Dimeric lipoprotein lipase is bound to triglyceride-rich plasma lipoproteins

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Abstract Lipoprotein lipase hydrolyzes the triglyceride-rich core of chylomicrons and very low density lipoproteins. It is also a ligand, in vitro, for binding of lipoproteins to the low density lipoprotein receptor-related protein and may play a central role in the receptor-mediated removal of triglyceride-rich lipoproteins. The aim of the present study was to determine to which lipoprotein subclass the enzyme is bound in preheparin plasma and when released into plasma by heparin injection. Tetrahydrolipstatin, a potent inhibitor of serine lipases, was used to block lipolytic activity, thereby preventing changes in plasma lipoproteins due to ex vivo lipolysis. To analyze the distribution pattern of lipoprotein lipase dimers among lipoprotein classes, a specific ELISA was used and gel filtration was performed in pre- and postheparin plasma from five subjects with triglyceride ranging from 69 to 522 mg/dl. When lipolytic activity was not inhibited, lipoprotein lipase dimers eluted in association with low and high density lipoproteins, reproducing results previously obtained by several groups of investigators. However, in pre- and postheparin samples treated with tetrahydrolipstatin, most of the dimeric enzyme was found associated with very low density lipoprotein particles. In conclusion in pre- and postheparin samples most of the lipoprotein lipase dimers are associated with very low density lipoproteins when ex vivo lipolytic activity is inhibited, which supports the hypothesis that, in vivo, lipoprotein lipase may affect the receptor-mediated removal of these particles. Moreover, it suggests that the association between lipoprotein lipase and cholesterol-rich lipoproteins might be an ex vivo phenomenon due to lack of inhibition of lipolytic activity.—**Zambon, A., I. Schmidt, U. Beisiegel, and J. D. Brunzell.** Dimeric lipoprotein lipase is bound to triglyceride-rich plasma lipoproteins. *J. Lipid Res.* 1996. **37:** 2394–2404.

Supplementary key words lipoproteins • lipoprotein lipase • triglyceride • gel filtration • tetrahydrolipstatin • heparin

Lipoprotein lipase (LPL) is a multifunctional protein and a key enzyme in lipoprotein metabolism (1–4). After synthesis in parenchymal cells, primarily adipose tissue and skeletal muscle, LPL is transported to the intimal surface of the vascular endothelium where it is noncovalently anchored to the heparan sulfate side chains of membrane proteoglycans (1, 5). At this site, LPL is responsible for the hydrolysis of the triacylglycerol(TG)-rich core of circulating chylomicrons (CM) and very low density lipoproteins (VLDL), generating free fatty acids (FFA) that can be either used immediately or stored away by peripheral tissues. The enzyme is active only in a dimeric configuration (6, 7). For its function, LPL binds the interface of TG-rich lipoproteins (8, 9). After reduction in TG content and size, CM and VLDL remnants detach from the endothelium into the circulation by mechanism(s) yet to be completely understood (10, 11).

There is growing evidence that LPL not only affects lipolysis but also acts as a ligand for binding of lipoprotein particles to cell surfaces and receptors. A number of studies indicate that LPL is associated with lipoproteins in human pre- and postheparin plasma, both in its inactive and active form (12–14). Felts, Itakura, and Crane (15) in 1975 suggested that LPL bound to lipoprotein remnants may be a marker for remnant uptake by the liver. The binding of LPL to lipoproteins is, however, very weak and is disrupted by high salt concentrations or by centrifugal forces as in the density gradient ultracentrifugation (12). Recent studies showed that LPL bound to apolipoprotein E-rich lipoproteins enhances their binding to the low density lipoprotein receptor-related protein (LRP), the putative receptor for chylomicron remnants (16), as well as to the VLDL receptor (17). Moreover, Nykjaer et al. (18) suggested that only the LPL dimer, and not the monomer, might be able to mediate the binding. Goldberg et al. (12) reported that lipolytic activity in pre- and postheparin plasma (PHP) is associated with cholesterol-rich particles slightly larger than LDL. Recent reports confirmed

Abbreviations: CM, chylomicrons; FFA, free fatty acids; LDL, low density lipoprotein; LPL, lipoprotein lipase; Mab, monoclonal antibody; PHP, post-heparin plasma; TG, triglycerides; THL, tetrahydrolipatatin (Orlistat™); VLDL, very low density lipoprotein.

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this observation by showing that LPL mass after gel filtration is associated with LDL both in preheparin and PHP (13, 14). In these studies, however, no steps were taken to inhibit lipolytic activity ex vivo. Recently, Zambon, Hashimoto, and Brunzell (19) demonstrated that a significant amount of the TG-rich core of CM and VLDL can be hydrolyzed in PHP stored ex vivo at 4°C if LPL activity is not effectively inhibited. It is, therefore, possible that, when heparin is administered, LPL is initially released associated with VLDL particles that undergo further hydrolysis by LPL ex vivo, resulting in lipoprotein in the LDL size range. In the present study, ex vivo lipolytic activity was blocked by tetrahydrolipstatin (THL), a potent inhibitor of serine lipases (20, 21), therefore preventing further changes in PHP lipoproteins due to lipolysis. To our knowledge the present study is the first to use this approach in the analysis of LPL binding to lipoproteins in PHP.

By inhibiting lipolytic activity ex vivo, this study aims to examine: *1*) which lipoprotein class LPL is associated with, in vivo, after heparin administration and in the presence of an enzyme inhibitor, and *2*) whether the association between LPL and cholesterol-rich lipoprotein previously observed in PHP reflects a physiologic condition or is an ex vivo phenomenon due to lack of inhibition of lipolytic activity.

MATERIALS AND METHODS

Subjects

Five healthy males aged 25 to 55 years and with different TG levels were selected for studies of the LPL distribution across the lipoprotein size range in PHP. None of the subjects were on drugs known to affect lipoprotein metabolism. They were all free of medical conditions that could alter their body weight or plasma lipids. The present study was approved by the Human Subjects Review of the University of Washington. Informed consent was obtained from all subjects.

Plasma handling

Pre- and postheparin plasma samples were obtained after a 12-h fast. Postheparin plasma was collected 5 min after i.v. administration of 60 U/kg body weight of heparin (Elkins-Sinn Inc., Cherry Hill, NJ), to allow heparin to be homogeneously distributed in the circulation on one hand, and to minimize in vivo lipolysis of lipoproteins and remnant clearance on the other. All blood samples were collected into prechilled heparin/lithium tubes (Beckton Dickinson, Rutherford, NJ).

Inhibition of ex vivo lipolytic activity

To inhibit lipolytic activity, one aliquot of PHP was mixed immediately with tetrahydrolipstatin (THL), (Hoffman-La Roche, Basel, Switzerland) from a stock solution of 1 mg/ml THL in ethanol. According to previously published data (20, 21), THL is an active-site inhibitor of mammalian lipases, including pancreatic lipase, LPL, and hepatic lipase (HL); the inhibition is due to covalent binding of THL to a Ser residue that is part of the catalytic triad of the pancreatic lipase, LPL, and HL molecular structure. THL concentration was selected to reach a THL/LPL molar ratio of approximately 6.0 (21) based upon the mean LPL protein concentration of a normal PHP (LPL mass = 345 ± 14 ng/ml, mean ± SEM, n = 70 normolipidemic subjects, measured by 5F9 ELISA as detailed in the following sections; J. D. Brunzell, unpublished results).

A second aliquot of PHP was transferred immediately into a tube containing 0.275 mg/ml Paraoxon, a known neurotoxic inhibitor of lipase activity (19, 22). This second aliquot was collected to compare the efficacy of Paraoxon and THL as LPL activity inhibitors, and to determine whether Paraoxon could be substituted by THL which is safer and easier to use on a gel filtration column. All blood samples were stored on ice and plasma was separated immediately from cells in a centrifuge at 4°C/3000 rpm per 15 min. Plasma samples were then stored at 4°C and gel filtration analyses were completed within 12 h from blood collection. Samples were centrifuged again to eliminate fibrinogen and were filtered through a 0.22-μm filter shortly before gel filtration.

Gel filtration chromatography

Gel filtration chromatography of pre- and postheparin samples was performed at 4°C by fast protein liquid chromatography (FPLC) using a Superose 6 HR 10/30 column (Pharmacia LKB, Sweden) to separate lipoproteins in phosphate-buffered saline (PBS) containing 10 U/ml heparin (Sigma H-7005). One ml of 0.22-μm-filtered plasma was applied to the column. Chromatography was carried out with a flow rate of 0.2 ml/min. Sixty fractions of 500 μl were collected into 200 μl of 50% glycerol (10 mM NaH_2PO_4, pH 7.4) and mixed. Samples for the LPL-ELISA were mixed with fetal bovine serum (FBS, HyClone, Utah) to a final concentration of 10% FBS immediately after elution from the column and applied to ELISA plates within 8 h. Heparin, glycerol, and fetal bovine serum were added to stabilize LPL during analysis. All samples were collected and stored on ice. Cholesterol and TG levels were measured by enzymatic methods in each of the 60 fractions eluted from the column. Mean cholesterol recovery in the chromatographic procedure was 96.5% (n = 12, range 90.3–103.1%).

A control experiment was carried out to assess the lipoprotein class elution profiles from the FPLC column (see Fig. 2). Briefly, a blood sample was collected, after

an overnight fast, from subject #2 (see Table 1). Lipoprotein classes were first isolated using standard sequential ultracentrifugation (23). Isolated VLDL, LDL, and HDL particles were then separately applied to the Superose 6 HR 10/30 column and analyzed as described in the previous paragraph. The experiment was performed in duplicate.

To determine the elution profile of LPL in a lipoprotein-free medium, 10 μl of a solution of purified bovine milk LPL was used, because extensive homology between human and bovine milk LPL (24, 25) has been previously reported. Bovine milk LPL was stored at −20°C, in 50% glycerol (10 mM NaH_2PO_4, pH 7.4), and diluted with 15% glycerol–PBS–0.15% Tween–1 mg/ml heparin prior to application to the column.

Postheparin plasma lipase activity

Total lipolytic activity was measured in PHP as described previously (26). The substrate containing tri [1-^{14}C]oleoyl glycerol (Amersham, Arlington Heights, IL) and lecithin was incubated with aliquots of PHP for 60 min at 37°C, and the liberated free fatty acid (FFA) radioactivity was extracted and counted. Lipase activity is expressed as nanomoles of fatty acids released per minute per milliliter of plasma (nmol FFA/min/ml). Selective measurements of LPL and hepatic lipase activity were achieved by inhibition of LPL activity by 5D2 monoclonal (Mab) antibodies (27). For each assay, a bovine milk LPL standard and a human postheparin plasma standard were included to adjust for interassay variation.

5F9 ELISA

To detect LPL dimers in PHP and gel-filtration fractions, a sandwich ELISA using two Mab raised against two different epitopes on LPL was used (7). The 5F9 Mab was used to coat the microtiter plate wells and therefore capture the antigen, and the 5D2 Mab, conjugated to horseradish peroxidase, was used for detection of the antigen. Specifically, to coat microtiter plates (Maxisorp, Nunc Inc.), 200 μl of the appropriate Mab (5 μg/ml) in PBS was added to each well and the plate was sealed and incubated at 37°C for 4 h. The plates were then washed four times with PBS/Tween 0.1%, sealed, and stored at 4°C. Coated plates were used within 1 week. Aliquots from a human PHP standard, stored at −70°C, were used as positive control in all experiments. A volume of 200 μl of diluted sample was added to each well and the plates were sealed. After 16 h incubation at 4°C, the wells were washed four times. 5D2 Mab conjugated to horseradish peroxidase in PBS/Tween (0.1% v/v) was added and the plate was incubated at room temperature for 4 h. After 5 washes with PBS/Tween and then *o*-phenylenediamine, substrate containing H_2O_2 was added. Plates were developed for 7 min. Samples, standards, and controls were always run in quadruplicate.

The sensitivity of the 5F9 ELISA allows for the detection of as little as 10 ng/ml of LPL (7). The coefficient of variation between assays was 10% and within assays less than 2%. Recovery of the dimeric LPL in the chromatographic procedure was between 55.3 and 63.7% in samples assayed without THL (n = 5) and between 52.5 and 64.1% in samples with THL (n = 5). Elevated levels of plasma TG did not interfere with the 5F9 ELISA assay (J. D. Brunzell, unpublished data).

The 5F9 Mab recognizes LPL when the epitope is exposed by partial denaturation. Therefore the epitopes of the intact, dimeric, active LPL dimers become detectable by the 5F9 ELISA only after protein denaturation with 1 M guanidine hydrochloride (+GuHCl). The background immunoreactivity originally present in the sample (−GuHCl) has not been carefully characterized and could be due to LPL monomers, fragments, or other unknown material (7). The difference between immunoreactivity +GuHCl (dimeric LPL + background) and immunoreactivity −GuHCl (background) represents the measurement of the active LPL dimers in the sample and is also called Delta 5F9 mass. In the present study, the measurements of dimeric LPL (Delta 5F9 mass) will be utilized.

Immunoprecipitation and Western blot

Two 0.5-ml fractions, from a normolipidemic subject (#3), eluted from the FPLC column in the VLDL size range were pooled, normalized for their lipid content, and delipidated with ethanol–acetone 1:1. The precipitated protein was dissolved in 200 μl of a Laemmli sample buffer and 50 μl was applied to a 10% sodium dodecyl (SDS) gel. Fifty ng of purified bovine LPL and prestained molecular weight markers (Gibco/BRL Life Technology Inc., Gaithersburg, MD) were used as standards. Proteins were electrophoretically transferred to a nitrocellulose membrane (Schleicher & Schuell Inc., Keene, NH). The membrane was incubated with 5D2 anti LPL Mab (5 μg/ml) (27). 5D2 Mabs bound to LPL on the membrane were then detected with a mouse antibody detection kit (Amersham International, Amersham U.K.) using an antimouse-streptavidin conjugate.

Lipid measurements

Very low density lipoproteins were isolated using standard preparative ultracentrifugation (23) and HDL were separated from LDL using dextran sulfate (28).

Triglyceride concentration in plasma, lipoprotein subclasses, and FPLC fractions was determined by enzymatic methods (Sigma Diagnostics Co., St. Louis, MO). Cholesterol concentration in plasma, lipoprotein sub-

classes, and FPLC samples was measured by enzymatic kits (Boehringer Mannheim, Mannheim, Germany).

RESULTS

Subjects

Lipid values in fasting, preheparin plasma of the five subjects selected for the study are presented in **Table 1.** The wide range of plasma TG (69–522 mg/dl), VLDL-cholesterol (14–104 mg/dl), LDL- (89–147 mg/dl) and HDL-cholesterol (35–54 mg/dl) was specifically chosen to assess LPL binding to lipoproteins under different substrate concentrations.

Inhibition of lipolytic activity

To compare the efficacy of THL as plasma lipolytic activity inhibitor, aliquots of pre- and postheparin plasma samples from normo- and hypertriglyceridemic subjects (n = 5) were collected: *1*) with THL, or *2*) with Paraoxon, or *3*) left untreated. The aliquots were then stored at 4°C for 24 h. Plasma TG was measured in each aliquot at baseline (before heparin administration), at sample collection (5 min after heparin administration), and after 24 h of incubation at 4°C (**Fig. 1**). Five minutes after heparin administration, about 11% of the baseline TG was hydrolyzed reflecting the in vivo lipolytic activity and remnant clearance. After 24 h of incubation an additional 1.7% and 3% of the baseline TG were hydrolyzed in samples with Paraoxon and THL, respectively, while a remarkable 36% increase was observed in the untreated samples. These values reflect the ex vivo lipolytic activity and confirm the ability of THL to inhibit both LPL and hepatic lipase (HL) activity as effectively as Paraoxon (19).

Plasma postheparin lipolytic activity was directly evaluated in aliquots of PHP from a normotriglyceridemic subject #3 (Table 1): aliquots collected with Paraoxon and THL showed, respectively, a 99.2% and 98.8% decrease in lipolytic activity as compared to the untreated aliquots. These results prompted us to choose THL, which is easier to handle and less toxic than Paraoxon, as the ex vivo inhibitor of both LPL and HL activity in all gel filtration experiments.

TABLE 1. Plasma lipids in the study subjects

	NormoTG			HyperTG	
Subject #	1	2	3	1	2
Triglycerides (mg/dl)	85.0	92.0	69.0	522.0	239.0
Cholesterol (mg/dl) Total	155.0	220.0	176.0	255.0	200.0
VLDL	17.0	18.0	14.0	104.0	49.0
LDL	89.0	147.0	110.0	113.0	119.0
HDL	49.0	54.0	52.0	35.0	37.0

Lipoprotein elution profiles by gel filtration chromatography

A preheparin, fasting plasma sample from subject #2 in the hyperTG group was also studied to test the FPLC elution profiles of lipoprotein classes pre-isolated by standard sequential ultracentrifugation (23). Each lipoprotein class was separately loaded onto the FPLC column. Cholesterol (**Fig. 2A**) and TG (Fig. 2B) elution profiles were determined for each lipoprotein subclass. VLDL particles eluted in fractions #16 to #21, LDL in fractions #22 to #29, and HDL in fractions #30 to #45. The VLDL lipid peak was in fraction #18, LDL peak in fraction #25, and the major HDL peak in fraction #33. Based on these results, dotted lines highlighting the lipoprotein elution ranges will be presented in the following figures.

Bovine milk LPL elution profile by gel filtration chromatography

Ten μg of purified bovine milk LPL was applied to the FPLC column. Sixty fractions were collected, and LPL mass was assayed by the 5F9 ELISA as previously described. Bovine LPL eluted in fractions #38 to #43 with a peak in fraction #40 corresponding to a molecular weight of approximately 110 kDa as determined by elution profiles of known molecular weight markers (low and high molecular weight gel filtration calibration kits, Pharmacia Biotech.). This finding is consistent with the expected molecular weight of the LPL dimer. Recovery of bovine milk LPL mass in the chromatographic procedure was 61.5%. THL was added to bovine milk LPL and the sample was subjected to the same procedure. Again LPL was detected in fractions #38 to #43 with the peak in fraction #40. Recovery of bovine milk LPL mass + THL in the chromatographic procedure was 59.8%.

Gel filtration of PHP

Effect of ex vivo lipolysis. To demonstrate the effect of the ex vivo lipolysis on the LPL binding to lipoproteins, PHP sample was obtained from subject #3 (Table 1) and analyzed by gel filtration. One aliquot of PHP was collected with THL and immediately applied to the FPLC column. A second aliquot was collected without THL and incubated at 4°C for 2 h; then THL was added to inhibit lipolytic activity and the sample was applied to the FPLC column. The experiment was performed in duplicate and the mean results are shown in **Fig. 3.**

When PHP sample with THL was immediately applied to the column, a major peak of the LPL dimer was observed in the VLDL elution range, with a minor

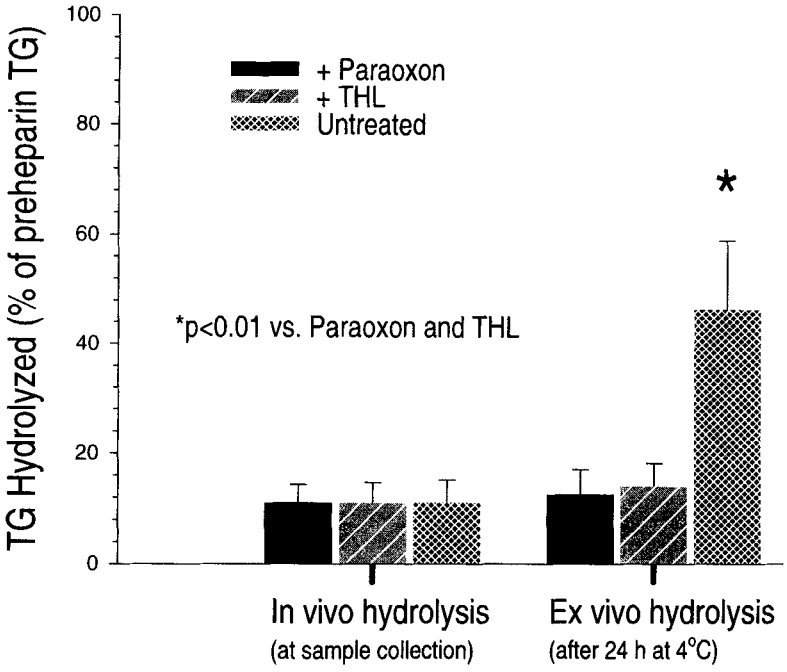

Fig. 1. Postheparin TG hydrolysis in samples collected with paraoxon or THL or left untreated. Values (mean and SD) are expressed as percentage of preheparin TG hydrolyzed (n = 5). Postheparin specimens were assayed: a) at sample collection, 5 min after heparin administration (in vivo hydrolysis), and b) after 24 h of incubation at 4°C (ex vivo hydrolysis).

shoulder in the LDL range and a small peak in the HDL range. After 2 h of incubation at 4°C, a small amount of LPL dimer was found in the VLDL elution range with major peaks in the LDL and HDL elution range. Quantitatively, 70% versus 9% of the LPL dimer was found in the VLDL range, 26% versus 60% in the LDL, and 4% versus 31% in the HDL, when THL was added immediately versus 2 h later.

As THL is highly lipophilic, we assessed the possibility that LPL binding to different lipoprotein subclasses was affected by the addition of THL to the specimen. One aliquot of PHP from the same subject (#3) was applied to the FPLC column after 2 h of incubation at 4°C with no addition of THL. As compared to the same sample with THL, tested under the same experimental conditions, no major differences were observed in the LPL distribution across the lipoprotein size range (Fig. 3). Specifically, 6% of the LPL dimer was in the VLDL elution range (9% with THL), 55% in the LDL (60% with THL), and 39% in the HDL range (31% with THL). Moreover, no differences in the FPLC albumin elution profiles were observed in plasma assayed both in presence and in absence of THL. Results are mean values from duplicate tests.

Gel filtration of normo- and hypertriglyceridemic PHP. LPL distribution across the lipoprotein elution range was studied in presence and absence of THL in PHP from three normotriglyceridemic and two hypertriglyceridemic subjects. Lipids and LPL mass were measured in the eluted fractions. LPL protein was measured in every other fraction.

Mean cholesterol and TG profiles of PHP samples collected with THL are presented in **Fig. 4A.** When LPL activity was not inhibited, LPL dimer in PHP was primarily found associated to LDL particles with an elution peak in fraction #26 (Fig. 4B). In presence of THL, however, most of the LPL dimers eluted in the VLDL range; both the VLDL TG and the LPL dimer peaks were on fraction #18. To compare the LPL dimer elution profiles in PHP samples with and without THL, a difference profile was calculated by subtracting the elution profile without THL from that with THL (Fig. 4C). When lipolytic activity was inhibited, a net increase of LPL dimers was observed associated with VLDL particles as well as a comparable decrease of the amount of protein present in the LDL and HDL elution range.

To evaluate whether the same results were reproducible in presence of increased plasma TG concentrations, LPL dimer elution profiles were measured, in presence and absence of THL, in PHP samples from two hypertriglyceridemic subjects. Plasma lipid profiles (**Fig. 5A**) demonstrate the increase in VLDL-TG and cholesterol as compared to normotriglyceridemic subjects (Fig. 4A). Again, the LPL dimer elution profiles with and without THL (Fig. 5B) and the difference profile between the two (Fig. 5C) entirely confirmed that, when lipolytic activity is adequately inhibited, most of the LPL dimers elute associated with VLDL particles. Moreover, the LPL dimer elution profile in PHP samples with THL suggests that both in normo- (Fig. 4B) and in hypertriglyceridemic subjects (Fig. 5B), similar amounts of LPL dimers were detected associated with

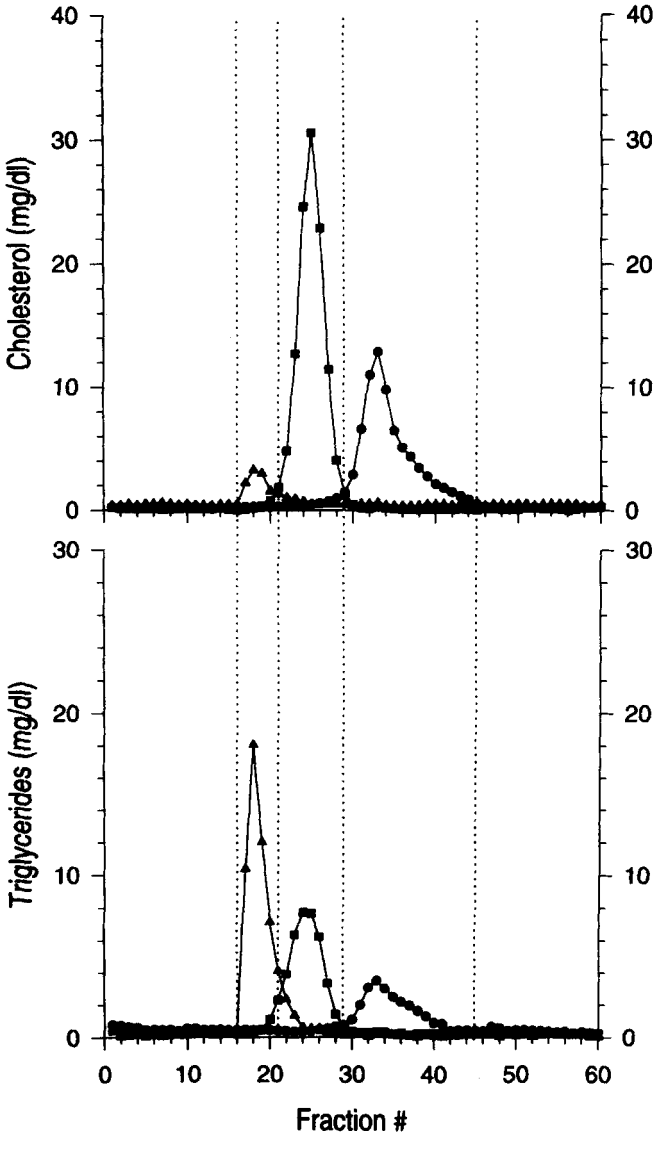

Fig. 2. Gel filtration elution profiles of lipoproteins isolated by ultracentrifugation. Cholesterol (panel A), and triglyceride (panel B) profiles of VLDL (▲-▲), LDL (■-■), and HDL (●-●). Vertical dotted lines mark the elution ranges of the different lipoprotein subclasses.

VLDL particles. In both normo- and hypertriglyceridemic subjects, the active LPL dimer accounted for 88% of the total immunoreactivity detected in PHP whereas the remaining 12% was background immunoreactivity. The background immunoreactivity eluted in association with large LDL particles.

Gel filtration of normotriglyceridemic preheparin plasma. To assess whether the association of LPL dimers with VLDL particles in PHP is also detectable under physiological conditions, LPL dimer elution profiles were measured, in presence and absence of THL, in preheparin samples from subjects #1 and #2 (**Fig. 6**). In preheparin plasma, when lipolytic activity is effectively inhibited, most of the LPL dimers elute associated with VLDL particles (Fig. 6B), confirming the observations reported in PHP samples from both normo- and hypertriglyceridemic subjects. The background immunoreactivity accounted for 77% of the total immunoreactivity detected in the preheparin specimen, with the remaining 23% being the active LPL dimers.

Western blot analysis of the immunoreactive material

To confirm that the immunoreactive material eluted in the VLDL size range was LPL, VLDL TG peak fractions #18 and #19 from samples collected with and without THL, were separately pooled, normalized for their lipid content, and analyzed by Western blot after protein separation on SDS gel (**Fig. 7**). A clear band with the approximate molecular weight of monomeric human LPL (55,000) and similar to bovine LPL, was detected in VLDL + THL samples. A similar but remarkably weaker band was detected in VLDL samples collected without THL in agreement with the LPL protein measurements from the gel filtration analysis.

DISCUSSION

The present work is the first report that investigates the LPL binding to lipoprotein particles in PHP after inhibition of ex vivo lipolytic activity. The study shows that: *1*) LPL dimers are mostly associated to TG-rich lipoproteins in PHP when the ex vivo lipolytic activity is inhibited, and *2*) the binding of LPL to cholesterol-rich lipoproteins might reflect an in vitro phenomenon due to lack of inhibition of ex vivo lipolytic activity.

It has long been known that lipolysis initiated intravascularly continues ex vivo (29), particularly in PHP, when the amount of active LPL increases several hundred fold. These results were recently confirmed by Zambon et al. (19), emphasizing the need for a complete inhibition of lipolytic activity in studies dealing with postheparin lipoprotein metabolism in order to minimize the risk of ex vivo artifacts.

In the present study, THL, an active-site inhibitor of pancreatic (20), LPL (21), and HL has been used. Tetrahydrolipstatin almost completely inhibited lipolytic activity in PHP (98.8% inhibition) and appeared to be as effective as Paraoxon (Fig. 1) and much safer to use in the FPLC system. Moreover, THL did not appear to affect the LPL elution profile by gel filtration when added to purified bovine milk LPL in a lipoprotein-free medium. Finally, THL had a minor effect on the LPL binding to lipoproteins (LPL dimer with THL vs. without THL: +3%, +5%, and −8% in the VLDL, LDL, and

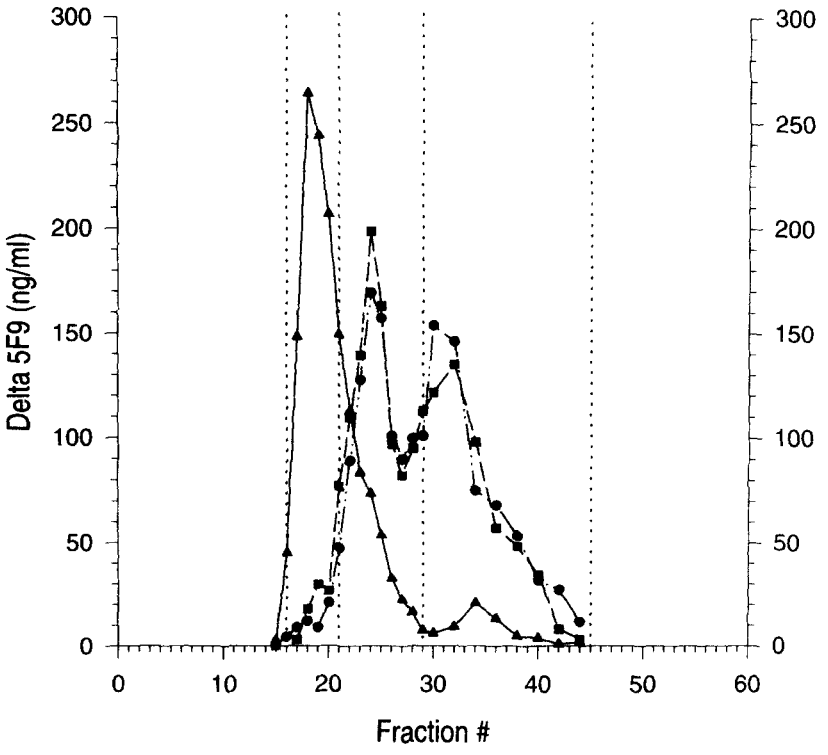

Fig. 3. Gel filtration elution profile of LPL dimer, reported as delta 5F9 mass, in a single PHP sample collected with THL and immediately applied to the column (▲-▲); incubated at 4°C for 2 h without THL; then THL was added and the sample was applied to the column (■-■); incubated at 4°C for 2 h without THL, then applied to the column (●-●). Vertical dotted lines mark the elution ranges of the different lipoprotein subclasses.

HDL elution ranges, respectively) when tested in PHP samples after a 2-h incubation at 4°C (Fig. 3). Therefore, THL had no significant effect on LPL binding to different lipoprotein subclasses under physiological lipid concentrations. Lookene, Skottova, and Olivecrona (21) suggested that LPL inhibition is due to covalent binding of THL to Ser 132 which is one of the residues in the catalytic triad of this enzyme. The binding of THL to LPL does not affect the dimeric structure of the enzyme as suggested by the detection of LPL protein by 5F9 ELISA in PHP samples with THL. This method, by combining measurements prior to and after protein denaturation with GuHCl, specifically detects LPL dimers originally present in the sample (7). Moreover, a similar recovery of the LPL mass in the chromatographic procedure was observed in the duplicate samples analyzed with and without THL.

For PHP collection, a time interval of 5 min after i.v. heparin administration was specifically chosen in order to minimize the in vivo lipolysis of lipoproteins, allowing, however, an homogeneous distribution of heparin in the circulation. In fact, Kern et al. (30) showed that up to 75% of the postheparin lipolytic activity is already detectable 5 min after heparin administration.

It is particularly relevant that THL effectively inhibited both LPL and HL activity. The aim of the present study was, in fact, to reproduce as accurately as possible the plasma lipoprotein profile after lipase displacement from the arterial wall by preventing not only TG-rich lipoprotein hydrolysis by LPL, but also remnant catabolism by HL. Direct evidence of the effect of selective HL inhibition on LPL association with VLDL particles is not available in the present study. We feel that a major influence is unlikely based on the evidence that VLDL are not the preferential substrate of HL. Specific studies, however, need to be performed to address this point.

Previous reports (12–14) suggested that in PHP, LPL is mostly associated with cholesterol-rich lipoproteins. To substantiate the hypothesis that these observations may be affected by ex vivo lipolysis, LPL distribution across the lipoprotein size range was evaluated in a PHP specimen after inhibition of lipolytic activity, performed either immediately or after a 2-h incubation at 4°C. After 2 h incubation at 4°C, LPL dimers were found associated with cholesterol-rich lipoproteins. These results (Fig. 3) match the observations first reported by Goldberg et al. (12) and then by Vilella et al. (14), that in PHP, LPL is predominantly associated with LDL and

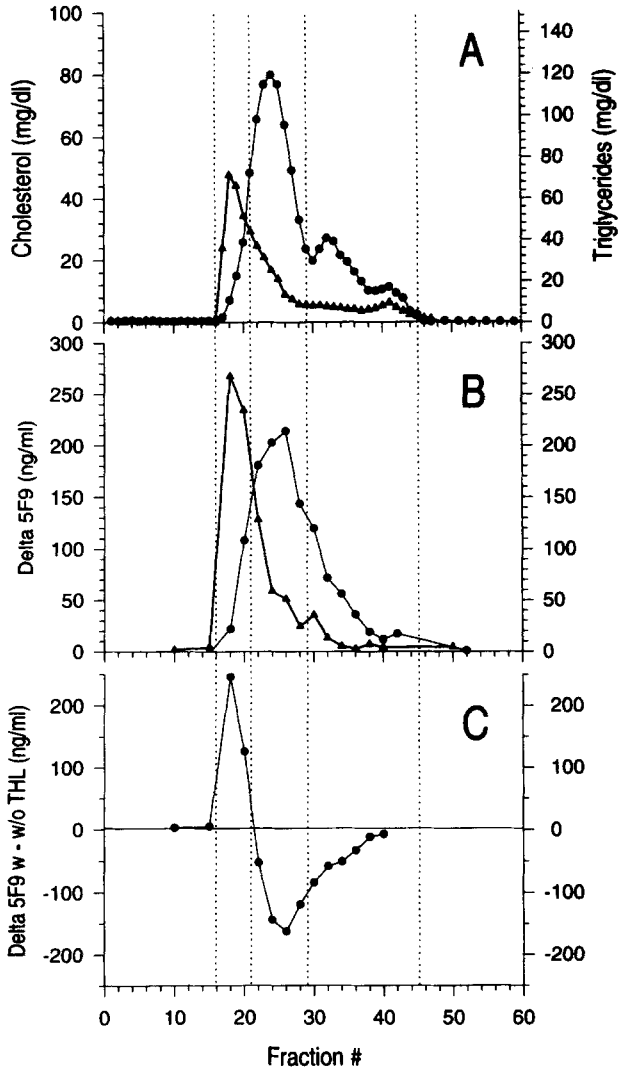

Fig. 4. Gel filtration elution profiles of PHP from normotriglyceridemic subjects (n = 3). Cholesterol (●-●) and triglyceride (▲-▲) distribution in PHP samples collected with THL (panel A). LPL dimer distribution, reported as delta 5F9 mass, in PHP samples collected with (▲-▲) and without (●-●) THL (panel B). Difference in LPL dimer distribution between samples with THL versus without THL (panel C). Vertical dotted lines mark the elution ranges of the different lipoprotein subclasses.

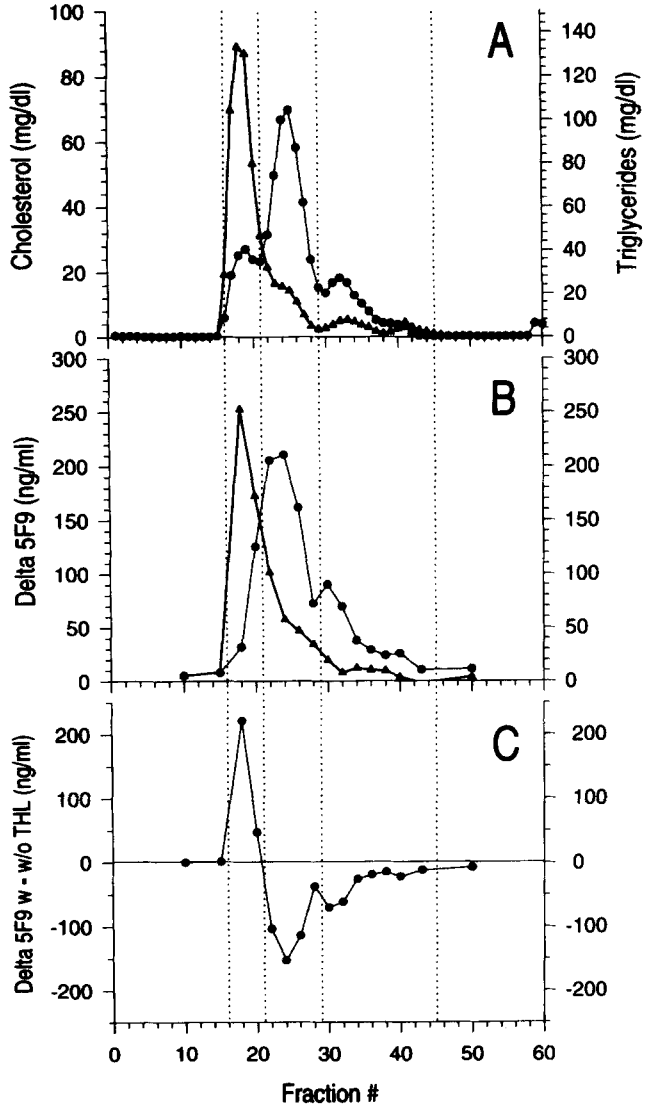

Fig. 5. Gel filtration elution profiles of PHP from hypertriglyceridemic subjects (n = 2). Cholesterol (●-●) and triglyceride (▲-▲) distribution in PHP samples collected with THL (panel A). LPL dimer distribution, reported as delta 5F9 mass, in PHP samples collected with (▲-▲) and without (●-●) THL (panel B). Difference in LPL dimer distribution between samples with THL versus without THL (panel C). Vertical dotted lines mark the elution ranges of the different lipoprotein subclasses.

HDL. However, after immediate inhibition of ex vivo lipolytic activity, most of the LPL dimers were found associated with TG-rich lipoproteins eluting in the VLDL range (Fig. 3). These results supported the hypothesis of a major role of the ex vivo lipolytic activity on previously reported studies of LPL-lipoprotein association in PHP. They also suggest that, in PHP, major changes in the TG-rich lipoproteins occurred in a very short time interval even when specimens were kept refrigerated (4°C) and the analysis was performed almost immediately after sample collection, confirming previous data by Zambon et al. (19).

To further expand and confirm this observation, we studied LPL-lipoprotein binding in PHP of three normo- (Fig. 4) and two hypertriglyceridemic subjects (Fig. 5). Postheparin plasma samples were analyzed by gel filtration immediately after collection with and without THL. Confirming the results of the previous experiment, when lipolytic activity was inhibited by THL, most of the LPL dimers eluted associated to TG-rich VLDL

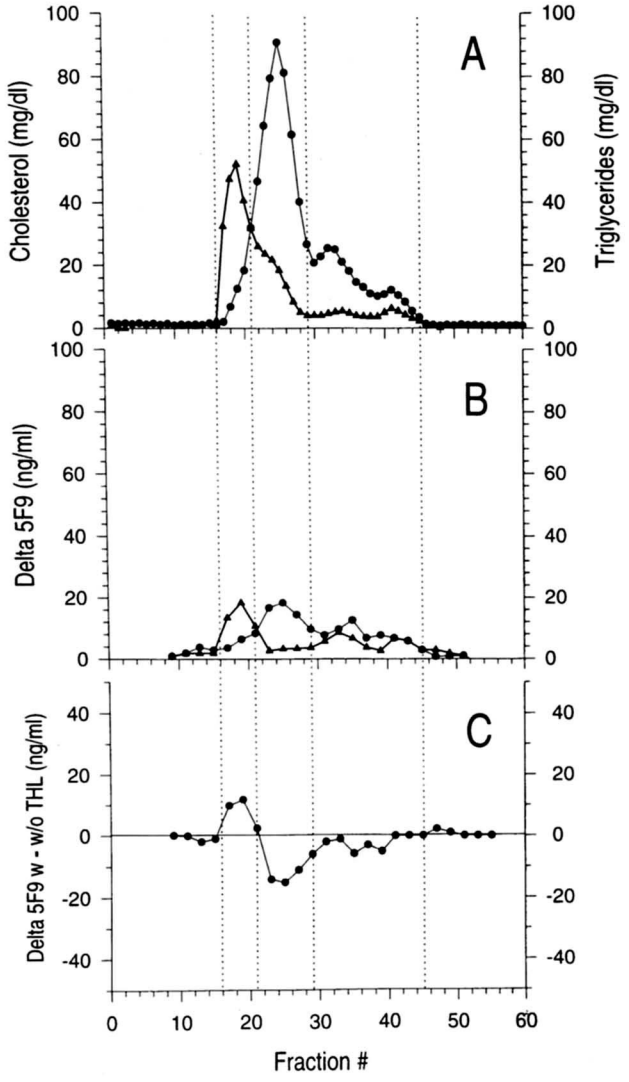

Fig. 6. Gel filtration elution profiles of preheparin plasma from normotriglyceridemic subjects (n = 2). Cholesterol (●-●) and triglyceride (▲-▲) distribution in PHP samples collected with THL (panel A). LPL dimer distribution, reported as delta 5F9 mass, in preheparin sample collected with (▲-▲) and without (●-●) THL (panel B). Difference in LPL dimer distribution between samples with THL versus without THL (panel C). Vertical dotted lines mark the elution ranges of the different lipoprotein subclasses.

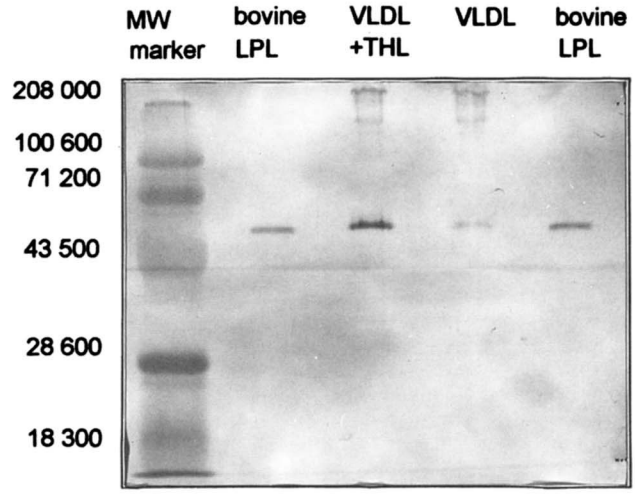

Fig. 7. SDS gel electrophoresis and Western blot on VLDL isolated by gel filtration of PHP sample from subject #3, collected with (VLDL + THL) and without THL (VLDL). Prestained molecular weight markers (MW marker) and purified bovine LPL (bovine LPL) were used as standards. LPL was detected using 5D2 Mab.

both in normotriglyceridemic (Fig. 4B) and in hypertriglyceridemic (Fig. 5B) individuals. A major redistribution of LPL dimers towards cholesterol-rich lipoproteins was observed in the same samples when lipolytic activity was not inhibited. These results suggest that in vivo, after heparin administration and LPL displacement from the endothelium, LPL dimers are mostly associated with VLDL. Recently, Carrero et al. (31) showed that LPL, when added to lipoproteins in vitro, has a higher affinity for VLDL than for LDL, supporting this hypothesis. If lipolysis is allowed to proceed, TG in the VLDL core are progressively hydrolyzed initiating the conversion of VLDL to LDL. Therefore, LPL dimers initially associated to VLDL particles are finally observed bound to lipoproteins in the LDL size range. Associated with the hydrolysis of TG in the VLDL core, some surface components, namely phospholipids, free cholesterol, and apolipoproteins, are transferred from VLDL to HDL (32). The presence of small amounts of LPL associated with HDL, as also shown by Villella et al. (14), suggests that during conversion of VLDL to LDL, some LPL dimers may be transferred to HDL, in association with lipoprotein surface components.

Based on the bovine milk LPL elution profile in a lipoprotein-free medium (elution range: fraction #38 to #43), no major amounts of free LPL dimers were found in the PHP elution profiles of the subjects studied either in presence or absence of THL. We speculate that in PHP all the LPL dimers are associated to lipoproteins, mostly VLDL. This hypothesis is supported by the lack of increase of LPL mass in presence of increased amounts of VLDL such as in the hypertriglyceridemic subjects (Fig. 5B), as compared to subjects with normal triglyceride levels (Fig. 4B). Comparable amounts of LPL dimers associated with VLDL particles were found in the two groups of subjects studied. It is plausible to hypothesize that if there were free LPL dimers in PHP from subjects with normal triglyceride levels, at least some of them should have become VLDL-bound in the presence of increased amounts of VLDL particles as in the hypertriglyceridemic TG individuals. This is clearly not the case (Fig. 5B). Although we believe that the LPL-lipoprotein interaction takes place at the endothelial site, we cannot rule out the possibility that heparin

releases LPL that then binds to lipoproteins in the bloodstream.

To further highlight the physiological implications of the LPL binding to TG-rich lipoproteins, preheparin samples from normotriglyceridemic subjects were also tested (Fig. 6). Confirming the results observed in PHP, when lipolytic activity was inhibited by THL most of the LPL dimers eluted associated with TG-rich VLDL (Fig. 6B). In samples where plasma lipolytic activity was not inhibited, LPL dimers eluted in association with LDL particles.

The 5F9 ELISA allows for the determination of the active, dimeric LPL protein. In PHP samples the large majority of LPL mass (88%) was represented by active dimers whereas only a small percentage (12%) was background, immunoreactive material. In preheparin samples, the proportion of active protein/inactive background material was reversed: the majority of total immunoreactivity was represented by background immunoreactivity (77%) with only a minor percentage of active dimers (23%). These findings are in agreement with previous observations (14, 33). The origin and the turnover of the background material is not clear. It is conceivable that it includes LPL monomers as well as fragments of LPL protein and possibly unknown components. In both pre- and postheparin plasma, in presence of THL, the background immunoreactivity eluted in association with large LDL particles.

The binding of LPL dimers to VLDL particles, detected in pre- and PHP, may have important implications in explaining the physiological role of this enzyme as a multifunctional protein. Because of its ability to simultaneously bind to the LDL receptor-related protein (LRP), lipoproteins, and heparin-like substances, LPL can efficiently mediate binding of lipoproteins to cell surfaces and to both the endocytotic receptor LRP (16, 34) and the VLDL receptor (17). Nykjaer et al. (18) found that the ability to mediate binding of lipoproteins to LRP resided only with the LPL dimer. Moreover, recent reports demonstrated that LPL enhances the binding of TG-rich lipoproteins and β-VLDL to cell-surface proteoglycans and to LRP (16, 34). It is therefore possible that LPL dimers associated with VLDL particles may play, in vivo, a physiological role in the receptor-mediated VLDL remnant catabolism by affecting the binding of TG-rich lipoproteins to the LRP and the VLDL receptor. Specific studies need to further address whether LPL monomers or their fragments might compete with other ligands (LPL dimers, apoE, hepatic lipase) for binding to LRP, and thereby affect remnant uptake and degradation.

It is interesting to note, from the data presented in Fig. 4B and Fig. 5B, that plasma TG concentrations do not affect the amount of LPL dimer eluting in the VLDL range nor its distribution across the lipoprotein size range. Hypertriglyceridemic subjects often have an increased amount of VLDL particles as indirectly confirmed in the present report by the increased amount of VLDL TG and cholesterol (almost 2-fold) observed in the two hyperTG individuals (Fig. 5A) as compared to the normo TG subjects (Fig. 4A). It appears, therefore, that in PHP the amount of LPL dimer associated with VLDL is not dependent on the VLDL particle number and has already reached the maximum level at normal plasma TG concentrations. Although indirectly, these results support the hypothesis that in PHP all the LPL dimers are bound to lipoproteins (mainly VLDL), as previously discussed, but more interestingly they suggest that in PHP only some VLDL particles carry LPL dimers. Furthermore, studies of the LPL/apoB molar ratio in the VLDL density range suggest that in PHP there is 1 LPL dimer for every 100 to 500 VLDL particles (J. D. Brunzell and A. Zambon, unpublished data). Whether this finding has any implication on the catabolism of triglyceride-rich lipoproteins remains to be investigated.

In summary, the present report first indicates that in PHP samples, after inhibition of ex vivo lipolytic activity, most of the LPL dimers are associated with VLDL particles. It also suggests that the association, previously observed, between LPL and cholesterol-rich lipoproteins in PHP might be an ex vivo phenomenon due to lack of inhibition of lipolytic activity. In conclusion, data from the present study and previously published reports support the hypothesis that during lipolysis a fraction of endothelial-bound LPL may dissociate attached to TG-rich lipoproteins, forming circulating LPL-lipoprotein complexes that subsequently bind to cell-surface proteoglycans and undergo receptor-mediated catabolism. Further studies are needed to test this hypothesis and the in vivo implications of these results.

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REFERENCES

1. Olivecrona, T., and G. Bengtsson-Olivecrona. 1987. Lipoprotein lipase from milk: the model enzyme in lipoprotein lipase research. *In* Lipoprotein Lipase. J. Borensztajn, editor. Evener Publishers, Inc., Chicago, IL. 15–77.

2. Eckel, R. H. 1989. Lipoprotein lipase: a multifunctional enzyme relevant to common metabolic diseases. *N. Engl. J. Med.* **320:** 1060–1068.
3. Glaser, D. S., T. J. Yost, and R. H. Eckel. 1992. Preheparin lipoprotein lipolytic activities: relationship to plasma lipoproteins and postheparin lipolytic activities. *J. Lipid Res.* **33:** 209–214.
4. Brunzell, J. D. 1995. Familial lipoprotein lipase deficiency and other causes of the chylomicronemia syndrome. *In* The Metabolic and Molecular Bases of Inherited Disease. 7th ed. C. R. Scriver, A. L. Beaudet, W. S. Sly, and D. Valle, editors. McGraw-Hill, New York. 1913–1932.
5. Pedersen, M. E., M. Cohen, and M. C. Schotz. 1983. Immunocytochemical localization of the functional fraction of lipoprotein lipase in the perfused heart. *J. Lipid Res.* **24:** 512–517.
6. Iverius, P-H, and A-M Ostlund-Lindqvist. 1976. Lipoprotein lipase from bovine milk: isolation procedure, chemical characterization, and molecular weight analysis. *J. Biol. Chem.* **251:** 7791–7795.
7. Peterson, J., W. Y. Fujimoto, and J. D. Brunzell. 1992. Human lipoprotein lipase: relationship of activity, heparin affinity, and conformation as studied with monoclonal antibodies. *J. Lipid Res.* **33:** 1165–1170.
8. Bengtsson, G., and T. Olivecrona. 1981. Lipoprotein lipase: modification of its kinetic properties by mild tryptic digestion. *Eur. J. Biochem.* **113:** 547–554.
9. Kobayashi, J., K. Shirai, Y. Saito, and S. Yoshida. 1989. Lipoprotein lipase with a defect in lipid interface recognition in a case with type I hyperlipidemia. *Eur. J. Clin. Invest.* **19:** 424–432.
10. Saxena, U., L. D. Witte, and I. J. Goldberg. 1989. Release of endothelial cell lipoprotein lipase by plasma lipoproteins and free fatty acids. *J. Biol. Chem.* **264:** 4349–4355.
11. Peterson, J., B. E. Bihain, G. Bengtsson-Olivecrona, R. J. Deckelbaum, Y. A. Carpentier, and T. Olivecrona. 1990. Fatty acid control of lipoprotein lipase: a link between energy metabolism and lipid transport. *Proc. Natl. Acad. Sci. USA* **87:** 909–913.
12. Goldberg, I. J., J. J. Kandel, C. B. Blum, and H. N. Ginsberg. 1986. Association of plasma lipoproteins with postheparin lipase activity. *J. Clin. Invest.* **78:** 1523–1528.
13. Vilella, E., and J. Joven. 1991. Lipoprotein lipase binding to plasma. *Med. Sci. Res.* **19:** 111–112.
14. Vilella, E., J. Joven, M. Fernández, S. Vilaró, J. D. Brunzell, T. Olivecrona, and G. Bengtsson-Olivecrona. 1993. Lipoprotein lipase in human plasma is mainly inactive and associated with cholesterol-rich lipoproteins. *J. Lipid Res.* **34:** 1555–1564.
15. Felts, J. M., H. Itakura, and R. T. Crane. 1975. The mechanism of assimilation of constituents of chylomicrons, very low density lipoproteins, and remnants—a new theory. *Biochem. Biophys. Res. Commun.* **66:** 1467–1475.
16. Beisiegel, U., W. Weber, and G. Bengtsson-Olivecrona. 1991. Lipoprotein lipase enhances the binding of chylomicrons to low density lipoprotein receptor-related protein. *Proc. Natl. Acad. Sci. USA.* **88:** 8342–8346.
17. Niemeier, A., M. Gåfvels, J. Heeren, N. Meyer, B. Angelin, and U. Beisiegel. 1996. VLDL receptor mediates the uptake of human chylomicron remnants in vitro. *J. Lipid Res.* **37:** 1733–1742.
18. Nykjaer, A., G. Bengtsson-Olivecrona, A. Lookene, S. K. Moestrup, C. M. Petersen, W. Weber, U. Beisiegel, and J. Gliemann. 1993. The alpha 2-macroglobulin/low density lipoprotein receptor-related protein binds lipoprotein lipase and beta-migrating very low density lipoprotein associated with the lipase. *J. Biol. Chem.* **268:** 15048–15055.
19. Zambon, A., S. I. Hashimoto, and J. D. Brunzell. 1993. Analysis of techniques to obtain plasma for measurement of levels of free fatty acids. *J. Lipid. Res.* **34:** 1021–1028.
20. Hadváry, P., W. Siedler, W. Meister, W. Vetter, and H. Wolfer. 1991. The lipase inhibitor tetrahydrolipstatin binds covalently to the putative active site serine of pancreatic lipase. *J. Biol. Chem.* **266:** 2021–2027.
21. Lookene, A., N. Skottova, and G. Olivecrona. 1994. Interaction of lipoprotein lipase with the active-site inhibitor tetrahydrolipstatin (Orlistat®). *Eur. J. Biochem.* **222:** 395–403.
22. Korn, E. D., and T. W. Quigley. 1957. Lipoprotein lipase of chicken adipose tissue. *J. Biol. Chem.* **226:** 833–839.
23. Verne, N. S., and D. L. Puppione. 1986. Sequential flotation ultracentrifugation. *Methods Enzymol.* **128:** 155–170.
24. Wion, K. L., T. G. Kirchgessner, A. J. Lusis, M. C. Schotz, and R. M. Lawn. 1987. Human lipoprotein lipase complementary DNA sequence. *Science.* **235:** 1638–1641.
25. Senda, M., K. Oka, W. V. Brown, P. K. Qasba, and Y. Furushi. 1987. Molecular cloning and sequence of cDNA coding for bovine lipoprotein lipase. *Proc. Natl. Acad. Sci. USA.* **84:** 4369–4373.
26. Iverius, P-H., and J. D. Brunzell. 1985. Human adipose tissue lipoprotein lipase: changes with feeding and relation to postheparin plasma. *Am. J. Physiol.* **249:** E107–E114.
27. Babirak, S. P., P-H. Iverius, W. Y. Fujimoto, and J. D. Brunzell. 1989. Detection and characterization of the heterozygote state for lipoprotein lipase deficiency. *Arteriosclerosis.* **9:** 326–334.
28. Warnick, G. R., J. Benderson, and J. J. Albers. 1982. Dextran sulfate-Mg^{2+} precipitation procedure for quantification of high density lipoprotein cholesterol. *Clin. Chim.* **28:** 1279–1288.
29. Grossman, M. I., H. C. Moeller, and L. Palm. 1955. Effect of lipemia and heparin on free fatty acid concentration of serum in humans. *Proc. Soc. Exp. Biol. Med.* **90:** 106–109.
30. Kern, P. A., R. A. Martin, J. Carty, I. J. Goldberg, and J. M. Ong. 1990. Identification of lipoprotein lipase immunoreactive protein in pre- and postheparin plasma from normal subjects and patients with type I hyperlipidemia. *J. Lipid Res.* **31:** 17–26.
31. Carrero, P., D. Gomez-Coronado, G. Olivecrona, and M. A. Lasuncion. 1996. Binding of lipoprotein lipase to apolipoprotein B-containing lipoproteins. *Biochem. Biophys. Acta,* **1299:** 198–206.
32. Havel, R. J., J. P. Kane, and M. L. Kashyap. 1973. Interchange of apolipoproteins between chylomicrons and high density lipoproteins during alimentary lipemia in men. *J. Clin. Invest.* **52:** 32–38.
33. Olivecrona, G., M. Hultin, R. Savonen, N. Skottova, A. Lookene, Y. Tugrul, and T. Olivecrona. 1995. Transport of lipiprotein lipase in plasma and lipoprotein metabolism. *In* Atherosclerosis X. F. P. Woodford, J. Davignon, and A. Snidermann, editors. Elsevier, Amsterdam. 250–253.
34. Chappell, D. A., G. L. Fry, M. A. Waknitz, L. E. Muhonen, M. W. Pladet, P-H Iverius, and D. K. Strickland. 1993. Lipoprotein lipase induces catabolism of normal triglyceride-rich lipoproteins via the low density lipoprotein receptor-related protein/alpha2-macroglobulin receptor in vitro. A process facilitated by cell-surface proteoglycans. *J. Biol. Chem.* **268:** 14168–14175.