Human lipoprotein lipase: relationship of activity, heparin affinity, and conformation as studied with monoclonal antibodies

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Abstract The objective of this study was to investigate how a conformational change in lipoprotein lipase (LPL) affects its molecular functions. Monoclonal antibodies (MAbs) were raised against purified bovine milk lipoprotein lipase. MAb 5D2 bound to human and bovine LPL both before and after denaturation of LPL. MAb 5F9 also recognized LPL from both species, but only after denaturation of the antigen, suggesting that a conformational change led to exposure of a previously hidden epitope. The MAbs were used in two sandwich enzyme-linked immunosorbent assays (ELISAs). One ELISA used the same MAb (5D2) to coat the plate and detect the bound antigen. This ELISA thus required the same epitope to be present in duplicate for detection (as would be the case with a dimeric antigen). The second ELISA used MAb 5F9 to coat the plate and MAb 5D2 to detect the antigen. This ELISA detected LPL only after it had been denatured. By measuring the same sample before and after denaturation with guanidine hydrochloride (GuHCl) in the 5F9 ELISA, and subtracting one from the other, a measure of native LPL was obtained. In inactivation experiments using human LPL, activity and the measure of LPL mass obtained in the 5D2 ELISA decreased and were related inversely to the measured mass obtained in the 5F9 ELISA which increased, indicating that loss of activity is closely linked to dimer dissociation and loss of native conformation. The effect of conformation and dimeric structure on LPL-heparin interaction was studied by heparin-Sepharose chromatography. LPL activity and LPL dimer co-eluted at a NaCl concentration of 1.1 M, and inactive, conformationally altered LPL eluted at 0.75 M NaCl. The data show that dissociation of human LPL dimer into monomer is closely coupled to loss of activity and a conformational change, and that the partially denatured LPL monomer has decreased affinity for heparin. With the present methods it was not possible to determine whether the conformational change recognized by 5F9 preceded or was secondary to dissociation of dimer to monomers.—**Peterson, J., W. Y. Fujimoto, and J. D. Brunzell.** Human lipoprotein lipase: relationship of activity, heparin affinity, and conformation as studied with monoclonal antibodies. *J. Lipid Res.* 1992. **33:** 1165–1170.

Supplementary key words lipoprotein lipase activity • mass monoclonal antibodies • ELISA

Lipoprotein lipase (LPL) hydrolyzes triacylglycerols (TG) from TG-rich lipoproteins, thereby making free fatty acids available for tissue uptake and subsequent utilization or storage. Through its action on TG-rich lipoproteins it gives rise to remnant particles and redundant surface material which is incorporated into high density lipoproteins (HDL). LPL exerts its main function at the capillary endothelium where it is bound to sulfated proteoglycans. Protruding into the blood stream, it interacts with substrate lipoproteins and its activator apolipoprotein C-II.

Analytical ultracentrifugation studies (1, 2) and a study using radiation inactivation (3) of LPL purified from bovine milk indicated that the smallest active enzyme species is a noncovalent homo-dimer. However, using LPL from human postheparin plasma for gel filtration (4), and monoclonal antibody studies, Ikeda et al. (5) concluded that LPL monomer is the active molecular species in humans. In the present study we have utilized a previously reported ELISA that recognizes LPL dimer (6) and a new ELISA, that only recognizes partially denatured LPL to determine which is the smallest active species and to probe the relationship of activity, native conformation, and dimer to monomer dissociation.

METHODS

Subjects

Pre- and postheparin plasma was obtained from normal adult human males, aged 30 to 55, on no medications.

Abbreviations: LPL, lipoprotein lipase; TG, triacylglycerol; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; HL, hepatic lipase; GuHCl, guanidine hydrochloride; TBS, Tris-buffered saline; MAb, monoclonal antibody.

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Monoclonals

LPL was purified from bovine milk as described (7). Mice were immunized and hybridomas prepared according to Campbell (8). Positive clones were detected by their ability to bind to immunoplates precoated with purified bovine LPL. The MAb-producing hybridomas were implanted intraperitoneally into nude mice and the ascites fluid that developed was collected and stored at −70°C. The antibodies used in this study (5D2 and 5F9) were chosen based on their ability to react with human LPL. On immunoblotting of spontaneously appearing bovine LPL fragments, the two MAbs reacted with different fragments indicating that they recognize two different epitopes.

ELISA

Monoclonal antibodies were purified from mouse ascites fluid by caprylic acid precipitation (8) and stored at an approximate concentration of 1 mg/ml in PBS at −70°C. To coat microtiter plates (Polysorp, 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 containing Tween-20 (0.05% v/v), sealed, and stored at 4°C. Coated plates were used within 2 weeks. Purified bovine LPL was stored in 50% glycerol at −20°C, and diluted in PBS containing glycerol (15% v/v), Tween-20 (0.1% v/v), BSA (50 μg/ml), and heparin (1 mg/ml) (dilution buffer), to obtain a standard curve from 0 to 40 ng/ml. Samples were also diluted in dilution buffer to fall within the range of the standard curve. Aliquots from a large pool of postheparin plasma, stored at −70°C, were used as positive controls in all experiments. A volume of 200 μl was added to each well, and the plates were sealed. After 16 h incubation at 4°C, the wells were emptied and washed four times. 5D2 MAb conjugated to horseradish peroxidase in PBS containing Tween-20 (0.1% v/v) was added and the plate was incubated at room temperature for 4 h. After 5 washes with *o*-phenylenediamine, substrate in citrate buffer (pH 5.5) containing H_2O_2 (0.006%) was added. Plates were developed for 7 min (5F9) or 20 min (5D2). Samples, standards, and controls were always run in at least triplicate. The coefficient of variation between assays was 10% for both ELISAs and within assays less than 2% for both ELISAs.

Lipase activity measurements

Lipase activity was measured against a phosphatidylcholine-stabilized radioactively labeled triolein emulsion; the released fatty acids were extracted and counted in a beta scintillation counter as described (7). Selective measurement of LPL and hepatic lipase activity was achieved by inhibition of LPL activity by 5D2 MAb (5).

Heparin-Sepharose chromatography

Chromatography was performed at 4°C. A 10-ml column of heparin-Sepharose was equilibrated with 10 mM phosphate buffer, pH 7.5, containing glycerol (30% v/v), CHAPS (0.1% w/v), and NaCl (0.5 M). Prior to application, the sample was made 0.1% (w/v) with respect to deoxycholate by addition of an appropriate volume of 10% deoxycholate. Solid NaCl was added to give a final concentration of 0.4 M. The sample was applied at a flow rate of 0.2 ml/min, the column was washed with equilibration buffer at a flow rate of 0.4 ml/min, and eluted with a linear NaCl gradient from 0.4 to 1.8 M. LPL mass, lipase activity, and conductivity were determined in each fraction. Recovery of lipase activity was between 65 and 85%.

Immunoprecipitation and Western blot

MAb 5D2 was coupled to a gel matrix (Actigel Ald, Stratogene Bioseparation, Arcadia, CA) according to the manufacturer's instructions. Coupling was carried out in PBS, pH 7.5, at room temperature. More than 80% of added protein was bound to the matrix, giving a MAb concentration of approximately 1 mg MAb/ml gel. Ten μl of gel was mixed with 1 ml of plasma. An equal volume of ammonia buffer containing detergents and protease inhibitors was added (9). The tubes were rotated end-over-end in the cold for 16 h. The antibody-gel was spun down,

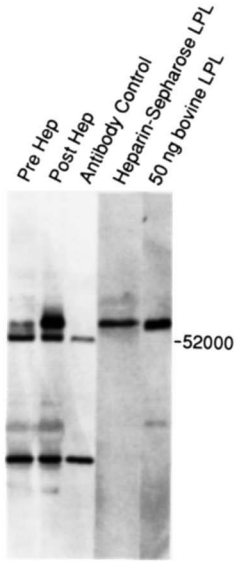

Fig. 1. Western blot of human LPL. LPL was immunoprecipitated from pre- and postheparin plasma, separated on SDS-PAGE, and detected with 5D2 MAb. Lanes: 1) preheparin plasma (corresponding to 1 ml of plasma); 2) postheparin plasma (corresponding to 1 ml of plasma); 3) MAb 5D2-gel only; 4) postheparin plasma LPL purified on heparin-Sepharose; and 5) purified bovine LPL.

the supernatant was discarded, and the gel was resuspended in 4 ml of the same buffer. After four wash cycles, 50 μl of Laemmli sample buffer was mixed with the gel and the samples were heated at 90°C for 5 min. The gel was then spun down, and the supernatant was applied to a 12.5% sodium dodecylsulfate (SDS) gel (10). After electrophoresis, the separated proteins were electrophoretically transferred to a nitrocellulose membrane (Schleicher & Schuell Inc., Keene, NH) at a constant voltage of 10 V. To verify the transfer of separated proteins, prestained molecular weight markers (BRL Life Technology Inc., Gaithersburg, MD) were run on each gel. After blocking in Tris-buffered saline (TBS) containing bovine serum albumin (BSA) (3% w/v) and gelatin (5% w/v) for 1 h at 42°C, blots were probed with 5D2 MAb (7.5 μg/ml) in TBS. MAbs bound to the membrane were then detected according to the manufacturer's instructions for the mouse antibody detection kit (New England Nuclear, Boston, MA), except that the antimouse-streptavidin conjugate was preabsorbed with human serum proteins (Sigma Chemical Co., St. Louis, MO) and diluted 1/500. After washing, the membrane was incubated with a streptavidin-alkaline phosphatase conjugate. After further washes, the LPL bands were visualized by addition of a chromogenic substrate for alkaline phosphatase.

RESULTS

The monospecificity of MAb 5D2 was first examined on Western blots. Immunoprecipitation was used as a purification step prior to electrophoresis and immunoblotting (**Fig. 1**). In this experiment, LPL was immunoprecipitated from postheparin plasma with 5D2 and was probed with the same antibody. A major band with an apparent molecular weight >52,000 (similar to bovine LPL) was detected. This band was also present in preheparin plasma but at a much lower level. Human LPL purified on heparin-Sepharose had an apparent molecular weight identical to immunoprecipitated LPL (Fig. 1). Two minor bands of greater mobility, conceivably representing degradation products, were also detected.

TABLE 1. Specificity of 5D2 MAb

	HL	LPL
	nmol/ml × min	
No addition	34.8	86.0
Sepharose	34.8	88.8
5D2-Actigel	34.2	0.3

The two activity peaks from heparin-Sepharose chromatography eluting at 0.70 M (HL) and 1.1 M (LPL) were incubated on ice with the indicated additions and vortexed every 15 min. After 2 h, the tubes were centrifuged at 200 *g* for 10 min. Activity was assayed on the supernatant as described in Methods.

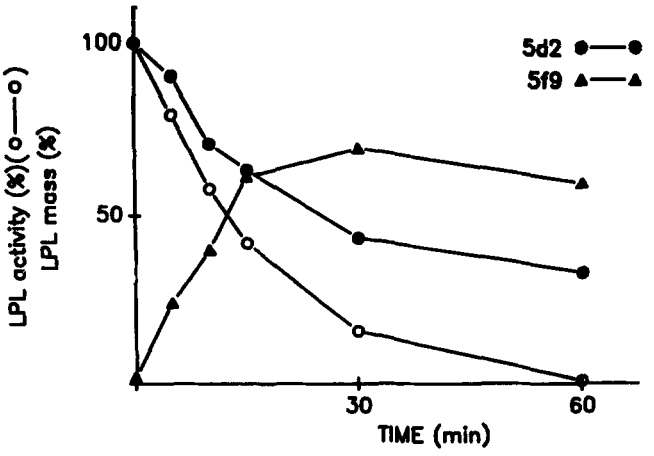

Fig. 2. Heat treatment of human LPL. Human postheparin plasma was chromatographed on heparin-Sepharose. The activity peak eluting at 1.1 M NaCl was incubated at 45°C for 60 min. At the indicated times aliquots were taken and assayed for activity and LPL mass in the two ELISAs. Activity and reactivity in the 5D2 ELISA are expressed as percentage of 0 time, 5F9 as percentage of a sample that was treated with 1 M GuHCL for 1 h. Each point represents the mean of triplicate determinations; the standard deviation was less than 8% of the mean in each case. For experimental details, see Methods.

Given the extensive degree of homology between hepatic lipase (HL) and LPL (11), it was important to rule out any cross-reactivity of 5D2 to HL. Postheparin plasma was chromatographed on heparin-Sepharose, and 5D2 MAb-gel was added to each of the two activity peaks (eluting at 0.70 M and 1.1 M for HL and LPL, respectively). After incubation for 2 h on ice, the MAb-gel was spun down and remaining activity in the supernatant was assayed (**Table 1**). Virtually all of the LPL activity was removed by this treatment, whereas HL activity was unaffected.

In this study two different ELISAs were used. One of them, referred to as 5D2 ELISA, utilizes the same MAb (5D2) both for coating the plate and detecting the antigen, thus requiring the same epitope to be present in duplication for detection. This ELISA has been used in studies previously conducted in this laboratory (6). The other ELISA (5F9 ELISA) utilizes MAb 5F9 to coat the plate and 5D2 MAb to detect the antigen. The two antibodies recognize different epitopes, as evidenced by their reaction with multiple spontaneous fragments (M_r< 30,000) of bovine LPL on Western blots (data not shown).

Preliminary experiments suggested that 5F9 MAb recognized only LPL that had undergone partial denaturation. To test whether this was the case, human LPL was heat-inactivated and the reactivities in the two ELISAs and LPL activity were measured (**Fig. 2**). The amount of denatured LPL, as measured in the 5F9 ELISA, was initially low. It increased with time and was strongly and negatively correlated to LPL activity (r = 0.95). Immunoreactivity in the 5D2 ELISA decreased with time.

At early time points, 5D2 mass and LPL activity decreased in parallel; however, unlike LPL activity, 5D2 reactivity did not disappear altogether. When human LPL was inactivated by exposure to 1 M GuHCl (**Fig. 3**), similar changes in LPL activity and immunoreactivity were also observed.

Studies were performed to determine whether the exposure of the 5F9 epitope was correlated to functional properties of LPL other than catalytic activity. To assess the heparin binding of inactivated LPL, human postheparin plasma was chromatographed on heparin-Sepharose and the eluate was assayed in the two ELISAs and in the activity assay (**Fig. 4**). In the 5D2 ELISA, two peaks were seen eluting at 0.75 M and 1.1 M NaCl. The first peak was also detected in the 5F9 ELISA. The second peak co-eluted with LPL activity and was not detected in the 5F9 ELISA. When the fractions from this peak were treated with 1 M GuHCl prior to assay in the 5F9 ELISA, they were detected, and co-eluted with the LPL activity.

To further establish the relationship between the two peaks detected by the 5F9 ELISA, the LPL activity peak (eluting at 1.1 M NaCl) was treated with 1 M GuHCl and rechromatographed on heparin-Sepharose (data not shown). No LPL activity was detectable in the GuHCl-treated LPL (Fig. 3) or in the eluted fractions. LPL content in the eluted fractions was measured in the 5F9 ELISA. Most (75%) of the GuHCl-inactivated LPL eluted as a broad peak with maximum at 0.75 M NaCl. A small proportion of LPL did not bind to the column. No LPL was detected in the fractions where active LPL would elute.

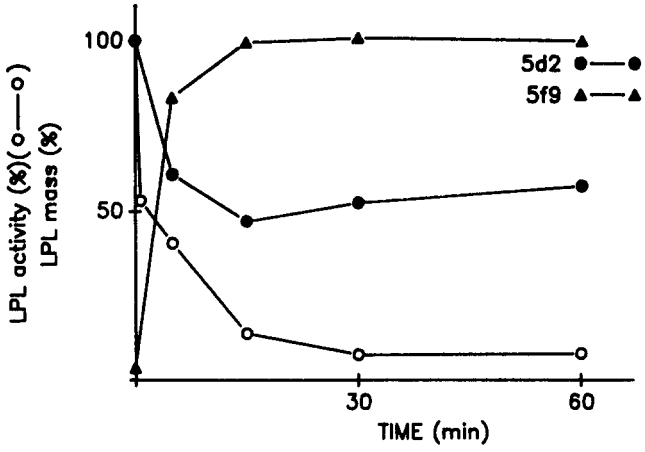

Fig. 3. GuHCl treatment of human LPL. Human postheparin plasma was chromatographed on heparin-Sepharose. The activity peak eluting at 1.1 M NaCl was made 1 M with respect to GuHCl by addition of a 6 M GuHCl solution, and incubated on ice. At the times indicated aliquots were taken and assayed for LPL activity and mass in the two ELISAs. Activity and reactivity in the 5D2 ELISA are expressed as percentage of 0 time, 5F9 as percentage of a sample that was treated with 1 M GuHCL for 1 h. Each point represents the mean of triplicate determinations; the standard deviation was less than 8% of the mean in each case. For experimental details, see Methods.

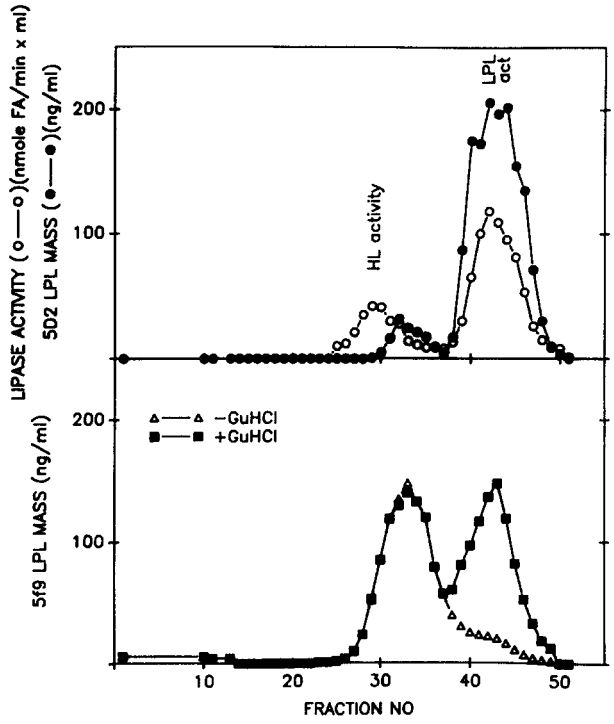

Fig. 4. Chromatography of postheparin plasma (PHPL) on heparin-Sepharose. Ten ml of a pool of PHPL from normolipemic donors was applied to a heparin-Sepharose column. After a wash, the column was eluted with a linear NaCl gradient. The fractions (1.8 ml) were assayed for lipase activity and mass in the two ELISAs. For details, see Methods.

DISCUSSION

The monospecificity of MAb 5D2 was first examined on Western blots. It was not possible to blot LPL when whole plasma was applied to an SDS-gel, possibly due to the massive amounts of albumin migrating in the same region of the gel. To overcome this problem, immunoprecipitation using MAb 5D2 bound to a gel matrix was used as a concentration step. Some of the MAb bound to the gel was apparently released when boiled in sample buffer prior to electrophoresis, and consequently light and heavy immunoglobulin chains were detected in both immunoprecipitates of plasma and of MAb gel without plasma. In addition to the major LPL band, two minor bands of greater mobility were also detected. These components possibly represent degradation products of LPL. The monospecificity of MAb 5D2 coupled to a gel matrix sedimented virtually all of the LPL activity but left HL unaffected.

A number of ELISAs for the quantification of LPL have been reported (5, 12–15). However, in most of these the molecular specificity in terms of native versus partially denatured LPL is not entirely clear. Two ELISAs were used in this study. The 5D2 ELISA utilizes the same MAb (5D2) for capturing and detecting the antigen. This requires the same epitope to be present at least in duplicate

for detection. Thus, the 5D2 ELISA measures dimers (and multimers, when present). The 5F9 ELISA utilizes MAb 5F9 for capturing and MAb 5D2 for detection. The two MAbs recognize different epitopes, and furthermore MAb 5F9 recognizes LPL only after it has undergone partial denaturation. The 5F9 ELISA thus gives a measure as partially denatured LPL present in a sample. The total amount of LPL can then be determined in the 5F9 ELISA following denaturation. The difference between these two measures would then represent the amount of native LPL originally present in the sample.

The native LPL measured by the 5F9 ELISA with and without GuHCl correlates with the LPL activity in the present study ($r = 0.95$). The incremental mass by the 5D2 ELISA appearing in plasma after intravenous heparin has previously been shown to correlate with LPL activity in 34 normal controls ($r = 0.83$) (6). In unpublished data (A. Zambon, and J. D. Brunzell), the LPL mass measured by the 5F9 and 5D2 ELISAs also is highly correlated in 71 control subjects ($r = 0.81$). Thus, each assay seems to be measuring the same catalytically active protein.

Data from analytical ultracentrifugation (1, 2) and radiation-inactivation (3) studies of bovine LPL have shown that the dimer is the smallest active molecular species. In view of the extensive homology between human and bovine LPL (16, 17), it seems probable that this would be the case with human LPL also. Recently, one group (4, 5) has suggested that in humans the LPL monomer is active. In the present study, human LPL was sandwiched between two identical MAbs (requiring the presence of the same epitope in duplicate) and the reactivity in this assay was closely correlated to LPL activity, supporting the view that LPL dimer is also the smallest active species in humans.

Osborne et al. (1) have proposed that active LPL dimer is in equilibrium with LPL monomer of native conformation, but that the monomer form is prone to undergo denaturation. The data presented here, obtained with MAbs to study human LPL, are in agreement with their proposal; LPL activity and LPL dimers disappear in parallel, concomitant with appearance of partially denatured material. In neither of these studies could an active monomer actually be detected, indicating that the proposed transformation occurs rapidly.

The capability of the 5F9 ELISA to distinguish between native and partially denatured LPL makes it a promising tool to evaluate LPL that lacks catalytic activity due to a missense mutation. For example, it might allow one to determine whether a nonfunctional LPL molecule lacks activity because a certain mutation affects overall native conformation, or whether the amino acid exchanged was crucial to enzyme function in a more specific sense. Preliminary studies using this methodology are under way in patients deficient in LPL activity (18) with known missense mutations to gain a better understanding of the structure-function relationships of LPL.

The presence of relatively low levels of LPL activity (19) and mass (20) in preheparin plasma has been reported. Western blot analysis in this study also indicates the presence in preheparin plasma of immunoreactive LPL of normal apparent molecular weight. The sensitivity of the 5F9 ELISA (down to approximately 10 ng/ml), and the fact that most of the LPL present is inactive (J. Peterson and J. D. Brunzell, unpublished data), makes this ELISA a convenient tool to evaluate the significance of this material.

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