% (v/v) ethanol at 70°C for 1 h. Retention of radioactivity in the tissues was measured per milligram of protein and corrected for the corresponding plasma-specific activities of [3H]TG and [14C]FFA.

**Statistical analysis**

The Mann-Whitney nonparametric test for two independent samples was used to define differences between vldlr$^{-/-}$ and vldlr$^{+/+}$ mice. The criterion for significance was set at $P < 0.05$. All data are presented as means ± SD.

**RESULTS**

**VLDLr deficiency increases fed TG levels**

To investigate the effect of VLDLr deficiency on postprandial lipid metabolism, plasma lipid levels were measured in fed and 4 h fasting male vldlr$^{-/-}$ and vldlr$^{+/+}$ mice (Table 1). Whereas no significant differences were found between these mice with respect to TG, FFA, and cholesterol levels after a 4 h fast, fed vldlr$^{+/+}$ mice did show significantly increased levels of TG (2.5-fold) and FFA (1.6-fold), but not cholesterol, compared with vldlr$^{-/-}$ mice. In fact, postprandial chylomicron-TG levels (i.e., the level in wild-type mice at 2 h and reached a maximum only after 8 h, vldlr$^{-/-}$ mice showed a severely increased and prolonged hypertriglyceridemia. The increase in TG was already 7-fold compared with the level in wild-type mice at 2 h and reached a maximum only after the fat load. Therefore, the postprandial TG response in vldlr$^{-/-}$ compared with vldlr$^{+/+}$ mice was 9-fold increased (AUC: 226 ± 188 mM/h vs. 25 ± 11 mM/h), demonstrating that VLDLr deficiency leads to severe impairment of postprandial TG clearance. Again, similar findings were observed for female mice.

**VLDLr deficiency decreases uptake of emulsion TG-derived FFA by adipose tissue**

Subsequently, we investigated whether VLDLr deficiency affects the delivery of postprandial lipoprotein-derived FFA to tissues with the highest LPL expression in the fed state [i.e., adipose tissue and heart (19)]. To exclude interindividual variations with respect to intestinal lipid absorption, [3H]TG-labeled emulsion particles that closely mimic chylomicrons were injected intravenously as a bolus dose and radioactivity in the tissues was measured per milligram of tissue.

**VLDLr deficiency increases postprandial TG response**

To investigate further the role of the VLDLr in postprandial lipoprotein metabolism, we administered an intragastric olive oil load by gavage to male vldlr$^{-/-}$ and vldlr$^{+/+}$ mice (Fig. 2). Strikingly, whereas wild-type mice showed only a mild increase in TG levels at 2 h after administration (from 0.3 ± 0.1 to 1.0 ± 0.4 mM), which reached baseline levels again by 8 h, vldlr$^{-/-}$ mice showed a severely increased and prolonged hypertriglyceridemia. The increase in TG was already 7-fold compared with the level in wild-type mice at 2 h and reached a maximum only after the fat load. Therefore, the postprandial TG response in vldlr$^{-/-}$ compared with vldlr$^{+/+}$ mice was 9-fold increased (AUC: 226 ± 188 mM/h vs. 25 ± 11 mM/h), demonstrating that VLDLr deficiency leads to severe impairment of postprandial TG clearance. Again, similar findings were observed for female mice.

**TABLE 1. Effect of VLDLr deficiency on fed and 4 h fasting plasma lipid levels**

| Mouse       | Fed TGs (mM) | Fasted TGs (mM) | Fed FFA (mM) | Fasted FFA (mM) | Fed Cholesterol (mM) | Fasted Cholesterol (mM) |
|-------------|--------------|-----------------|--------------|-----------------|-----------------------|------------------------|
| vldlr$^{+/+}$ | 0.47 ± 0.18* | 0.27 ± 0.06     | 0.64 ± 0.25* | 0.64 ± 0.15     | 2.29 ± 0.35            | 2.37 ± 0.37            |
| vldlr$^{-/-}$ | 1.20 ± 0.37* | 0.44 ± 0.20     | 1.02 ± 0.15* | 0.83 ± 0.29     | 2.08 ± 0.24            | 2.10 ± 0.23            |

TG, triglyceride; VLDLr, VLDL receptor. Vldlr$^{+/+}$ and vldlr$^{-/-}$ mice were fed a normal chow diet. Plasma levels of TGs, free fatty acids, and total cholesterol were measured in the fed state and after 4 h of fasting as described. Values represent means ± SD of eight vldlr$^{+/+}$ and eight vldlr$^{-/-}$ mice.

$^a P < 0.001$, $^b P < 0.05$.

**Fig. 1.** Effect of VLDL receptor (VLDLr) deficiency on plasma lipoprotein lipase (LPL) and hepatic lipase (HL) activity. Postheparin plasma was obtained from vldlr$^{+/+}$ (closed bars) and vldlr$^{-/-}$ (open bars) mice, and triglyceride (TG) hydrolyase activity was measured in the absence (i.e., LPL and HL) or presence (i.e., HL) of 1 M NaCl. Values represent means ± SD of 10 vldlr$^{+/+}$ and 8 vldlr$^{-/-}$ mice. * $P < 0.05$.**
plasma TG concentrations were determined in plasma and corrected for TG values. Blood samples were drawn at 0, 2, 4, 8, and 24 h after the bolus. TG concentrations were determined in plasma and corrected for TG values at time 0. Values represent means ± SD of seven vldlr+/+ and eight vldlr−/− mice. * P < 0.05.

To evaluate whether the VLDLr may function by transporting FFAs across the endothelial layer per se rather than by facilitating LPL-mediated lipolysis, [3H]TG-labeled chylomicron-like emulsion particles were continuously infused together with albumin-bound [14C]FFA palmitate in a high-fat diet. On this diet, similar increasing effects of VLDLr deficiency on fed plasma TG levels were observed compared with the chow diet (1.10 ± 0.27 mM for vldlr+/+ mice and wild-type littermates, respectively; P < 0.05). Plasma half-lives of both [3H]TG and [14C]FFA were calculated from steady-state kinetics (14). Whereas the plasma clearance of [3H]TG under these conditions was again delayed in vldlr−/− compared with vldlr+/+ mice, the plasma half-life of [14C]FFA was not affected significantly (Table 2). Accordingly, the uptake of [3H]TG-derived activity by adipose tissue per mass unit was 60% decreased in vldlr−/− mice (Fig. 4A). The uptake of [3H]TG-derived activity by the heart per mass unit was increased in vldlr−/− mice, the plasma half-life of [14C]FFA was 55% decreased uptake by skeletal muscle per mass unit (Fig. 3B). As depicted in Fig. 3B, the uptake of [3H]TG-derived radiolabel by the heart per mass unit was higher in vldlr−/− mice. However, it was noted that the total heart weight of vldlr−/− mice was 25% reduced compared with that of wild-type mice (0.74 ± 0.06 g vs. 0.98 ± 0.09 g; P < 0.05). Therefore, the total uptake of [3H] radioactivity by the heart was not different (P = 0.149) when corrected for total heart weight.

VLDLr deficiency does not affect the uptake of albumin-bound FFAs by adipose tissue

After bolus injection of [3H]TG-labeled chylomicron-like emulsion particles (described in the legend to Fig. 3) or during continuous infusion of these particles in the presence of [14C]FFA (described in the legend to Fig. 4), blood samples were taken and plasma half-lives (t1/2) were calculated from monoexponential decay curves (bolus) or from steady-state kinetics (infusion) according to Teusink et al. (14). Values represent means ± SD of four vldlr−/− and four vldlr+/+ mice (bolus) or four vldlr−/− and six vldlr+/+ mice (infusion).

| Mouse | Bolus Injection | Steady-State Infusion |
|-------|----------------|-----------------------|
|       | t1/2 [3H]TG | t1/2 [3H]TG | t1/2 [14C]FFA |
| vldlr+/+ | 5.5 ± 0.9a | 6.6 ± 1.2a | 2.0 ± 0.4 |
| vldlr−/− | 12.0 ± 2.6a | 9.2 ± 2.3a | 2.5 ± 0.2 |

After bolus injection of [3H]TG-labeled chylomicron-like emulsion particles (described in the legend to Fig. 3) or during continuous infusion of these particles in the presence of [14C]FFA (described in the legend to Fig. 4), blood samples were taken and plasma half-lives (t1/2) were calculated from monoexponential decay curves (bolus) or from steady-state kinetics (infusion) according to Teusink et al. (14). Values represent means ± SD of four vldlr−/− and four vldlr+/+ mice (bolus) or four vldlr−/− and six vldlr+/+ mice (infusion).

* P < 0.05.
A decreased VLDL catabolism in caused by increased hepatic VLDL production (10). A decreased LPL activity (12). These data were derived from mice that had fasted for 16 h. However, they observed no change in the uptake of VLDL-TG by adipose tissue and a reduced uptake by heart and muscle in VLDLr deficiency, whereas we find a significantly decreased uptake of chylomicron-like emulsion TG by adipose tissue with no consistent reduction in uptake of VLDL-TG by adipose tissue and heart (9). The de-

A decreased LPL activity appeared to be 20% decreased (P < 0.05) in female vldlr−/− mice, which is in agreement with recent observations in male vldlr−/− mice by others (12). The decreased postheparin LPL activity is not solely attributable to decreased adipose tissue stores, because Yagyu et al. (12) showed that VLDLr deficiency also reduces the specific LPL activity in adipose tissue per mass unit.

The involvement of a reduced peripheral lipolysis of postprandial lipoproteins as part of the mechanism underlying the disturbed postprandial TG clearance was established by performing kinetic studies with [3H]TG-labeled chylomicron-mimicking emulsion particles. These emulsions have previously been shown to be true chylomicron mimics with respect to rapid apoE-mediated uptake from plasma, LPL-mediated remnant formation, and efficient apoE-mediated hepatic uptake of core remnants by the LDLr and LDLr-related protein (17). Both upon intravenous bolus injection and continuous infusion, the plasma half-life of TG was prolonged in vldlr−/− compared with vldlr+/- mice in the fed state. Accordingly, the distribution of [3H]TG-derived FFAs into adipose tissue was 60% decreased, irrespective of the mode of administration, bolus or continuous infusion. Taking into account that the total fat pad mass is reduced in vldlr−/− mice compared with wild-type littermates (9, 10), the difference in total uptake of radiolabel by adipose tissue is even more pronounced. Our findings complement the data of Yagyu et al. (12), who showed decreased VLDL-TG catabolism in vldlr−/− mice that had fasted for 16 h. However, they observed no change in the uptake of VLDL-TG by adipose tissue and a reduced uptake by heart and muscle in VLDLr deficiency, whereas we find a significantly decreased uptake of chylomicron-like emulsion TG by adipose tissue with no consistent reduction in uptake of TG by heart or muscle. Although substrate variation (i.e., emulsion particles vs. native VLDL) may have contributed to the observed discrepancy, the differences between the two studies can be fully explained by the natural regulation of tissue LPL levels by the feeding state, as prolonged

**DISCUSSION**

Although earlier studies have reported that the VLDLr is able to bind and internalize TG-rich lipoproteins as mediated via apoE and/or LPL in vitro (4, 5), vldlr−/− mice were initially reported to have no phenotype with respect to lipid and/or lipoprotein levels (9). Placing FFA metabolism under stress by feeding vldlr−/− mice a high-fat diet or cross-breeding these mice onto a hyperlipidemic background (10, 11) or by prolonged fasting (10, 12) did lead to a hypertriglyceridemic phenotype, which was not caused by increased hepatic VLDL production (10). A recent study has shown that a decreased VLDL catabolism in vldlr−/− compared with vldlr+/- mice was related to decreased LPL activity (12). These data were derived from mice that underwent a prolonged 16 h fasting period (12). However, because LPL is more crucial in postprandial lipoprotein metabolism, we hypothesized that the role of the VLDLr may be most prominent under postprandial conditions, during which the flux of TG-derived FFAs to adipose tissue is greatly enhanced (14). Therefore, we performed as yet unaddressed studies to evaluate a function of the VLDLr in chylomicron catabolism using vldlr−/− mice and wild-type littermates. Indeed, we found that the VLDLr has a prominent physiological function in the lipolysis of postprandial lipoproteins, with a subsequent decreased delivery of particle TG-derived FFAs to adipose tissue, and not in the uptake of FFAs per se.
fasting induces a gradually decreased expression of LPL by adipose tissue whereas that of heart and muscle is gradually increased (13, 19–23). Furthermore, under fed conditions, the flux of TG-derived FFAs to adipose tissue is greatly enhanced compared with the fasting state (14). The finding that the VLDR is crucially involved in the uptake of chylomicron-TG-derived FFA by adipose tissue appears to fully explain the prevention of high-fat-diet-induced obesity in vldlr−/− mice as we described previously (10). Clearance of albumin-bound FFAs from the circulation did not seem to be affected by VLDR deficiency, because the plasma half-life of albumin-bound FFAs was not significantly different between fed vldlr−/− mice and their littermate controls. However, a tendency toward a 25% increased half-life of FFAs in fed vldlr−/− mice was observed (Table 2), which is in agreement with the reduced FFA turnover we previously reported for fasted vldlr−/− mice (10). Taken together with the observation that plasma FFA levels were also significantly increased in fed mice (Table 1), we cannot exclude that the VLDRs may have an additional function in FFA turnover per se.

The effect of reduced peripheral lipolysis in VLDR deficiency is thus likely to be one of the reasons underlying the postprandial hypertriglyceridemia, confirming previous reports on the crucial involvement of LPL in TG metabolism. First, LPL knockout mice do not survive, most probably because of extreme hypertriglyceridemia (24). Conversely, mice that overexpress LPL show a decreased postprandial TG response (25). Similarly, transgenic mice overexpressing the main endogenous LPL inhibitor apoC-III develop hypertriglyceridemia (26), whereas apoCIII−/− mice exhibit reduced plasma TG levels and are protected from postprandial hypertriglyceridemia (26, 27).

Mice heterozygous for a mutation in the LPL gene (lpl+/−) were shown to have a 43% decreased postheparin LPL activity (28). In these mice, plasma TG levels were increased during ad libitum feeding and after fasting for 12 h (28). In addition, the reaction to orally administered vitamin A-containing corn oil was impaired in lpl+/− mice compared with wild-type littermates, as shown by a 3-fold increased vitamin A response (24). Although comparison of vldlr−/− mice with lpl+/− mice reveals similarities with respect to its impeding effects on TG metabolism, the effects of VLDR deficiency on chylomicron catabolism are much more severe and cannot be explained solely by a modest 20% reduced LPL expression. Despite less affected LPL levels, the increasing effects of VLDR deficiency on the postprandial TG response is much more dramatic (i.e., 9-fold) than the effects of LPL heterozygosity (i.e., 5-fold) (our unpublished observations). These data indicate that besides affecting LPL expression by its chaperone function (7) or LPL binding properties (4), the VLDR is likely to participate in chylomicron catabolism by bridging of particles to the endothelial surface (“docking function”), thereby facilitating the LPL-particle interaction. In addition, the involvement of the VLDR in lipoprotein internalization, as has been shown by VLDR-expressing cells in vitro (4, 5) and by hepatocytes after recombinant adenovirus-mediated transfer of the VLDR in vivo (29), cannot be conclusively eliminated as an additional factor contributing to TG clearance.

To summarize, we have shown that VLDR deficiency leads to a disturbed clearance of postprandial lipoproteins into peripheral tissues such as adipose tissue, which can explain our previous findings that vldlr−/− mice are protected from diet-induced obesity. We conclude that the VLDR plays a major physiological role in postprandial lipoprotein metabolism by enhancing LPL-mediated TG hydrolysis, with the subsequent delivery of FFAs to adipose tissue. The authors are grateful to Marijke Voskuilen for excellent technical assistance. The research described in this paper is supported by the Leiden University Medical Center (Gisela Thier Fellowship to P.C.N.R.), the Netherlands Organization for Scientific Research [Netherlands Scientific Research Council (NWO) Grant 903-39-192/194, NWO VIDI Grant 917.36.351, and NWO VENI Grant 916.36.071], and the Netherlands Diabetes Foundation (Dutch Diabetes Research Foundation Grant 96.604).

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