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The majority of lipoprotein lipase in plasma is bound to remnant lipoproteins: A new definition of remnant lipoproteins

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ABSTRACT

Background: Lipoprotein lipase (LPL) is a multifunctional protein and a key enzyme involved in the regulation of lipoprotein metabolism. We determined the lipoproteins to which LPL is bound in the pre-heparin and post-heparin plasma.
Methods: Tetrahydrolipstatin (THL), a potent inhibitor of serine lipases, was used to block the lipolytic activity of LPL, thereby preventing changes in the plasma lipoproteins due to *ex vivo* lipolysis. Gel filtration was performed to obtain the LPL elution profiles in plasma and the isolated remnant lipoproteins (RLP).
Results: When *ex vivo* lipolytic activity was inhibited by THL in the post-heparin plasma, majority of the LPL was found in the VLDL elution range, specifically in the RLP as inactive dimers. However, in the absence of THL, most of the LPL was found in the HDL elution range as active dimers. Furthermore, majority of the LPL in the pre-heparin plasma was found in the RLP as inactive form, with broadly diffused lipoprotein profiles in the presence and absence of THL.
Conclusions: It is suggested that during lipolysis *in vivo*, the endothelial bound LPL dimers generates RLP, forming circulating RLP-LPL complexes in an inactive form that subsequently binds and initiates receptor-mediated catabolism.

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1. Introduction

Lipoprotein lipase (LPL) hydrolyzes the triglyceride-rich core of chylomicrons (CM) and very low density lipoproteins (VLDL). It is also known as a ligand, *in vitro* or *in vivo*, for the binding of lipoproteins to the low density lipoprotein receptor-related protein 1 (LRP-1) and may play a central role in the receptor-mediated removal of triglyceride rich lipoproteins [1–3]. After synthesis in parenchymal cells, primarily in adipose tissue and skeletal muscle, LPL is transported to the intimal surface of the vascular endothelium, where it is non-covalently anchored to the heparan sulfate side chains of membrane proteoglycans and the recently discovered GPI-HBP1 [1,4,5]. At this site, LPL is responsible for the hydrolysis of the TG-rich core of circulating CM and VLDL, generating free fatty acids that can be either used immediately for energy or stored, primarily in adipose tissue under normal circumstances. The enzyme is known to be active only in a dimeric configuration [6,7]. To carry out its functions, LPL binds the interface of TG-rich lipoproteins [8,9]. After reduction in TG content and size, CM and VLDL remnants are believed to detach from the endothelium and released into the circulation by mechanism(s) that have yet to be fully understood [10,11].

The aim of this study was to clarify the characteristics of the interaction between LPL and remnant lipoproteins (RLP) in both pre-heparin

Abbreviations: CM, chylomicrons; VLDL, very low density lipoproteins; HTGL, hepatic triglyceride lipase; LRP-1, low density lipoprotein receptor-related protein 1; LPL, lipoprotein lipase; RLP, remnant-like lipoprotein particles; RLP-C, remnant-like lipoprotein particles-cholesterol; RLP-TG, remnant-like lipoprotein particles-triglyceride; TC, total cholesterol; TG, triglyceride; THL, tetrahydrolipstatin; VLDL, very low density lipoprotein.

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and post-heparin plasma. Tetrahydrolipstatin (THL) was used to block the *ex vivo* lipolytic activity of LPL in the post-heparin plasma in this study. The relationship between LPL activity and its inhibitors (apoC1 and apoC3) in RLP was also investigated.

There is growing evidence that LPL not only affects lipolysis, but also acts as a ligand for the binding of lipoprotein particles to receptors on the cell surface. A number of studies have reported that LPL is associated with lipoproteins in human pre- and post-heparin plasma, in both its inactive and active form [12–14]. Felts, Itakura, and Crane [15] in 1975 suggested that LPL binds to lipoprotein remnants and may be a marker for uptake of remnant lipoproteins by the liver. However, the isolation of LPL bound remnants from TG-rich lipoproteins or VLDL, namely the LPL-remnant complex, in plasma has not been demonstrated at this time.

Previous studies showed that LPL bound to apolipoprotein E-rich lipoproteins enhances their binding to the LRP-1 *in vitro*, the putative receptor for CM remnants [16], as well as to the VLDL receptor [17,18]. Moreover, Nykjaer et al. [19] suggested that only the LPL dimer, and not the monomer, was able to mediate this binding. Goldberg et al. [12] reported that lipolytic activity in the pre- and post-heparin plasma is associated with cholesterol-rich particles slightly larger than LDL. Other reports confirmed this observation by showing that the LPL protein after gel filtration is associated with LDL and HDL in both pre-heparin and post-heparin plasma [13,14]. In these studies, however, no steps were taken to inhibit lipolytic activity *ex vivo*. Zambon, Hashimoto, and Brunzell [20] demonstrated that a significant amount of the TG-rich core of CM and VLDL can be hydrolyzed in the post-heparin plasma stored *ex vivo* even at 4 °C if the 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 lipoproteins with an LDL and HDL size range. However, more recently Zambon et al. [21] reported that dimeric LPL is bound to triglyceride-rich plasma lipoproteins in the presence of THL [22,23] as the result of an inhibition of *ex vivo* lipolysis.

The present study, we analyzed LPL binding to RLP isolated from post-heparin and pre-heparin plasma by immunoaffinity gel separation. By inhibiting lipolytic activity *ex vivo*, this study aimed to examine: 1) lipoprotein subclass with which LPL is associated *in vivo* before and after heparin administration as well as in the presence and absence of THL, 2) whether the LPL bound to RLP isolated by immunoaffinity gel are dimers or monomers and with an active or inactive form, and 3) whether the association between LPL and lipoproteins previously observed in the post-heparin plasma reflects a physiological or non-physiological condition. To clarify these biochemical and physiological characteristics of the LPL and RLP interaction will provide the critical new insight into the definition of remnant lipoproteins.

2. Materials and methods

2.1. Subjects

Randomly selected type 2 diabetes mellitus (T2DM) and metabolic syndrome (MetS) patients (aged 23–77 y; males 101, females 94) who were under treatment at Gunma University Hospital, Maebashi, Japan were studied in cases with plasma TG levels above and below 150 mg/dl. The study had the approval of the Ethical Committee of the Gunma University School of Medicine. Written informed consent was obtained from all of the participants.

The post-heparin plasma study was conducted as part of a trial investigating the metabolic effects of sugar consumption at University of California, Davis [24,25]. The participants, who were young and generally healthy, received intravenous administration of heparin for measurements of LPL and hepatic triglyceride lipase (HTGL) activity. The 29 samples analyzed for the current study were from 15 male and 14 females who were overweight or obese and susceptible to be postprandial remnant hyperlipoproteinemia (median age 24 y, with an average BMI of 27 kg/m^2). The post-heparin plasma was collected in the fasting state at 8:00 a.m. in the morning. The UC Davis Institutional Review Board approved the experimental protocol and all of the subjects provided written informed consent to participate in the study. All of the plasma samples were kept frozen at −80 °C until analysis.

2.2. Plasma handling

The pre- and post-heparin plasma samples were obtained after 12-h fasting. The post-heparin plasma was collected at 15 min after i.v. administration of 50 U/kg body weight of heparin at UC Davis. In addition, a normotriglyceridemic (male aged 60 y) and a hypertriglyceridemic Japanese subject (male aged 32 y) were injected with 30 U/kg of heparin and the plasma was collected at 0, 15, 30, 60 min at Hidaka Hospital, Takasaki, Japan.

To inhibit lipolytic activity *ex vivo*, 1 aliquot of post-heparin blood was mixed with tetrahydrolipstatin (THL) (Orlistat, Sigma-Aldrich) immediately after blood withdrawal at a final concentration of 1 μg/ml [21]. Plasma was separated from the THL-added blood by centrifugation (1800*g*, 10 min at room temperature).

2.3. Assay procedure for sandwich LPL-ELISA

The LPL concentration in plasma was measured using the LPL-ELISA newly developed at Immuno-Biological Laboratories (IBL). The assay used 2 different monoclonal antibodies against human recombinant LPL (57A5 and 88B8) for the sandwich ELISA. Tetra methyl benzidine (TMB) was used as the coloring agent (chromogen). Briefly, 100 μl of plasma or standard LPL diluted >100 fold was incubated with a solid phase antibody (57A5) for 60 min at 37 °C using a plate lid. After washing the plate with phosphate buffer, another antibody (88B8) labelled with horse radish peroxidase was added and incubated for 30 min at 4 °C with the plate lid. After washing, chromogen was added and incubated for 30 min at room temperature. The plate was read at 450 nm against a reagent blank within 30 min of the addition of 1 N H_2SO_4 solution to stop the reaction. The measurement range of the assay was 0.02–1.5 ng/ml. The CV was <10% in both intra- and inter-assay

2.4. LPL activity assay

The LPL activities were determined by an assay developed by Imamura et al. [26] for measuring the increase in absorbance at 546 nm due to the quinoneine dye. Reaction mixture-1 (R-1) contained dioleoylglycerol solubilized with lauryldimethylaminobetaine, monoacylglycerol-specific lipase, glycerolkinase, glycerol-3-phosphate oxidase, peroxidase, ascorbic acid oxidase and apolipoprotein C-II (apoC-II). R-2 contained Tris-HCl (pH 8.7) and 4-aminoantipyrine. Assay of lipase activity was performed with a chemistry analyzer (H7700P). In the assay for LPL activity, 160 μl of R-1 was incubated at 37 °C with 2 μl of sample for 5 min, and 80 μl of R-2 was added and incubated for additional 5 min. HL activities were measured under the same conditions without apoC-II.

2.5. Isolation of remnant lipoproteins with the immunoaffinity gel

Immuno-separation method [27] was used to isolate RLP containing both apoB-48 and apoB-100 from the plasma using specific antibodies so as to isolate the RLP as an unbound fraction. Briefly, 0.5 ml aliquots of plasma were applied to 5 ml of immunoaffinity mixed gel containing 2 monoclonal antibody clones, Mab JI-H raised against human apoB-100 and Mab H-12 raised against human apoA-I (JIMRO) with gentle shaking for 2 h at room temperature. The unbound fraction was concentrated to the same volume of plasma used for the preparation of RLP with an Amicon Ultra filter (Millipore) for the gel filtration analysis.

2.6. Western blot analysis

The isolated RLP sample or plasma was subjected to 12% SDS-polyacrylamide gel electrophoresis and the proteins in the gel were transferred to a polyvinylidene difluoride membrane (ProBlott; Applied Biosystems) by electroblot. The membranes were blocked with 5% dry milk for 2 h and incubated with primary antibodies for 16 h. The membranes were then incubated with a second antibody conjugated with alkaline phosphatase for 2 h, then visualized using the nitro blue tetrazolium/5-bromo-4-chloro-3-indolylphosphate *p*-toluidine salt system. Anti-apolipoprotein A-I antibody (600-101-109) was from Rockland and anti-apolipoprotein B-100 antibody (20H-G1b) was from the Academy Bio-Medical Co.

2.7. Gel filtration chromatography

Gel filtration chromatography of the pre- and post-heparin plasma and RLP was performed at room temperature by fast protein liquid chromatography (FPLC) (Pharmacia LKB) to separate the lipoproteins in phosphate-buffered saline (PBS). The system was programmed (Liquid Chromatography Controller LCC-500 Plus) to load and separate samples on a 50-cm column (16 mm internal diameter) packed with cross-linked agarose gel (Superose 6B grade, Pharmacia). The column was eluted with PBS at a rate of 1.0 ml/min, and 25 min after addition of sample, 50 × 1 ml fractions were collected sequentially. Sample elution was monitored spectrophotometrically at optical density 280 nm. VLDL-, LDL-, and HDL-sized fractions were pooled according to the optical density elution profile.

2.8. Lipid and lipoprotein measurements

After a 12-h overnight fast, blood samples were taken, then plasma was isolated and stored at 4 °C. The following analytes were measured within 48 h of drawing the blood: total cholesterol (TC), triglycerides (TG), direct low density lipoprotein cholesterol (LDL-C), HDL-C and small dense LDL- cholesterol (sdlDL-C) (Denka-Seiken). Aliquots of serum were stored at −80 °C for subsequent determination of parameters. RLP-C and RLP-TG assay (JIMRO II) was obtained from Otsuka (Tokyo, Japan) [28]. ApoB100 ELISA from IBL and apoA1, apoC1 and apoC3 ELISA were purchased from Abnova. Lipid parameters were measured with a Hitachi 917 Analyzer.

2.9. Statistical analysis

Statistical analyses were conducted with SPSS II (ver. 11.0.1 J) and StatFlex ver. 6 (Artech). All values are expressed as means with standard deviations. Differences between groups were tested using the unpaired Student's *t*-test for normally distributed data and the Mann-Whitney *U* Test for skewed data. Pearson or Spearman analysis was used for parametric or non-parametric variables to determine the correlations between parameters. A $P < 0.05$ was considered statistically significant.

3. Results

3.1. Subjects

Table 1 shows the lipid and lipoprotein concentrations, RLP-TG/RLP-C ratio, apo CIII and LPL concentration in pre- heparin plasma. The data are presented as mean values with standard deviations. The results indicated that over 80% of the LPL in circulating pre-heparin plasma was found in the RLP fraction. The LPL concentration was significantly higher in plasma with TG < 150 mg/dl than TG ≧ 150 mg/dl ($p < 0.05$). This shows that the ratio of LPL bound to RLP (RLP-LPL/RLP-TG ratio) was significantly higher in the normal TG cases than in the high TG cases ($p < 0.01$). A larger number of LPL was bound to RLP in the normal TG plasma and hydrolyzed RLP more effectively than in high TG plasma.

Table 1
LPL concentration in plasma and RLP in cases with normal and high TG concentration.

	TG < 150 mg/dl (n = 154)	TG ≧ 150 mg/dl (n = 41)	*p* Value
TC (mg/dl)	200 ± 39	217 ± 41	<0.01
TG (mg/dl)	107 ± 30	253 ± 100	<0.01
LDL-C (mg/dl)	83 ± 27	98 ± 25	<0.01
HDL-C (mg/dl)	57 ± 13	46 ± 11	<0.01
sdldl (mg/dl)	23 ± 10	42 ± 14	<0.01
RLP-C (mg/dl)	4.9 ± 1.7	12.2 ± 4.9	<0.01
RLP-TG (mg/dl)	15.6 ± 10.5	63.2 ± 60.2	<0.01
Plasma LPL (ng/ml)	98 ± 27	80 ± 28	<0.05
RLP-LPL (ng/ml)	86 ± 25	67 ± 23	<0.05
RLP-LPL/Plasma-LPL (%)	87 ± 7	85 ± 13	N.S.
RLP-LPL/RLP-TG (%)	5.5	1.1	<0.01
ApoC-III (mg/dl)	7.6 ± 2.1	12.0 ± 3.3	<0.01

Table 2 presented the lipid and lipoprotein levels in the fasting, pre-heparin and post-heparin plasma (15 min after heparin administration) in the 29 subjects. Lipids, lipoproteins and the LPL concentration in plasma and in RLP were determined. Approximately 80% of the LPL concentration was found in RLP derived from circulating plasma (pre-heparin), while <30% of the LPL concentration was found in RLP in the post-heparin plasma. This means that >70% of the LPL concentration was detected in non-RLP fraction in the post-heparin plasma. The LPL concentration in post-heparin RLP increased approximately ≥2 fold compared to LPL in pre-heparin RLP. Significantly increased LPL bound to RLP (RLP-LPL) was found in the post-heparin plasma associated with a significant decrease in RLP-TG ($p < 0.01$). This means that LPL detached from the endothelium bound to RLP directly in circulating blood and generated smaller RLP particles (a reduced RLP-TG/RLP-C ratio).

3.2. Establishment of 2 kinds of highly sensitive and specific LPL sandwich ELISA methods

Two kinds of antibodies were used for the ELISA, the 57A5 and 88B8 antibodies against human recombinant LPL. Both of the 57A5 and 88B8 antibodies recognize the C-terminal region of the LPL protein. Neither monoclonal antibody by itself detects the monomeric recombinant LPL by the sandwich ELISA method, recognizing only the dimeric (or polymeric) LPL that is known to be the active form. Fig. 1 shows the significantly high correlation between the LPL activity & LPL concentration (mass) by 2 MoAb (57A5-88B8) ELISA (Fig. 1 (A) ($p < 0.001$) and 1 MoAb (88B8-88B8) ELISA (Fig. 1 (B) ($p < 0.001$). Also as shown in Fig. 1 (C), a significantly high correlation was found between the 1 MoAb and 2 MoAb assay in the post-heparin plasma ($p < 0.001$), indicating that the single MoAb-ELISA detected the dimeric or polymeric LPL in the active form. The 2 antibody ELISA detected all of the LPL, *i.e.* both the monomeric and dimeric LPL. As the recombinant LPL with a monomeric form did not react with this 1 MoAb (88B8-88B8) ELISA, this method allowed a specific assay for dimeric LPL detection in the plasma. Fig. 1 (D) shows the significantly high correlation between the 1 MoAb and

Table 2
LPL concentration in plasma and RLP fraction in pre- and post-heparin plasma of 29 healthy volunteers.

	Pre-heparin	Post-heparin	*p* Value
TC (mg/dl)	174 ± 33	169 ± 32	N.S.
TG (mg/dl)	139 ± 78	103 ± 58	<0.01
LDL-C (mg/dl)	102 ± 28	100 ± 27	N.S.
HDL-C (mg/dl)	49 ± 13	47 ± 12	N.S.
RLP-C (mg/dl)	7.2 ± 4.6	6.7 ± 4.1	N.S.
RLP-TG (mg/dl)	45 ± 37	26 ± 24	<0.01
RLP-TG/RLP-C (%)	6.0 ± 1.7	3.5 ± 1.4	<0.01
Plasma-LPL (ng/ml)	81 ± 27	438 ± 95	<0.01
RLP-LPL (ng/ml)	62 ± 26	117 ± 30	<0.01
RLP-LPL/Plasma-LPL (%)	77 ± 11	27 ± 5	<0.01
RLP-LPL/RLP-TG (%)	1.4 ± 0.7	4.5 ± 0.8	<0.01

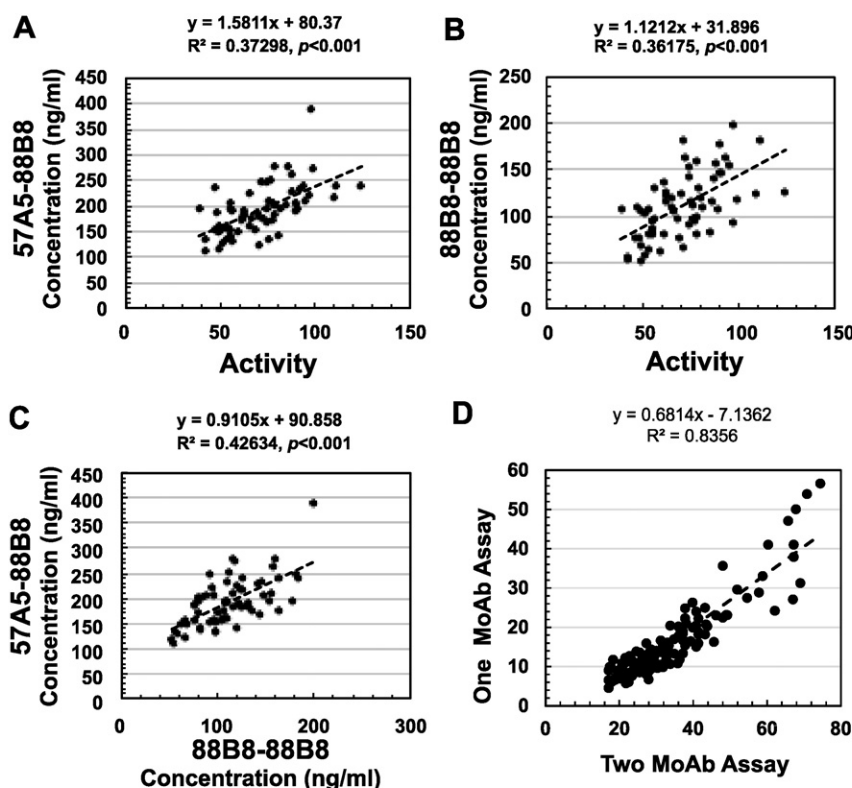

Fig. 1. Correlation between LPL activity and the LPL concentration (mass) along with a comparison of LPL-ELISA between the 1 MoAb and 2 MoAb systems. A. LPL activity *vs* 2 MoAb sandwich assay (57A5 and 88B8) in the post-heparin plasma. B. LPL activity *vs* 1 MoAb sandwich assay (88B8-88B8) in the post-heparin plasma. Comparison between 2 MoAb sandwich assay (57A5 and 88B8) and 1 MoAb sandwich assay (88B8-88B8) in the post-heparin plasma (C) and in the pre-heparin plasma (D). Two MbAb and 1 MoAb LPL sandwich assay were highly correlated ($p < 0.001$) in both pre-heparin and post-heparin plasma.

2 MoAb ELISA in the pre-heparin plasma as well ($p < 0.001$). Those data strongly suggest that most of the LPL in the pre-heparin plasma was in a dimeric form, although the LPL activity in pre-heparin plasma was undetectable (*i.e.* very low activity) as known by previous literatures. However, the absolute concentration of LPL determined by the 2 methods was not exactly the same, approximately 60% of LPL concentration was determined by the 1 MoAb-ELISA compared with the 2 MoAb-ELISA using the same LPL calibrator. The different reactivity of the antibodies to the LPL calibrator may have caused this difference, but the result showed significantly high correlation between the 2 LPL-ELISA methods. Therefore, we have concluded that most of the LPL in the post-heparin and pre-heparin plasma was in the dimeric form, as previously reported by Brunzell et al. [21].

3.3. Characteristics of the LPL dimers in plasma and in RLP after heparin administration in the presence and absence of THL

LPL dimers in the post-heparin plasma and in RLP fraction of a normal (Case 1) and a hypertriglyceridemic (Case 2) subject as representative cases were demonstrated in a time dependent manner after heparin administration. LPL dimers in pre-heparin plasma were >2-fold higher in Case 1 (low RLP-C) compared to Case 2 (high RLP-C). Post-heparin plasma was collected from the 2 cases at 0, 15, 30, 60 min and the LPL dimers (Fig. 2) were determined along with the lipid and lipoprotein profiles (Table 3). The plasma LPL dimers and activity (data not shown) increased most significantly at 15 min and then decreased in parallel to the basal levels (RLP-LPL levels). The *in vitro* addition of THL immediately after blood withdrawal did not affect the LPL dimer concentration, but it did completely inhibit the LPL activity. The TG and RLP-C levels significantly decreased, followed by an increase in the LPL dimers after heparin administration in Case 2, but did not show significant change in Case 1 because of the low concentrations of TG and RLP-C (Table 3). The LPL dimers in the RLP were also increased at 15 min (Fig. 2) as in the case of the plasma LPL, but they did not decrease as rapidly as LPL in plasma. Therefore, the half-life of LPL in RLP was much longer than that of the LPL bound to non-RLP fraction in plasma.

The LPL dimer immunoreactivity (concentration) determined in the post-heparin plasma (active form) was significantly decreased at 15 and 30 min after 2 h pre-incubation at 37 °C in the absence of THL (Fig. 3 (A)), which decreased almost to the LPL dimer levels in RLP. However, in the presence of THL, the immunoreactivity of the LPL dimers (inactive form) did not change after 2 h pre-incubation at 37 °C (Fig. 3 (B)). These results suggest LPL dimers in post-heparin plasma having activity *ex vivo* is easily degraded to the level in RLP in plasma, while the LPL dimers in RLP was resistant to degradation (Fig. 3 (C)). LPL dimers in the RLP remained unchanged at 37 °C pre-incubation for 2 h in the presence and absence of THL. Therefore, those results suggest that LPL dimers in RLP were inactive because of no effect of THL on degradation of LPL dimers. These results clarified that there were 2 kinds of LPL dimers in post-heparin plasma, active form in non-RLP fraction and inactive form in RLP.

Furthermore, LPL dimers in RLP in pre-heparin plasma were consistently increased approximately 10 to 30% after 37 °C incubation for 2 h. These results suggest the possibility that there were approximately 20% of LPL dimers bound to non-RLP in pre-heparin plasma, and shifted to RLP after 2 h incubation at 37 °C.

3.4. The LPL distribution and lipoprotein elution profiles obtained by gel filtration chromatography

An immuno-separation method was used to isolate RLP from the post-heparin and pre-heparin plasma in the presence or absence of THL to investigate which lipoprotein subclass LPL dimers bound. RLP

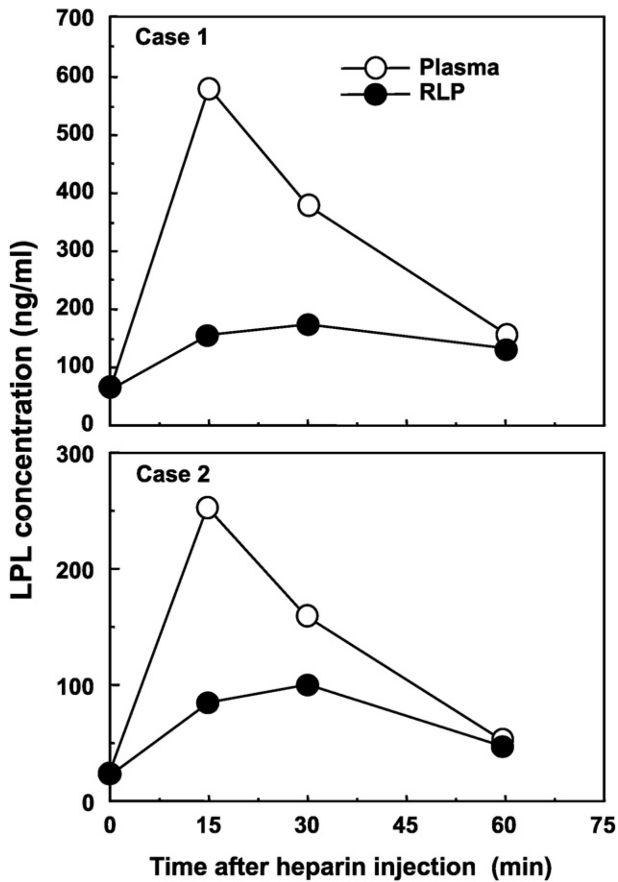

Fig. 2. LPL concentration in the post-heparin plasma and RLP at 0, 15, 30, 60 min after heparin administration in normal (case 1) and hypertriglyceridemic (case 2) cases. Plasma LPL and LPL in the RLP significantly high in case 1 than in case 2 and increased at 15 min after heparin administration, but decreased nearly to the basal level in 60 min.

contained apoB-100 and apoB-48 with a minor amount of apoA1, as shown by SDS-PAGE electrophoresis and Western blot analysis in the presence or absence of heparin administration (Fig. 4 A)

To demonstrate the effect of *ex vivo* lipolysis in the presence and absence of THL on the LPL distribution across the entire lipoprotein elution range, the post-heparin plasma was analyzed by Superose 6B gel filtration. An aliquot of post-heparin plasma was collected in the presence of THL and applied to the FPLC column. As shown in Fig. 5 (A), a large amount of the LPL dimers was found in the VLDL elution range, with minor peaks in the LDL and HDL elution range. However, in another aliquot of post-heparin plasma in the absence of THL (Fig. 5 (A)), a small amount of the LPL dimers was found in the VLDL elution range, with major peaks shifted to the HDL elution range. No significant amount of free LPL protein was found in plasma both in the presence and absence of THL.

The RLP fractions isolated from the post-heparin plasma by immunoaffinity gel in the presence or absence of THL were also applied to the FPLC column (Fig. 5 (B)). In the presence of THL, a large amount of the LPL dimer was found in the VLDL elution range with minor LDL and HDL elution range peaks, a pattern which was very similar with the elution profile in the post-heparin plasma. However, in the absence of THL (Fig. 5 (B)), no LPL dimers were found in the VLDL elution range, with a certain amount in the HDL elution range. These results suggest that THL inhibited LPL activity *ex vivo* and significantly affected the elution profiles of the LPL dimers in RLP as in plasma. The reason why the LPL dimers in RLP were significantly decreased in the absence of THL was probably due to the conformational changes of RLP during the isolation procedure from plasma at room temperature; the dissociation of apoE or other apolipoproteins from RLP, which blocks the epitope of anti-apoB100 (JI-H) antibody [27]. Therefore, LPL dimers in RLP decreased significantly with the decrease of RLP (apoB100) shown as gel filtration profile (Fig. 4 C).

The pre-heparin plasma and RLP isolated from the pre-heparin plasma in the presence and absence of THL were applied to the FPLC column (Fig. 5 (C) and (D)). A small amount of the LPL dimers both in the plasma and RLP in the presence and absence of THL were found very similar in the VLDL, LDL and HDL elution range with a broad profile of diffusion. These elution profiles were similar to the ones of the RLP isolated from the post-heparin plasma in the absence of THL. No effect of the presence of THL on *ex vivo* lipolysis was found both in the pre-heparin plasma and RLP. Because the LPL dimers in the pre-heparin plasma had negligible LPL activity *ex vivo*, the isolation procedure of the RLP by the immunoaffinity gel did not affect the elution profiles of the LPL dimers. Free LPL dimers, unbound to the lipoproteins, were not found in the elution range of low molecular weight by this gel filtration method in pre-heparin plasma.

3.5. Inhibition of LPL activity by apoC1 and C3 in the post-heparin plasma

RLP has been reported to be apoC3 and apoE-rich in cases of increased RLP-C, but low apoC3 level in RLP in cases of normal RLP-C [29]. Fig. 6 shows that apoC1 (A), apo C3 (B) and apoE (C) in RLP were found in the VLDL and apoE-rich HDL elution ranges by gel filtration chromatography. Although apoE-rich HDL is contained in RLP, LPL dimers were not found in this fraction in the presence of THL as show in Fig. 5 (B). Also the presence of THL did not affect the elution profiles of these apolipoproteins.

LPL activity was not detected in RLP isolated from the post-heparin plasma as well as in pre-heparin plasma (Table 3). Table 3 also indicates that the addition of apoC1 and apoC3 in the LPL activity assay system [26] with the same concentration (5 μg) of apoC2 (activator) inhibited LPL activity, specifically strong inhibition was observed by apoC1. These results suggest the possibility that the LPL dimers in RLP are inactive because of the presence of co-localized inhibitors such as apoC1 and C3 in the remnant lipoprotein particles (Fig. 7).

4. Discussion

We report LPL binding directly to plasma remnant lipoprotein particles (RLP-LPL complex) which we have reported as RLP since 1993 [27–31]. In particular, we used THL for the inhibition of the *ex vivo* lipolytic activity of LPL to minimize the risk of artifacts after blood withdrawal. The results show the following. 1) More than 80% of circulating LPL in pre-heparin plasma was found in RLP as RLP-LPL complex. 2) The circulating LPL found in RLP in the pre- and post-heparin plasma was shown as LPL dimers but in the inactive form. 3) When lipolytic activity was inhibited by THL in the post-heparin plasma, most LPL dimers were found in the VLDL elution range, specifically in the RLP. When the *ex vivo* lipolytic activity was not inhibited by THL, the LPL dimers became transitional in the post-heparin plasma and shifted rapidly to the HDL elution range. 4) Two different type of LPL dimers were found in post-heparin plasma; one is active form found mostly in HDL elution range, the other is inactive form found in RLP. 5) The proposed model of the RLP-LPL complex shows that when LPL is detached from the endothelium surface, LPL is mostly released as the complex with RLP and inhibitors (apoC1, C3) into the blood. The results reported

Table 3
Plasma TG and RLP-C concentration in post-heparin plasma at 0, 15, 30, 60 min after heparin injection.

	Plasma-TG (mg/dl)				RLP-C (mg/dl)			
Time (min)	0	15	30	60	0	15	30	60
Case 1	64	48	44	50	3.3	3.0	3.2	3.4
Case 2	213	100	120	160	25.3	11.1	14.2	20.4

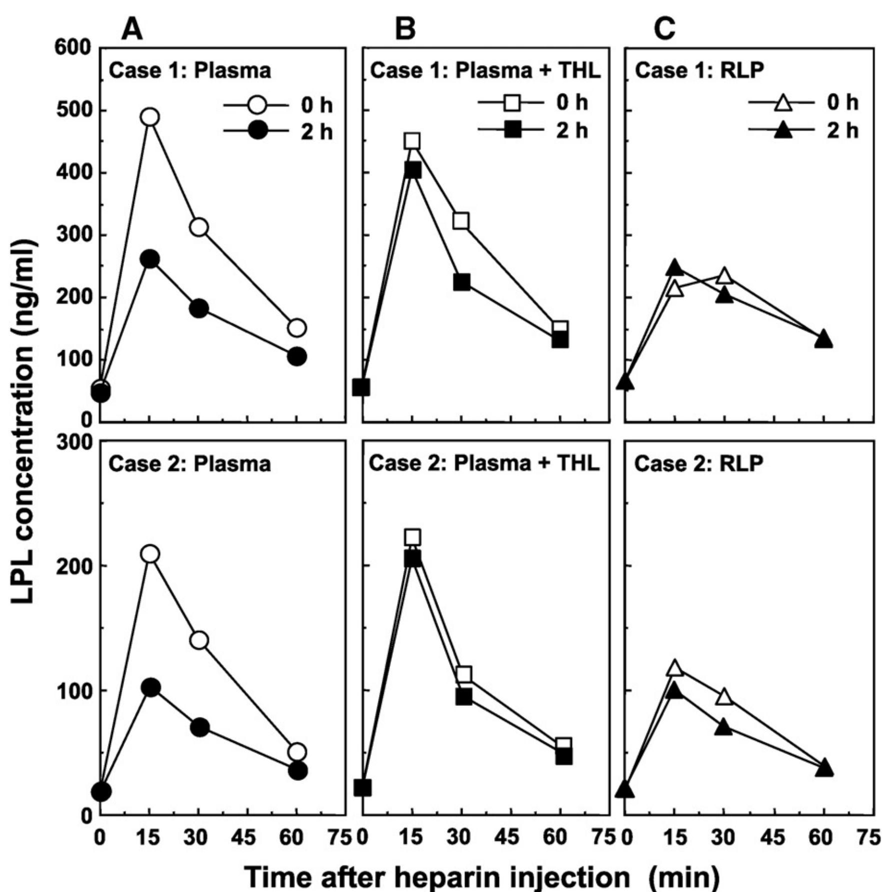

Fig. 3. The LPL concentration in the post-heparin plasma and RLP with and without pre-incubation at 37 °C for 2 h. As shown in (A), LPL in total plasma, after 2 h 37 °C incubation, LPL levels significantly decreased at 15 and 30 min. However, in the present of THL, the decrease of plasma LPL was inhibited (B). LPL in RLP did not change in the presence or absence of THL after 2 h 37 °C incubation (C). Plasma LPL levels after 2 h 37 °C incubation decreased almost to the same level with LPL in RLP.

here provide a new insight into LPL in the circulating plasma and suggest the need of a new definition of remnant lipoproteins in the plasma. There has been no report that LPL is a specific component of remnant lipoproteins isolated from both pre- and post-heparin plasma.

Post-heparin plasma has been convenient means of measuring LPL activity for a diagnostic purpose, because it has been difficult to measure the LPL protein or identify its different plasma forms until recently. Therefore, previous studies have been focused on the measurement of LPL catalytic activity after heparin administration. However, the LPL activity in the post-heparin plasma has been known to be generally unstable and non-physiological compared to the LPL protein concentration in pre-heparin plasma. Recently, Shirakawa et al. [32,33] reported a comparative study of the LPL activity and mass (concentration) in the pre- and post-heparin plasma, suggesting that the determination of the LPL concentration in the pre-heparin plasma may take the place of LPL activity and mass in the post-heparin plasma for clinical diagnostic purposes. This is also supported by other previous reports as well [34,35].

In this study, we first investigated the protein structure of LPL, *i.e.*, whether LPL is dimer or monomer, in the pre-and post-heparin plasma, using a newly developed sandwich ELISA made up of a 1 MoAb and 2 MoAb system. In the post-heparin plasma, the majority of the LPL protein is known to be in the active, dimeric form, but most of the LPL protein in the pre-heparin plasma has been known to be inactive form [36, 37]. A single MoAb sandwich ELISA was unable to detect monomeric LPL, detecting only dimeric (or polymeric if any) LPL, which is known to be the active form. A significantly high correlation was found between the 1 MoAb and 2 MoAb ELISA and activity assays in the post-heparin plasma. Moreover, as shown in Fig. 1 (D), even in the pre-heparin plasma, the 1 MoAb ELISA was significantly correlated with the 2 MoAb ELISA. These findings suggest that most of the LPL in the pre-heparin plasma as well as the post-heparin plasma are in a dimeric form. These findings are important because no reports have previously described the predominant presence of monomer, polymer or degraded LPL in plasma under physiological conditions. These results are also in agreement with those of Brunzell et al. [21,36], who reported previously that most LPL in plasma was in the dimeric form by using an ELISA with 5D2 and 5F9 monoclonal antibodies. Although the antibodies (57A5 and 88B8) we used in this study were different from Brunzell's antibodies, the recognition site of antibodies were similar at C-terminal. Therefore, we have used the term “LPL dimers” to represent the “LPL concentration” in this manuscript. Furthermore, we used mostly the 2 MoAb sandwich ELISA in this study, because the assay was highly sensitive and was able to determine an LPL concentration of >20 pg/ml, which made it possible to determine the LPL dimers in the fractionated gel filtration chromatography aliquots.

RLP isolation was performed by an immunoaffinity gel separation method [27]. The lipoproteins isolated by this method exhibit the typical characteristics of remnant lipoproteins [31], but there has been a need to explain more specific characteristics of remnant lipoproteins. As predicted by Felts et al. [15], the direct binding of LPL with RLP in plasma would provide the most convincing proof that RLP is identical with the circulating remnant lipoproteins in the plasma. RLP as “Remnant like-Lipoprotein Particles” were first named by Havel (1993), a term meant to designate TG-rich lipoproteins with apoE-rich, apoC3-rich and cholesteryl-ester rich VLDL [27–31]. The presence of LPL has never been included as a component of remnant lipoproteins. Therefore, this study has added a new characteristic of remnant lipoproteins that majority of the LPL in the circulating plasma is RLP-bound, particularly in the pre-heparin plasma. As LPL is known to bind to apoB-containing lipoproteins with a higher affinity in VLDL than LDL [21,

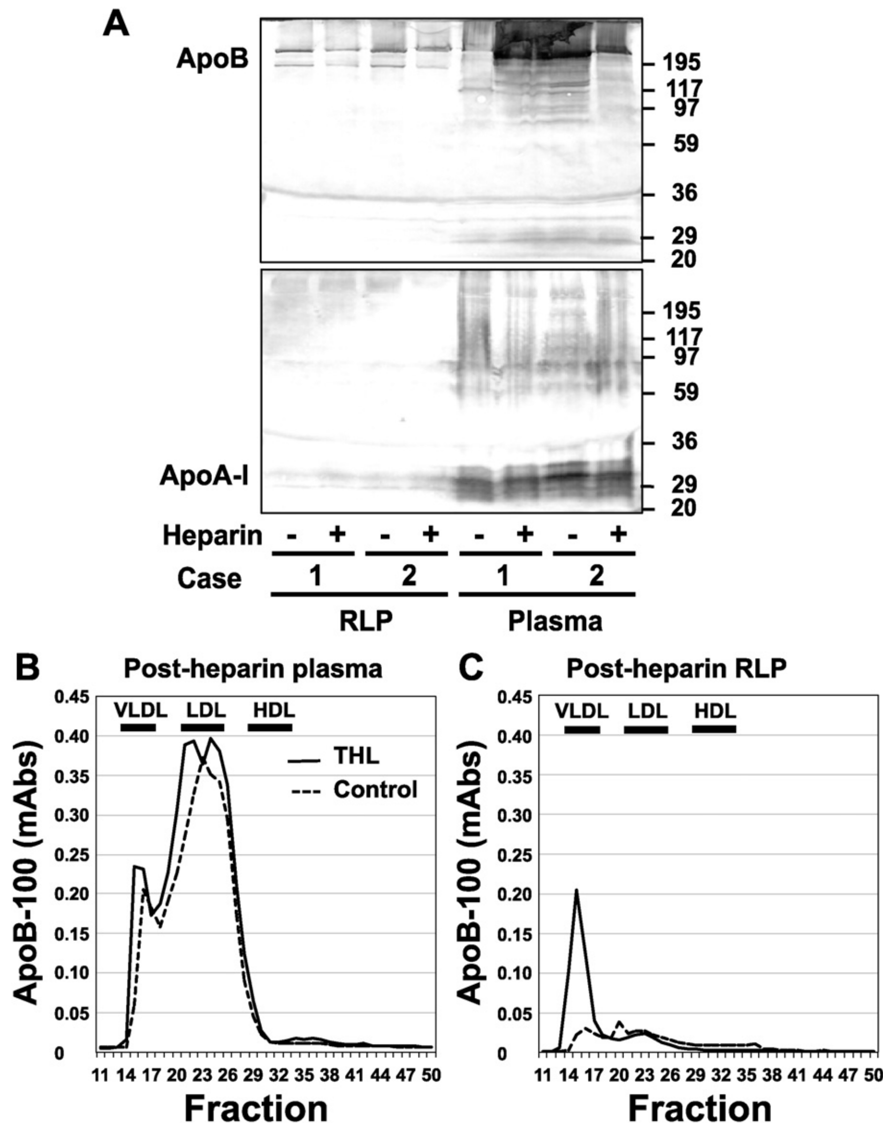

Fig. 4. Isolation of remnant lipoproteins (RLP) by immuno-affinity gels and Western blot analysis. A. The isolated RLP sample or plasma was subjected to 12% SDS-polyacrylamide gel electrophoresis and the proteins in the gel were transferred to a polyvinylidene difluoride membrane by electroblot. The membranes were incubated with primary antibodies for 16 h. The membranes were then incubated with a second antibody conjugated with alkaline phosphatase for 2 h, then visualized using the nitro blue tetrazolium/5-bromo-4-chloro-3-indolylphosphate *p*-toluidine salt system. Anti-apoA-I antibodies and anti-apoB-100 antibodies were used for the immunostaining. As shown in (A), apoB100 and apoB48 were mainly detected in RLP and a small amount of apoA1 was also detected in RLP in the presence and absence of heparin. B and C. Gel filtration chromatography of the post-heparin plasma (B) and RLP (C) in the presence and absence of THL was performed to separate the lipoproteins and monitored by apoB100 ELISA. ApoB100 was detected largely in LDL elution range in post-heparin plasma in the presence and absence of THL. However, apoB100 in RLP was found as the major peak in the VLDL elution range in the presence of THL as previously reported.

37], the presence of LPL in RLP could account for the characteristics of remnant lipoproteins in general. However, as the binding of LPL to TG-rich lipoproteins in plasma is very weak and labile *in vitro*, it was difficult to detect LPL bound to RLP directly by an electrophoretic method even with THL. LPL was easily dissociated from RLP under electrophoresis. Therefore, gel filtration chromatography was used to isolate the RLP and determine the presence of LPL bound to RLP by a highly sensitive ELISA method.

The main aim of the present study was to reproduce as accurately as possible the RLP profile after lipase displacement from the arterial wall by preventing *ex vivo* TG-rich lipoprotein hydrolysis by LPL. Previous reports [12–14] have suggested that LPL in the post-heparin plasma is mostly associated with cholesterol-rich lipoproteins such as LDL and HDL. To investigate the hypothesis that these observation may be affected by *ex vivo* lipolysis, the LPL distribution across the entire lipoprotein size range was evaluated in the post-heparin plasma. LPL dimers were found associated with cholesterol-rich lipoproteins such as are typically found in the HDL elution range. The results shown in Fig. 5 in the absence of THL match the observations first reported by Goldberg et al. [12] and then by Vilella et al. [14], which indicate that, in the post-heparin plasma, LPL is predominantly associated with LDL and HDL. It has long been known that lipolysis is initiated intravascularly and continues *ex vivo* [36], particularly in the post-heparin plasma, when the amount of active LPL is increased several hundred fold. These results were also confirmed by Zambon et al. [20,21], emphasizing the need for a complete inhibition of lipolytic activity in studies dealing with post-heparin lipoprotein metabolism in order to minimize the risk of *ex vivo* artifacts. In the present study, the active-site inhibitor of LPL and THL was used. THL almost completely inhibited lipolytic activity in the post-heparin plasma. Moreover, THL did not affect the lipoprotein elution profile by gel filtration when added to the post-heparin plasma [21]. Hadvary et al. [22] and Looken et al. [23] suggested that LPL inhibition is due to the covalent binding of THL to Ser 132, which is 1 of the residues of the catalytic triad of this enzyme. Looken et al. [23] also reported that the binding of THL to LPL makes more stable and causes the enzyme to form tetramers. However, we did not find the significant

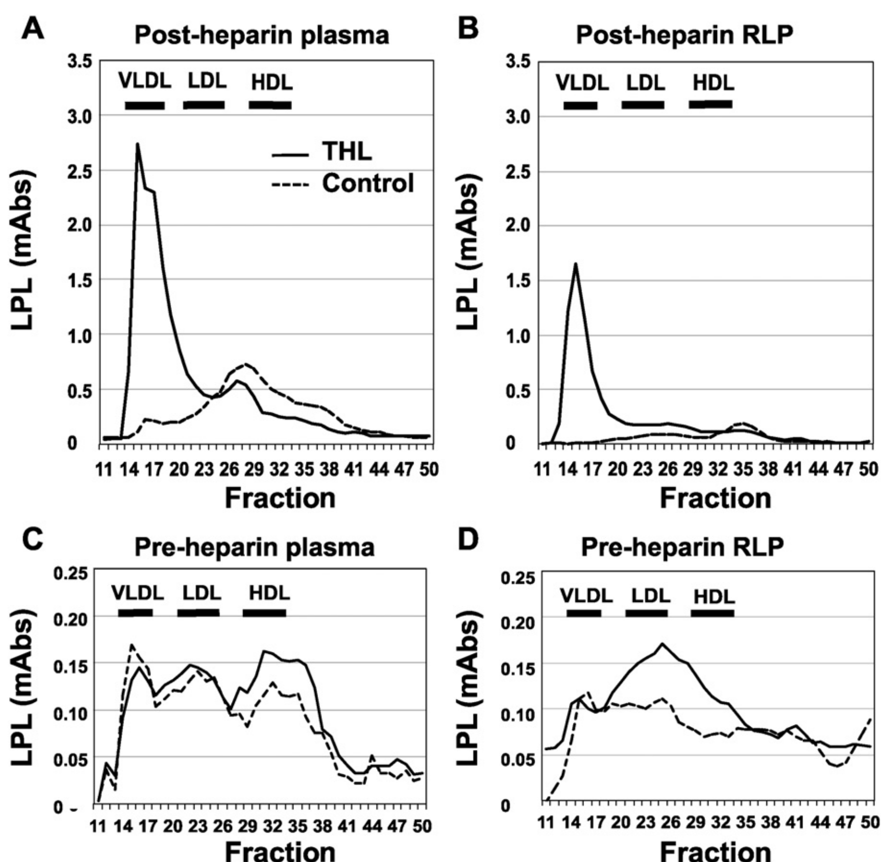

Fig. 5. Effect of an LPL inhibitor (tetrahydrolipstatin: THL) on the gel filtration profiles of LPL in the post-heparin plasma and RLP. A. A large amount of the LPL dimers was found in the VLDL elution range, with minor peaks in the LDL and HDL elution range in the presence of THL. However, in the absence of THL, a small amount of the LPL dimers was found in the VLDL elution range, with major peaks in the HDL elution rang. B. The RLP, in the presence of THL, a large amount of the LPL dimer was found in the VLDL elution range with minor LDL and HDL elution range peaks, a pattern which was very similar with the elution profile in the post-heparin plasma in the absence of THL, however no LPL dimers were found in the VLDL elution range, with a certain amount in the HDL elution range. A small amount of the LPL dimers in the plasma (C) and RLP (D) in the presence and absence of THL were found in the VLDL, LDL and HDL elution range with a broad pattern of diffusion. These elution profiles are similar to the ones of the RLP isolated from the post-heparin plasma in the absence of THL.

effect of THL on the determination of LPL concentration by ELISA in the pre- and post-heparin plasma. Further studies by Heeren et al. [38] and Zeng et al. [39] used THL for the analysis of postprandial TG-rich lipoproteins and LPL.

As shown in Fig. 5, the elution profile of the RLP-LPL complex was significantly different between the post-heparin and pre-heparin plasma as well as RLP in the presence of THL. However, RLP-LPL elution profile looked similar in all the cases in the absence of THL. Those cases seemed to show the final metabolized RLP profiles predominantly existed in the LDL and HDL elution range. THL inhibited *ex vivo* LPL activity in the post-heparin plasma maintained an elution profile of RLP-LPL complex in the VLDL elution range. These results show that active LPL dimers are easily dissociated from VLDL and shifted to the HDL elution range, having *ex vivo* lipolytic activity continuously. LPL dimers having the inactive form of LPL remained in the RLP. Therefore, the LPL dimers in the RLP have no further activity for lipolysis, as shown with the isolated RLP in the *in vitro* LPL activity assay (Table 4). As no significant effect of THL on the elution profile in the pre-heparin plasma or RLP was observed, it was suspected that the pre-heparin plasma or RLP do not contain active LPL dimers. However, approximately 20% of LPL dimers in pre-heparin plasma seemed to be active form. Because after 2 h incubation of pre-heparin plasma at 37 °C, LPL dimers in RLP increased consistently together with the increase of FFA (data not shown). As Brunzell et al. described [21,37], there are still a small amount of active LPL dimers in pre-heparin plasma.

RLP-LPL complex elution profile in the post-heparin plasma in the presence of THL was similar to the elution profiles for TC and TG [27, 28]. However, the elution profile of RLP-LPL in the pre-heparin plasma was evidently different, as shown Fig. 5 (C, D). A greater amount of LPL dimers in RLP detected in the HDL range than in the VLDL range, indicating that smaller RLP particles carried a larger amount of the LPL dimers than larger RLP particles, which is the opposite profile for TC and TG. Unlike LPL, we have not found the presence of hepatic triglyceride lipase (HTGL) in the form of RLP-HTGL complex in the VLDL elution range in the post-heparin plasma. HTGL was found mostly at apoE-rich HDL range in the presence and absence of THL (manuscript in preparation). Together with our previous report [32,40], it appears that HTGL may not play as important a role in remnant metabolism as LPL, although it has been generally believed that HTGL hydrolyzes small remnants to LDL.

After the reduction in the TG content by LPL, the CM and VLDL particles have been generally believed to become smaller VLDL particles such as intermediate density lipoproteins (IDL) as remnants. However, several studies, including our own [32,39,41], reported that large VLDL particles were the major structural components of remnant lipoproteins. If RLP were homogeneously metabolized TG-rich lipoproteins, the particle size would be smaller than VLDL. However, RLP was predominantly of large VLDL size, which remained without further hydrolysis by the dimeric but inactive LPL. As RLP bears apoC1 and C3 [29], LPL activity may be inhibited by the presence of these apolipoproteins on the particles as shown in Table 4. ApoC1 and C3 may be transferred from HDL particles with apoE at the time or after remnant generation on the endothelium. Although the inhibition of LPL by apoC1 and C3 has been reported [42–50], it is not yet clear whether LPL dimers in RLP are rendered inactive by those inhibitors *in vivo*. Once the inhibitors on RLP disturb the lipolytic activity of LPL, large and fixed sized VLDL

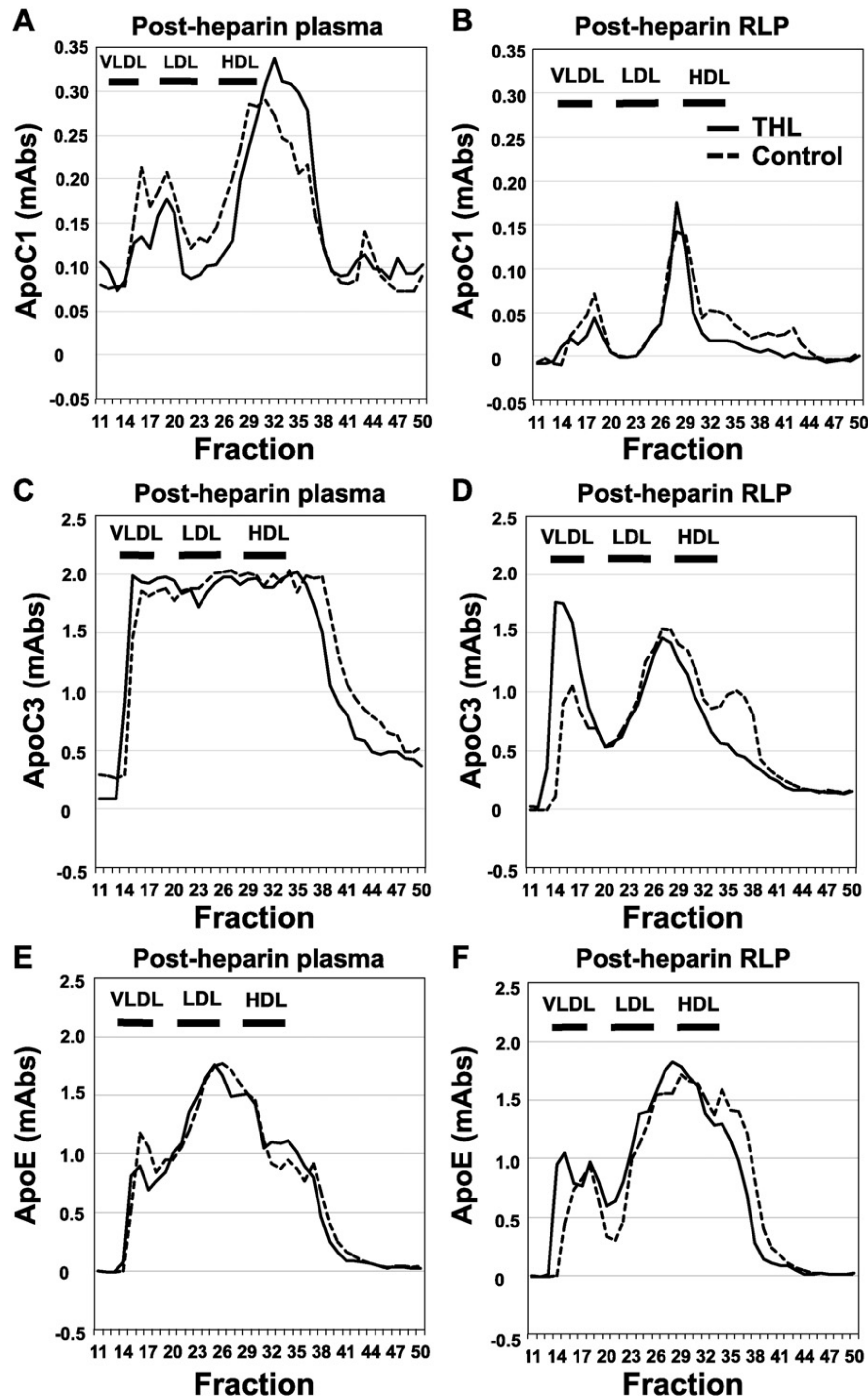

Fig. 6. ApoC1, apoC3 and apoE distribution across the lipoprotein elution range in the post-heparin plasma and RLP, with and without tetrahydrolipstatin (THL). A. ApoC1 elution range in post-heparin plasma, B. ApoC1 elution range in post-heparin RLP, C. ApoC3 elution range in post-heparin plasma, D. ApoC3 elution range in pre-heparin RLP, E. ApoE elution range in post-heparin plasma, F. ApoE elution range in post-heparin RLP.

particles may remain without being fully metabolized and are then released into the circulation as remnant lipoproteins along with the inactive LPL dimers and its inhibitors. Inhibition of the lipolytic activity of LPL by THL seems to be a similar phenomenon as that of the inhibition of LPL by apoC1 or apoC3 in RLP, specifically high inhibition of LPL activity by apoC1.

Table 1 showed that the cases with TG > 150 mg/dl carry significantly less amount of LPL than in cases with TG < 150 mg/dl. Namely, a smaller amount of LPL dimers are carried by larger RLP particles compared to a smaller RLP particles which carry a larger amount of LPL dimers *per se*. We reported previously that the LPL dimers in plasma were inversely correlated with the RLP concentration and particle size in patients with diabetes and MetS [32,33]. As the RLP-TG/RLP-C ratio reflects the RLP particle size [51], we also reported the finding of a significant increase of the RLP-TG/RLP-C ratio after a fat load [52,53]. This may be associated with the fact that LPL activity in the postprandial plasma did not increase subsequent to the increase of RLP-C and RLP-TG [24]. This means that the RLP particle size significantly increased after the fat load because of the insufficiency of LPL enzyme activity, *i.e.* an inadequate capacity to hydrolyze the increased lipoprotein particles such as CM and VLDL on the endothelium in the postprandial plasma.

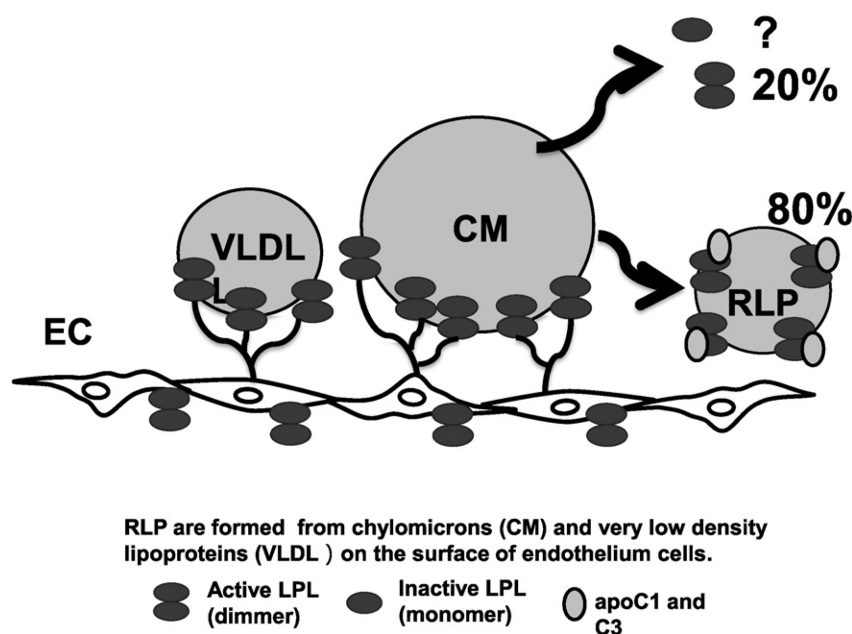

Fig. 7. After the hydrolysis of CM and VLDL, most of the LPL is dissociated from endothelium into circulation as the LPL- RLP complex with dimeric but inactive form, possibly by the presence of apoC1 and C3. A small amount (approximately 20%) of LPL bound to non-RLP fraction (possibly an active form) were also found in pre-heparin plasma.

The binding of LPL dimers to RLP with inactive form in the pre-heparin plasma likely has important implications for the physiological role of this enzyme as a multifunctional protein. Because of its ability to simultaneously bind to the receptors and heparin-like substances, LPL is able to efficiently mediate binding of lipoproteins to the cell surface and to both of the endocytotic receptors, LRP-1 [16,17] and the VLDL receptor [18]. Nykjaer et al. [19] found that the mediation of the binding of lipoproteins to LRP-1 resided only with the LPL dimer. Moreover, other reports have demonstrated that inactive LPL dimers enhances the binding of TG-rich lipoproteins and β-VLDL to cell-surface proteoglycans as well as LRP-1 [19,54,55]. It is therefore possible that the LPL dimers associated with RLP particles play a physiological role *in vivo* in the receptor-mediated remnant catabolism by affecting the binding of RLP to LRP-1 and the VLDL receptor. RLP is known to be incorporated into macrophages and the VLDL receptor without the additional oxidation like as has been reported for LDL [56,57].

On the contrary as shown in Table 2, significantly increased LPL levels in RLP compared to the pre-heparin levels were found in 15 min after heparin administration associated with the decrease of RLP-TG and the RLP-TG/RLP-C ratio. This means that active LPL dimers detached from endothelium after heparin administration bound to circulating RLP directly and hydrolyze RLP in blood, not on endothelium. Because under such circumstances, RLP-C levels did not change, indicating that LPL dimers were not additionally detached from the endothelium with newly generated RLP. This RLP-LPL complex found in circulating blood may be the first enzyme-substrate complex isolated from the post-heparin plasma.

We have further investigated the biochemical characteristics of LPL dimers in plasma and RLP in the post-heparin plasma. The plasma LPL dimers were increased significantly 15 min after heparin administration and returned to nearly the basal level (the pre-heparin level) after 60 min in Japanese subjects. The LPL concentration and activity in plasma increased and decreased in parallel, but LPL activity in the presence of THL was inhibited completely without concentration change in the LPL dimers. The half-life of LPL in RLP was much longer than that of total LPL in plasma. Interestingly, the immunoreactivity (or concentration) of the LPL dimers detected in the post-heparin plasma was significantly decreased after the addition of 2 h pre-incubation at 37 °C in the absence of THL, while in the presence of THL the immunoreactivity of the LPL dimers did not change under the same conditions. However, the immunoreactivity of the LPL dimers in RLP did not change both in the presence and absence of THL after the addition of 2 h pre-incubation at 37 °C. These results suggest that LPL dimers in the active form in the post-heparin plasma are easily degraded *ex vivo*, while the LPL dimers in RLP in the inactive form are resistant to *ex vivo* degradation. Therefore, it has become clear that 2 different types of LPL dimers exist in post-heparin plasma; one is active form mostly in HDL elution range, the other is inactive form in RLP. Inactive LPL dimers in RLP with apoC1 and C3 *in vivo* may become stable as in the presence of THL and play the role of interacting with the LRP-1 and/or VLDL receptor effectively. Active LPL dimers bound to non-RLP may not exist in plasma, because the active LPL seems to be unstable at 37 °C *in vivo*. These results suggest the possibility that the measurement of the LPL activity or concentration in the post-heparin plasma does not appropriately reflect the physiological conditions, because there are 2 types of LPL dimers and the active form of LPL dimers is easily degraded *in vivo* and *in vitro*.

In conclusion, the present study shows that most of the LPL in the pre-heparin plasma was in the dimeric form and bound to RLP particles as the inactive form. We isolated the newly generated RLP-LPL complex from the plasma after heparin administration by inhibiting the *ex vivo* lipolysis of LPL in the presence of THL. Furthermore, the RLP-LPL complex isolated from the pre-heparin and post-heparin plasma was found inactive, bearing apoC1 and apoC3. Inactive LPL dimers in RLP may be important ligand to interact with receptors such as LRP-1 or VLDL efficiently *in vivo*.

Therefore, we propose that the TG-rich lipoproteins bound to LPL dimers with inactive form is the new definition of remnant lipoproteins.

Table 4
Inhibition of LPL activity by apoC1 and C3 in post-heparin plasma.

	LPL activity (%)
Control	100
Apo C1	10.3 ± 4.3
Apo C3	68.4 ± 9.8
RLP	Undetected

Also the pre-heparin plasma LPL measurement may be more clinically appropriate for the diagnosis of TG-rich lipoprotein metabolism than LPL in the post-heparin plasma.

Acknowledgements

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