Lipoprotein Lipase Bound to Apolipoprotein B Lipoproteins Accelerates Clearance of Postprandial Lipoproteins in Humans

Chunyu Zheng, Susan J. Murdoch, John D. Brunzell, Frank M. Sacks

Objectives—Experiments in cells and animal models show that lipoprotein lipase (LpL) bound to apolipoprotein (apo)B lipoproteins enhances their uptake by receptor mediated pathways. It is unknown whether this pathway is important in humans.

Methods and Results—apoB lipoproteins with LpL were isolated from normal subjects after oral fat loading by immunoaffinity chromatography and were further separated into apoB100 and apoB48 lipoproteins. Postprandially, apoB lipoproteins with LpL had significantly greater increases (4- to 10-fold) and faster rates of clearance (5- to 8-fold) percentage-wise than those without LpL. apoB lipoproteins with LpL had enhanced clearance regardless of whether they also contained apoE. LpL was particularly important for the clearance of apoB48 lipoproteins, of which 25% (range, 11% to 31%) could be removed from circulation together with LpL during the postprandial state. apoB lipoproteins with LpL were larger in size and were enriched in triglyceride, cholesterol, and apoE compared with those without LpL. However, neither size nor apoE content explained the faster clearance rates of LpL-containing lipoproteins.

Conclusion—Plasma LpL may act like an apolipoprotein to enhance the clearance of apoB lipoproteins in humans, a mechanism particularly important for intestinal lipoproteins in the postprandial state. (Arterioscler Thromb Vasc Biol. 2006;26:000-000.)

Key Words: lipoprotein lipase ■ ApoB lipoproteins ■ apoliprotein E ■ postprandial response ■ dietary fats

Lipoprotein lipase (LpL) is the key enzyme that hydrolyzes triglyceride (TG) in TG-rich lipoprotein (TRL) such as chylomicrons and very-low-density lipoprotein (VLDL), thereby regulating the uptake of free fatty acids by peripheral tissues, especially adipose tissue, skeletal muscle, and cardiac muscle.1,2 After being synthesized in parenchymal cells of these tissues, LpL is transported to the luminal surface of capillary vascular endothelium and attaches to heparan sulfate side chains of proteoglycans (HSGP), where it binds to TRL and hydrolyzes its TG content.3 Growing evidence suggests that LpL may also be involved in further metabolism of TRL remnants aside from its lipolytic function. A low concentration of LpL has been detected in human blood, and it is mainly associated with apoB lipoproteins.4,5 As early as 1975, Felts et al proposed that LpL could bind to lipoproteins in vivo and might serve as a marker for remnant uptake by the liver.6 In cell culture experiments, LpL is able to enhance the uptake and degradation of chylomicrons, VLDL, and their remnants. Beisiegel et al reported that the addition of LpL increased the cellular binding of chylomicrons to HepG2 cells by up to 30-fold and also increased the cellular binding of β-VLDL to low-density lipoprotein (LDL) receptor-negative fibroblasts up to 40-fold, through either directly binding to LDL receptor related protein (LRP) or enhancing the binding of apolipoprotein (apo)E to LRP.7 Interestingly, the lipolytic function of LpL is not required in LpL-mediated clearance because the enhanced uptake of LpL-associated lipoproteins is not affected by inhibitors of its lipolytic activity, and a carboxy-terminal fragment of LpL, which lacks the catalytic domain, is also able to enhance the binding and uptake of lipoproteins by LDL receptor-negative fibroblasts in a manner that mimics the native enzyme.8—10 In addition, Skottova et al discovered that catalytically inactive LpL added to the perfusate significantly increased the clearance of chylomicrons by perfused rat liver.11 However, there is only limited information on the importance of LpL-mediated clearance of apoB lipoproteins in vivo. Transgenic mice that overexpressed catalytically inactive LpL in muscle have decreased plasma TG concentrations,12 and injected human TRL are removed by the liver to a significantly larger extent than in wild-type mice,13 suggesting LpL may be able to enhance the clearance of lipoprotein
from circulation in vivo in mice. If this pathway is also present in humans, it may have important physiological implications because plasma LpL was found mainly on triglyceride-rich lipoproteins, including VLDL and chylomicrons.

The current project aims to extend the findings from in vitro experiments and animal studies to humans to determine whether apoB lipoproteins with LpL are cleared from circulation faster than those without LpL, and whether they account for an important amount of postprandial lipoprotein flux.

Methods
A detailed description of methods and materials is available online at http://atvb.ahajournals.org.

Nine healthy volunteers (6 men and 3 women) between 25 and 38 years of age completed the study. Blood was first drawn after an overnight fast (baseline). Participants then ingested a fat load in the form of a milkshake (1339 kcal with 70% of energy from fat) and had blood drawn every hour between 3 and 8 hours after oral fat-loading. Tetrahydrolipstatin (THL), an active inhibitor of LpL, was immediately added to inhibit further hydrolysis occurring in vitro after blood sampling.

Sequential immuno-affinity chromatography, developed from a procedure previously described and validated, was used to separate lipoproteins according to their contents of LpL, apoB, or apoE. Plasma samples were first applied to Sepharose 4B resin coupled with a monoclonal antibody against LpL (5D2 M Ab). The fraction without LpL (LpL unbound, or LpL−) was collected by gravity flow, and the fraction with LpL (LpL bound, or LpL+) was collected by briefly incubating the resin with 3 mol/L NaSCN solution. LpL+ and LpL− fractions were then immediately applied to anti-apoB100 or anti-apoE immuno-affinity chromatography to separate apoB100+ and apoE+ lipoprotein fractions. Anti-apoB100 resin was made from a monoclonal antibody that recognizes apoB100 but not apoB48, and anti-apoE resin was made from an affinity purified polyclonal antibody. Efficiency of binding for anti-LpL, anti-apoB100 and anti-apoE resin was all excellent (>95%). Western blotting against LpL, apoB100, and apoE did not detect their presence in respective unbound fractions. Recovery rates of lipids (triglyceride and cholesterol) and apolipoproteins (apoB, apoE, apoCI, and apoCII) were >90% for anti-LpL columns and were >80% for anti-apoB100 and anti-apoE columns. Total recovery rate of lipids and apolipoproteins after sequential columns (anti-LpL followed by anti-apoB100 or anti-apoE) were between 65% and 85%. The recovery rate of LpL dimers was between 55% and 80% for the anti-LpL column.

In certain experiments, fractions from immuno-affinity chromatography were further separated by ultracentrifugation into TRL density (<1.025 g/mL) and into LDL (1.025–1.063 g/mL). Concentrations of apolipoproteins were determined by sandwich enzyme-linked immunosorbent assay (ELISA) using affinity purified antibodies. LpL dimer concentrations were measured by a monoclonal antibody-based ELISA. All ELISAs were highly sensitive with signal-to-background absorbance ratios of 40 and higher. Intraclass correlation coefficients of variances for lipid and apolipoprotein measurements were between 2% and 6%, and interassay coefficients of variances were between 4% and 10%.

Transmission electron microscopy experiments were performed on 3 subjects’ fasting plasma to evaluate particle size distribution of apoB lipoproteins without LpL. All data presented here have been corrected for loss during immuno-affinity chromatography and/or ultracentrifugation. Postprandial clearance rates (mg/dL per hour), ie, the change in concentration per hour of a lipid or apolipoprotein after its postprandial peak, were calculated by fitting data in the SAS software. Percentage clearance rates (%/h) were calculated by dividing postprandial clearance rates of the lipid or apolipoprotein by respective postprandial peak concentrations. The difference between fractions with and without LpL was analyzed by paired t tests. P<0.05 was considered as statistically significant.

Results
Participants of this study are all healthy, nonsmoking, non-diabetic, and not taking medications that may affect lipids, with mean age 30 years and mean fasting total plasma cholesterol 175 mg/dL and fasting TG 108 mg/dL (Table). Electron microscopy results showed that apoB lipoproteins with LpL comprised a wide spectrum of particle sizes and that the majority was big triglyceride-rich particles. Particle size distributions of LpL+ and LpL− lipoproteins were studied in 3 subjects’ fasting plasma, and in subfractions of apoB lipoproteins in 2 of them. On average, the diameter of the apoB lipoproteins with LpL was 47±17 nm, which was >70% larger than those without LpL, 27±7 nm, (P<0.01). This is also the case in light VLDL (LpL+ versus LpL−: 62±11 versus 45±7 nm), dense VLDL (LpL+ versus LpL−: 43±10 versus 30±4 nm), and intermediate-density lipoprotein (IDL) (LpL+ versus LpL−: 33±5 versus 24±4 nm) fractions (all P<0.05). Thus, the average diameter of LpL bound lipoproteins was 40% to 75% larger than apoB lipoproteins without LpL. Assuming a spherical shape of apoB lipoproteins, this corresponds to a 100% to 200% larger surface area and 170% to 440% bigger volume of LpL+ compared with LpL− particles. Fast protein liquid and density gradient ultracentrifugation conducted on one subject’s plasma also confirmed a significant percentage of LpL bound lipoproteins was VLDL-sized particles and the major-

![Table](http://atvb.ahajournals.org/)

<table>
<thead>
<tr>
<th>Characteristics of Participants (N=9)</th>
<th>Mean ± SD</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>30 ± 4</td>
<td>25–38</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
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<td>19–28</td>
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<tr>
<td>Fasting lipids and apolipoproteins, mg/dL</td>
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<tr>
<td>Total cholesterol</td>
<td>175 ± 21</td>
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<tr>
<td>TRL cholesterol</td>
<td>30 ± 10</td>
<td>11–42</td>
</tr>
<tr>
<td>LDL cholesterol</td>
<td>96 ± 27</td>
<td>45–135</td>
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<tr>
<td>HDL cholesterol</td>
<td>49 ± 18</td>
<td>25–74</td>
</tr>
<tr>
<td>Total triglyceride</td>
<td>108 ± 40</td>
<td>39–158</td>
</tr>
<tr>
<td>TRL triglyceride</td>
<td>80 ± 37</td>
<td>20–134</td>
</tr>
<tr>
<td>LDL triglyceride</td>
<td>16 ± 4</td>
<td>10–22</td>
</tr>
<tr>
<td>Total apoB</td>
<td>64 ± 16</td>
<td>38–97</td>
</tr>
<tr>
<td>TRL apoB</td>
<td>11 ± 4</td>
<td>3–17</td>
</tr>
<tr>
<td>LDL apoB</td>
<td>53 ± 11</td>
<td>26–66</td>
</tr>
<tr>
<td>Total apoE</td>
<td>3.6 ± 0.8</td>
<td>1.8–4.8</td>
</tr>
<tr>
<td>TRL apoE</td>
<td>1.0 ± 0.5</td>
<td>0.3–1.7</td>
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<tr>
<td>LDL apoE</td>
<td>0.54 ± 0.2</td>
<td>0.26–0.95</td>
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<tr>
<td>Total apoCI</td>
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<td>Total apoCII</td>
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<tr>
<td>Total apoCIII</td>
<td>11 ± 3</td>
<td>6–15</td>
</tr>
<tr>
<td>LpL dimers, ng/mL</td>
<td>61 ± 31</td>
<td>15–180</td>
</tr>
</tbody>
</table>

TRL indicates triglyceride-rich lipoproteins.

Density <1.025 g/mL.

This is also the case in light VLDL (LpL+ versus LpL−: 62±11 versus 45±7 nm), dense VLDL (LpL+ versus LpL−: 43±10 versus 30±4 nm), and intermediate-density lipoprotein (IDL) (LpL+ versus LpL−: 33±5 versus 24±4 nm) fractions (all P<0.05). Thus, the average diameter of LpL bound lipoproteins was 40% to 75% larger than apoB lipoproteins without LpL. Assuming a spherical shape of apoB lipoproteins, this corresponds to a 100% to 200% larger surface area and 170% to 440% bigger volume of LpL+ compared with LpL− particles. Fast protein liquid and density gradient ultracentrifugation conducted on one subject’s plasma also confirmed a significant percentage of LpL bound lipoproteins was VLDL-sized particles and the major-
The concentration of apoB100 lipoproteins without LpL changed little. Interestingly, LDL with LpL exhibited a postprandial response pattern more like TRL than typical LDL. In fact, LDL with LpL had significantly greater postprandial increments than TRL without LpL (P < 0.05 for apoB and TG). During these 2 hours, 23% of the peak level of apoB and 75% of the peak level of TG in TRL with LpL disappeared from circulation. In contrast, apoB and TG concentrations in TRL without LpL decreased much more slowly. Only 5% and 28% of peak levels of apoB and TG disappeared per hour and it took 3 to 5 hours after the peaks for the concentrations to reach near-fasting levels (P<0.05 between LpL+ and LpL−). apoB in LDL with LpL also disappeared rapidly from circulation, with 75% of peak concentration disappearing within 1 hour, whereas apoB in LDL without LpL changed little.

LpL was found on both apoB48 and apoB100 lipoproteins, and both had significantly bigger postprandial increments percentage-wise than their LpL free counterparts (Figure 3). The concentration of apoB48 lipoproteins with LpL increased 10-fold, and TG 6-fold postprandially, significantly higher than the respective 2.4-fold and 2-fold increments of apoB and TG in apoB48 lipoproteins without LpL (P<0.05 for both). apoB100 lipoproteins with LpL also significantly increased their apoB and TG concentrations more than those without LpL (LpL+ versus LpL−: apoB100, 4.5-fold versus 16%; TG, 4-fold versus 2-fold; P<0.05).

The bulk of apoB48 lipoproteins with LpL (79%) disappeared from circulation within one hour after their concentration peaked postprandially (Figure 3). In contrast, apoB48 lipoproteins without LpL were cleared much more slowly: three hours after their peak, the plasma concentration declined only 25%. The percentage clearance rate was 9-times greater for apoB48 lipoproteins with LpL than those without LpL (P<0.05). Similar to apoB48 lipoproteins, most of apoB100 lipoproteins with LpL (71%) disappeared from circulation within 1 hour after the peak, whereas the concentration of apoB100 lipoproteins without LpL changed little during the 8-hour postprandial period.

apoB48 lipoproteins with LpL had a bigger postprandial response after fat ingestion than apoB100 lipoproteins with LpL. In the fasting state, the concentration of apoB48 lipoprotein with LpL (expressed in molar units: 0.12 nmol/L),
Postprandial response of LpL− fractions and LpL+ light VLDL. 

(A) apoB in LpL+ TRL and LpL− light VLDL (Sf>60); (B) apoB in LpL+ LDL and LpL− light VLDL; (C) apoB48 in LpL+: apoB lipoproteins and LpL− light VLDL; (D) apoB100 in LpL+: apoB lipoproteins and LpL− light VLDL.

Electron microscopy experiments showed that on average apoB lipoproteins with LpL were bigger than those without LpL, and this disparity in particle size might contribute to the faster clearance of the former. Thus, we further isolated TRL into light VLDL, dense VLDL, and IDL (Figure 1), and studied the postprandial response between LpL+ and LpL− lipoproteins of similar size distributions. This would help address the potential confounding effect of particle size on postprandial response. Results showed that concentrations of LpL− light VLDL increased ∼44% (from 1.01 to 1.45 mg/dL) after oral fat-loading, which was significantly lower than the 3- and 5-fold increase observed in LpL+ TRL and LDL (Figure 4A and 4B). After its concentration peaked at 5 hours, LpL− light VLDL was removed at a percentage clearance rate of 9%/h, which was significantly lower than the respective 23%/h and 20%/h percentage clearances of LpL+ TRL and LDL. We further separated LpL− light VLDL into apoB100 and apoB48 lipoproteins. Results also showed that concentrations of apoB48 and apoB100 in LpL− light VLDL increased ∼90% (from 0.084 to 0.16 mg/dL) and 50% (from 0.96 to 1.46 mg/dL) postprandially, which were also significantly lower than the 10- and 5-fold increase observed in LpL+ apoB48 and apoB100 lipoproteins (Figure 4C and 4D).apoB48 in LpL− light VLDL was gradually cleared after its concentration peaked at 3 hours with a percentage clearance rate of 10%/h, which was significantly slower than apoB48 in LpL+ apoB lipoproteins (79% peak concentration disappeared within 1 hour). However, 33% apoB100 in LpL− light VLDL was cleared in 1 hour after it peaked at 5 hours after oral fat loading, a percentage clearance rate less than half of apoB100 lipoproteins with LpL (71%/h). Thus, lipoprotein size, per se, did not account for the fast metabolism of apoB lipoproteins with LpL.

apoB lipoproteins with LpL have high contents of apoE. apoE has a well-established role in enhancing lipoprotein removal through receptor-mediated pathways. Therefore, apoE could also be a confounding factor when we evaluate the independent contribution of LpL to the enhanced clearance of apoB lipoproteins from circulation. To distinguish the effect of LpL from that of apoE, we separated lipoproteins with and without LpL further according to their apoE content with anti-apoE immuno-affinity chromatography. We found that regardless of apoE, TRL with LpL decreased significantly faster than those without LpL. TRL that contained both LpL and apoE was removed ∼2.5-times as fast as TRL with apoE and not LpL (19%/h versus 7.5%/h; P<0.05) (Figure 5, upper panel). However, TRL that contained LpL and not apoE was cleared ∼2.3-times as fast as that of TRL with neither LpL nor apoE (27%/h versus 12%/h; P<0.05) (Figure 5, lower panel). Overall, LpL enhanced the clearance of triglyceride-rich lipoproteins by 2- to 3-fold, whether apoE coexisted with it.

It has been suggested that the binding between plasma LpL and apoB lipoproteins might not be as strong as other apolipoproteins such as apoE, mainly because of the observation that LpL dissociates from lipoproteins during ultracentrifugation in a high-gravity, high-salt environment. This raised the question whether a circulating LpL lipoprotein could become an LpL− lipoprotein by transferring LpL to a circulating LpL− lipoprotein. To investigate this, we labeled all protein contents of LpL+ lipoproteins with biotin, and incubated the biotin labeled LpL+ fraction with biotin free LpL− fraction at room temperature for 30 minutes. Then, the mixture was separated again into LpL+ and LpL− with anti-LpL immuno-affinity chromatography. If significant transfer of LpL from LpL+ lipoproteins to LpL− lipoprotein did happen during the incubation period creating new LpL− apoB lipoprotein particles, biotin-labeled apoB would then appear in the LpL− fraction after incubation caused by the loss of LpL. However, Western blotting against biotin with avidin–alkaline phosphatase conjugates did not show a biotin labeled apoB band in the LpL− lane (Figure II, available
online at http://atvb.ahajournals.org), suggesting the transfer of LpL from LpL+ to LpL− is negligible under these conditions.

Discussion

Lipids in the postprandial state may be more closely related to risk for coronary heart diseases than levels in the fasting state, and impaired clearance of postprandial lipoproteins may be an underlying risk factor for coronary heart diseases.21 As a pivotal regulator of postprandial lipid metabolism, LpL could accelerate TRL clearance by receptor-mediated pathways in addition to its role as a lipase. The relevance of this apolipoprotein-like function in an in vivo metabolic setting has not been studied before and is the goal of this study. Our results strongly suggest that postprandially, apoB lipoproteins with LpL have significantly faster rates of clearance than those without LpL, regardless of the apoE content or particle size.

Plasma concentrations of LpL among the 9 participants are similar to what have been observed by others.1,4,18–20 The majority of apoB lipoproteins with LpL are large, indicating high core lipid contents. Although low in concentration, LpL+ lipoproteins carry a disproportionately higher percentage of plasma triglyceride in the postprandial state (average 4.5%; range 1% to 14%). In all types of apoB lipoproteins that we examined, those with LpL show a distinctive pattern of postprandial response with a sharper increase and faster decline than those without LpL, suggesting plasma LpL may be particularly important postprandially. In this regard, apoB48 lipoproteins with LpL have a 2-fold bigger postprandial increment percentage-wise than apoB100 with LpL, suggesting that LpL is more involved with the metabolism of intestinal than hepatic lipoproteins in the fed state. It has been proposed that chylomicrons and VLDL compete for the same hydrolysis pathway,22 and thus it is possible that higher postprandial response of chylomicrons may inhibit the binding of VLDL to LpL. More importantly, this study demonstrates that a physiologically significant percentage of apoB48 lipoproteins could be removed from circulation together with LpL. We estimate that 25% of chylomicrons and their remnants could be cleared by the LpL mechanism. The computation assumes that the production of chylomicron is low after its concentration peaks (4 to 5 hours after fat ingestion) postprandially and that the response of apoB48 to a single fatty meal is a close approximation to a biological tracer. However, although apoB100 lipoproteins with LpL were removed from circulation much faster than those without LpL, the overall effect of LpL on apoB100 clearance may be less important than on apoB48 because LpL is present on a smaller percentage of apoB100 lipoproteins.

The enrichment of apoE in apoB lipoproteins with LpL does not account for their enhanced clearance. We found that the presence of LpL in TRL is associated with fast clearance whether apoE was also present with LpL in the particles. The contents of C apolipoproteins, which could inhibit receptor-mediated lipoprotein removal,22 were similar in apoB lipoproteins that do or do not contain LpL. Particle size also did not seem to be a major factor contributing to the faster removal of lipoproteins with LpL. When compared with the light VLDL without LpL, which have a similar particle size distribution, apoB lipoproteins with LpL still have greater postprandial response and faster clearance. Taken together, this study strongly suggests that LpL per se accelerates clearance of apoB lipoproteins in vivo in humans.

Our results also suggest that LpL on an apoB lipoprotein marks it for clearance by hepatic receptors, and that apoB lipoproteins with LpL are remnants of triglyceride-rich lipoproteins having undergone intravascular metabolism. apoB lipoproteins with LpL are heterogeneous in size with 2 peaks on electron micrographs corresponding to typical chylomicron remnants and VLDL remnants.23 The key events causing the production of these types of remnants may first be lipolysis of nascent triglyceride-rich chylomicrons and VLDL, then uptake of LpL from the capillary endothelium by these lipoproteins, and finally release into the circulation. These events may constitute normal processing of chylomicrons and VLDL to form their specific remnant particles, which then are rapidly cleared by hepatic receptors.

The mechanisms that cause triglyceride-rich postprandial apoB lipoproteins undergoing lipolysis to remove LpL molecules from the capillary endothelium are unknown. This study suggests that enrichment of triglyceride, cholesterol, and apoE are possible contributors. Particle size may also be important. Chylomicrons compete with VLDL for LpL binding and triglyceride hydrolysis in vivo,21 and it has been shown that LpL binds larger particles better than smaller ones in vitro.24 It is unclear how much lipolytic processing occurred before the production of particles with LpL and their release into the circulation. Removal of LpL by a partially metabolized triglyceride-rich lipoprotein may also involve direct interaction between LpL and other lipid or protein components of the lipoprotein, such as apoA-V.25 Nevertheless, once an apoB lipoprotein re-enters circulation with LpL, it is rapidly cleared.

In conclusion, our study extends to humans the findings of cell culture experiments and animal studies, and demonstrates that a significant amount of postprandial apoB lipoproteins, especially chylomicrons, can be removed from circulation with the help of LpL. LpL is not only responsible for hydrolyzing apoB lipoproteins at the endothelium but also able to function like an apolipoprotein by facilitating receptor-mediated uptake during circulation. The apolipoprotein function of LpL may constitute a normal “low-concentration, high speed” pathway for clearance of triglyceride-rich lipoproteins.

Acknowledgments

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Online Figure 2: LpL transfer evaluated by Western blots against biotin (A) and LpL (B) and protein staining of apoB (C).

Methods:

Subjects:

Ten people participated in the study and nine of them completed the protocol. They were between 25 and 38 years of age (6 men and 3 women). They were all healthy, nonsmoking, nondiabetic and not taking any medication that could affect blood lipids. The study was approved by the Human Subjects Committee at Harvard School of Public Health and all participants gave informed consent.

Oral Fat Loading and Collection of Blood Samples:

Blood was first drawn after a twelve-hour fast (baseline). Participants then ingested a fat load in the form of a milkshake (1339 kcal with 70% of energy from fat, 23% from carbohydrate, and
7% from protein) and had blood drawn at three, four, five, six, seven and eight hours afterwards. Between blood draws, participants were prohibited from taking any food that contained calories.

**Handling of Blood Samples:**

Blood was drawn into EDTA coated vacuum tubes. Tetrahydrolipstatin (THL, or Orlistat, an active-site inhibitor of mammalian lipases including LpL [1], from Roche, Switzerland) was immediately added to a final concentration of 100 ng/ml. At this concentration, THL did not interfere with cholesterol and TG assays used in this project. Blood was then centrifuged at 2000 RPM on a Beckman GS-6R centrifuge (Beckman Coulter Inc., Fullerton, CA) for 25 min at 4 ºC to isolate plasma. Preservatives, consisting of Protease Inhibitor Cocktail (10 ul/ml), phenylmethanesulfonyl fluoride (PMSF) (17.5 ug/ml), benzamidine (2 uM), and gentamicin (50 ug/ml) (all from Sigma, St. Louis, MO), were added to plasma. Samples were then stored at -80 ºC in 1.8 ml aliquots until they were analyzed one to six months later. We found that freezing and storage did not affect the binding efficiency or recovery of anti-LpL and anti-apoB100 immuno-affinity columns. The efficiency of the columns (the percentage of ligand, i.e. LpL or apoB100, measured in the bound fraction compared to that in the unbound fraction) was higher than 95% for both fresh and frozen plasma. Furthermore, the percent apoB bound, and the apoB concentrations of the bound and unbound fractions were not affected.

**Sequential Immuno-affinity Chromatography:**

Separation of lipoproteins according to content of LpL and apoB100 was carried out by immuno-affinity chromatography following a similar procedure as previously described and validated [2-4]. CNBr-activated Sepharose 4B resin (Amersham Pharmacia, Piscataway, NJ) was

An aliquot of 1.5 ml plasma was added to an anti-LpL immuno-affinity column. The column was incubated at 4 ºC overnight with constant mixing on a rotator (VWR Intl., West Chester, PA). The fraction without LpL (LpL-) from the column was collected by gravity flow into a Vivaspin 20 concentrator (Vivascience AG, Edgewood, NY). Afterwards, LpL column was washed with 20 ml PBS solution with THL (at 10 ng/ml), and the eluted liquid was collected into the same concentrator. LpL column was washed by another 20 ml of PBS/THL, and this washing was discarded. In order to collect LpL bound fraction (LpL+), the column was then incubated with 3 ml 3M NaSCN for 3 minutes at room temperature on a rotator, and the eluted fraction was collected by gravity flow. Sodium thiocyanate was immediately removed from the eluted fraction by gel filtration chromatography (PD-10 columns, Amersham Pharmacia, Piscataway, NJ) with PBS-EDTA as the washing buffer. LpL column was then incubated with NaSCN for four more times, and the desalted samples from all five steps were pooled into a Vivaspin 20 concentrator. Concentrators that contained collected LpL+ and LpL- fractions were centrifuged at 2500 RPM on a Beckman GS-6R centrifuge until the volume of concentrates reached around 1.5 ml.

LpL+ and LpL- fractions obtained from the above anti-LpL immuno-affinity chromatography were immediately applied to anti-apoB100 immuno-affinity columns. The procedure for anti-apoB100 immuno-affinity chromatography was similar to that of anti-LpL. ApoB lipoproteins in both bound and unbound fractions were obtained by ultracentrifugation (density < 1.063 g/ml). In the end, four fractions of apoB lipoproteins were obtained according to
the presence/absence of LpL and apoB100: those with both LpL and apoB100 (LpL+apoB100), with LpL and not apoB100 (LpL+apoB48), with apoB100 and not LpL (LpL-apoB100), and with neither LpL nor apoB100 (LpL-apoB48).

In certain experiments, LpL+ and LpL- fractions from the anti-LpL column were further separated by anti-apoE immuno-affinity chromatography [2, 3]. To obtain enough lipoprotein material for measurements on the isolated fractions, plasma samples from the nine subjects were pooled at each time point. Pooled plasma samples were first applied to anti-LpL columns according to the above procedure to separate LpL+ from LpL-, and LpL+ and LpL- fractions (0.8 ml each) were applied to anti-apoE columns, made from a polyclonal antibody against apoE (Academy-Biomedical, Houston, TX). In the end, around 0.8 ml of concentrates was obtained for each of the four fractions: LpL+apoE+, LpL+apoE-, LpL-apoE+, and LpL-apoE-. TRL (TG-rich lipoprotein, density < 1.025 g/ml) of these fractions was isolated by ultracentrifugation.

Efficiency of binding for LpL, apoB100 and apoE columns, computed by dividing the mass of LpL, apoB100, or apoE in bound fractions by the respective total mass in both bound and unbound fractions, was excellent (>95%). Western blotting against LpL, apoB100 and apoE didn’t detect their presence in the unbound fractions. The recovery rate of LpL dimers was between 55%~85% for the anti-LpL column. Recovery rates of lipids (triglyceride and cholesterol) and apolipoproteins (apoB, apoE, apoCl, apoCII and apoCIII) were above 90% for anti-LpL, anti-apoB100 and anti-apoE columns. Total recovery rate after sequential columns (anti-LpL followed by anti-apoB100 or anti-apoE) were between 65%~85%.

Separation of Lipoprotein by Ultracentrifugation:
Fractions from plasma obtained by immuno-affinity chromatography were separated by ultracentrifugation into TRL (density < 1.025 g/ml), which contains chylomicrons, VLDL and IDL, and into LDL (1.025 g/ml < density <1.063 g/ml) in a Beckman TLA 120.2 fixed angle rotor on a Beckman TLX Optima centrifuge (Beckman Coulter Inc., Fullerton, CA). To prepare TRL, 500 ul of samples was transferred to thick-wall polycarbonate ultracentrifuge tubes (No. 343778, Beckman). The density of the samples was first adjusted to 1.025 g/ml by adding 41.0 ul 32.25% KBr (density = 1.285 g/ml) and 59.0 ul double-distilled water. Samples were then overlayed with 600 ul 3.66% KBr solution (density = 1.025 g/ml), and submitted to ultracentrifugation (1 hour 51 minutes, 15°C, with a centrifugal field of 537,000 g). The top 400 ul of each tube was carefully harvested by aspiration. To prepare LDL, we added 134 ul of 32.35% KBr solution to the infranatant to bring its density to 1.063 g/ml, and overlayed with 266 ul 8.62% KBr solution (density = 1.063 g/ml). The top 300 ul of the supernatant, containing LDL, was collected after centrifugation (2 hours 30 minutes, 15°C, 537,000 g). The run time and speeds were standardized by comparison with the Lindgren method using a Beckman SW41 swinging bucket rotor [8]. Density of KBr solutions was verified regularly on an Auto Abbe Refractometer (Leica, Bannockburn, IL).

Electron Microscopy:

Transmission Electron Microscopy (EM) experiments were performed on three subjects’ fasting plasma. Plasma samples were first applied to anti-LpL immunoaffinity chromatography to separate LpL+ and LpL- fractions. For 2 subjects, LpL+ and LpL- fractions were further applied to ultracentrifugation to isolate light VLDL, dense VLDL and IDL. EM experiments were carried out at the EM Core Facility at Harvard Medical School (Boston, MA), on a model
JEOL 1200EX (JOEL Inc., Tokyo, Japan), with a magnification of X 30,000. Diameters of 100 particles were measured for fraction.

**Separation of Lipoproteins by Gel Filtration Chromatography (FPLC) and Density Gradient Ultracentrifugation (DGUC):**

FPLC and DGUC experiments were performed on fasting plasma of one subject who was included in the EM experiments. Plasma was first applied to anti-LpL immuno-affinity chromatography to separate LpL+ and LpL- fractions.

For FPLC, LpL bound fraction was passed through a 0.4 um filter, and 1.0 ml of the filtrate was immediately applied to a single Superose 6 HR 10/30 Y column (10 X 300 mm, Pharmacia Fine Chemicals, NJ). The fractions were separated at 4°C by elution with 0.05 M phosphate, 0.15 M NaCl, 0.01% EDTA, 0.02% NaN₃, pH 7.4, pumped at a rate of 0.2 ml/min. Sixty fractions of 500 ul were collected. The column was standardized using the following molecular weight markers: thyroglobulin, ferritin, catalase, albumin and LDL. Previous experiments showed that VLDL corresponded to fractions 16-21, LDL corresponded to fractions 22-29, and HDL corresponded to fractions 30-45 [1].

For DGUC, 1.0 ml LpL bound fraction was combined with 1.5 ml of a NaCl solution of density 1.006 g/ml and 1.5 ml of a solution of NaCl and KBr of density 1.21 g/ml to obtain a final density of 1.0825 g/ml [9]. A solution of NaCl density 1.006 g/ml, 8.5 ml, was placed in a Beckman ultracentrifuge tube (No. 344322) and was underlayed with the 4 ml of the sample solution. It was centrifuged at 65,000 RPM for 70 min (total \( w^2 t = 1.95 \times 10^{11} \)) in a Beckman VTi 65.1 vertical rotor. Thirty seven 0.34 ml fractions were collected from the bottom of the tube and the lipid concentration was determined for each fraction. Previous experiments showed
that fractions 28-37 corresponded to VLDL, fractions 17-31 corresponded to IDL, fractions 8-17 corresponded to LDL and fractions 1-8 corresponded to HDL [9].

**Biotinylation of Apolipoproteins:**

In order to study the possible transfer of LpL among apoB lipoproteins, one subject’s postprandial plasma was applied to anti-LpL immuno-affinity column. LpL+ fraction was labeled with biotin by using a EZ-Link Sulfo-NHS-Biotin kit (Pierce, Rockford, IL). Biotin non-selectively labels surface proteins of apoB lipoproteins including LpL and apoB. Biotin labeled LpL+ fraction was then incubated with biotin free LpL- fraction at room temperature for 30 minutes, and the mixture was re-applied to an anti-LpL immuno-affinity column. Western blotting with avidin-alkaline phosphatase conjugates (Pierce, Rockford, IL) was carried out to examine the transfer of biotin labeled apolipoproteins from the LpL+ to LpL- fraction. 3%-8% Tris-Acetate gradient gels were used (Invitrogen, Carlsbad, CA), and silver staining was also performed (OWL Separation Systems, Portsmouth, NH).

**Determination of Lipids and Apolipoproteins:**

Triglyceride and cholesterol concentrations were determined enzymatically on a Cobas MIRA Plus Autoanalyser (Roche, Nutley, NJ). LpL dimer concentrations were determined by sandwich ELISA using a monoclonal antibody (5D2) against LpL for plate coating, and horseradish peroxidase (HRP) conjugated 5D2 for detecting [1].

Concentrations of apoB were determined by ELISA using a monoclonal antibody that recognizes both apoB100 and apoB48 (U.S. Biological, Swampscott, MA) for the coating antibody and an Avidin-conjugated polyclonal anti-apoB for detection. We also evaluated apoB
concentrations in TRL and LDL with LpL or without LpL using SDS-PAGE with high sensitivity silver stain. In brief, the lipoprotein fractions with LpL or without LpL (TRL with or without LpL; LDL with or without LpL) were diluted according to their apoB concentrations determined by ELISA to have the same final apoB concentration. 20 ul samples were applied to 3-8% Tris-Acetate gels (Invitrogen, Philadelphia, PA) after boiling for 10 minutes with reducing agent and lithium dodecyl sulfate sample buffer (LDS buffer, Invitrogen). After electrophoresis at 120 V for 1 hour, protein bands were stained using a silver stain kit (Owl Separation Systems, Portsmouth, NH). Optical density of apoB100 bands was measured by a gel scanner. The ratio of the optical density between lipoprotein fractions with and without LpL was similar to that obtained by the apoB ELISA (± 20%), demonstrating that the ELISA was accurately determining apoB across a very wide range of concentrations, and that apoB immunoreactivity was preserved in the LpL bound fraction during the column and ultracentrifugation procedures.

ApoE, apoCI, apoCII and apoCIII concentrations were determined by ELISA using affinity purified goat or rabbit polyclonal antibodies for coating and HRP conjugated polyclonal antibodies for detecting (coating antibodies and HRP conjugates are from Academy-Biomedical, Houston, TX.). Intra-assay coefficients of variance for TG, cholesterol, apoB, apoE, apoCI, apoCII, apoCIII and LpL measurements were between 2% and 6%. Inter-assay coefficients of variances were between 4% and 10%. All data have been corrected for loss during immunoaffinity chromatography and/or ultracentrifugation.

Inhibition of in vitro hydrolysis by THL:

Due to the extensive analysis of LpL containing apoB lipoproteins of this study, tetrahydrolipstatin (THL), an active inhibitor of LpL, was immediately added to inhibit further
hydrolysis occurring in vitro after blood sampling. We have previously shown that if THL was not present, apoB lipoproteins containing LpL would differ from the authentic particles in vivo due to continued lipolysis by LpL in vitro [1]. THL, a small molecule (molecular weight 496), inhibits LpL by binding covalently to the serine residue of the active site of LpL [10]. The active site of LpL is in the amino terminal of the molecule whereas the carboxyl terminal contains the region that mediates the initial binding of LpL to the lipoprotein [11]. Therefore, in the case of the biotin-labeled LpL being transferred to another lipoprotein, THL should not interfere with the binding of LpL to either the donor or acceptor lipoprotein and thus not interfere in LpL movement between particles. Similarly, THL would not affect the binding of apoE and other apolipoproteins to lipoprotein particles. In fact it is advisable that THL be present, since inhibiting further lipolysis would prevent the transfer of apoE that could occur with lipolysis. At the low concentration used in this study (100 ng/ml), THL did not affect the measurement of triglyceride and cholesterol.

Statistical Analysis:

Postprandial clearance rates (mg/dL/hour), i.e. the change in concentration per hour of a lipid or apolipoprotein after its postprandial peak, were calculated by fitting data in the SAS software (Version 8 for Windows, SAS Institute, Cary, NC). Percentage clearance rates (%/hour) were calculated as dividing postprandial clearance rates of the lipid or apolipoprotein by their postprandial peak concentrations. The difference between fractions with and without LpL was analyzed by paired T-tests. A p-value ≤ 0.05 (2-sided) was considered as statistically significant.
References for online supplement:


Online Figure 1:

**FPLC**

![Graph showing FPLC analysis of triglyceride (TG) in LpL+ fraction.](image)

**DGUC**

![Graph showing DGUC analysis of TG in LpL+ fraction.](image)
Online Supplement Figure 1: Triglyceride distribution in Fast Protein Liquid Chromatography (FPLC) and Density Gradient Ultracentrifugation (DGUC) fractions of LpL+ lipoproteins. Dotted lines indicate cholesterol distribution of in FPLC/DGUC fractions of whole plasma, and serve as reference for the position of VLDL, IDL and LDL peaks.
Online Supplement Figure 2: LpL transfer evaluated by Western blots against biotin (A) and LpL (B) and protein staining of apoB (C). LpL bound (LpL+) lipoproteins were first isolated by anti-LpL immunoaffinity chromatography. Proteins in the LpL+ fraction were labeled with biotin. Biotin labeled LpL+ lipoproteins (Lane 1) were then incubated with biotin free LpL- fraction (Lane 2) for 30 min at room temperature. The mixture was re-separated into LpL+ (Lane 3) and LpL- (Lane 4) fractions by anti-LpL immuno-affinity chromatography. Alkaline phosphatase conjugated Avidin was used for western blotting against biotin in A. Alkaline phosphatase conjugated antibodies against LpL and apoB were used for western blotting in B and C. “LpL” lane in B and “apoB” lane in C are purified proteins of LpL and apoB100.