

# Identification of lipoprotein lipase immunoreactive protein in pre- and postheparin plasma from normal subjects and patients with type I hyperlipoproteinemia

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**Abstract** Postheparin plasma is a convenient source for the measurement of lipoprotein lipase (LPL) in humans. Previous studies have focused on the measurement of LPL catalytic activity, and have been unable to conveniently measure the LPL protein or identify possibly different plasma forms of the enzyme. Pre- and postheparin plasma was treated with a highly specific antibody raised against bovine milk LPL and the immunoprecipitate was analyzed by Western blotting. In normal subjects there were several species of LPL in plasma. A 56 kD protein increased after heparin injection, and likely represented active LPL. The anti-LPL antibody reacted specifically with this 56 kD protein, and also reacted specifically with proteins at 52 kD, 69 kD, as well as a 20 kD breakdown product. In addition, using peptide mapping, the 56 kD protein was structurally similar to the 52 and 69 kD LPL proteins. The antibodies were affinity purified, biotinylated, and used to quantitate LPL immunoreactive mass using an enzyme-linked immunosorbant assay (ELISA). LPL immunoreactive mass was present in all subjects in preheparin plasma. In postheparin plasma, five patients with type I hyperlipoproteinemia displayed decreased LPL immunoreactive mass when compared to normal subjects, although there was a wide range of specific activity of the small amount of enzyme present. When the LPL from the plasma of the patients was immunoprecipitated and Western blotted, there was considerable heterogeneity in the appearance of the LPL forms, and an overall decrease in LPL protein. Thus, several different immunoreactive LPL proteins were present in pre- and postheparin plasma. In preheparin plasma, as well as in patients with type I hyperlipoproteinemia, there was decreased immunoreactive LPL protein, and the LPL protein that was present was of low specific activity. — Kern, P. A., R. A. Martin, J. Carty, I. J. Goldberg, and J. M. Ong. Identification of lipoprotein lipase immunoreactive protein in pre- and postheparin plasma from normal subjects and patients with type I hyperlipoproteinemia. *J. Lipid Res.* 1990. 31: 17–26.

**Supplementary key words** bovine milk lipoprotein lipase • ELISA • peptide mapping

Lipoprotein lipase (LPL) is an enzyme found in a number of tissues and is responsible for the hydrolysis of

triglyceride-rich lipoproteins (1). After LPL is produced by parenchymal cells, the enzyme is transported to the capillary endothelium, where it is bound to glycosaminoglycans. It has long been known that an injection of heparin releases LPL activity from its glycosaminoglycan binding sites, and postheparin plasma LPL activity has been a useful measurement in clinical studies. The measurement of LPL activity, however, only detects catalytically active LPL protein, which may not be representative of actual LPL protein mass if alterations in LPL specific activity occur. Enzyme-linked immunosorbant assays (ELISA) for human postheparin plasma LPL have been reported previously (2–4) using monoclonal antibodies to LPL. However, a monoclonal antibody may not recognize an LPL molecule that is modified due to degradation, mutation, or denaturation. Hence, an assay that detects both active and inactive LPL protein has been needed for the study of LPL regulation, and to characterize the different forms of circulating LPL.

The absence of measurable LPL activity is essential for the diagnosis of familial LPL deficiency (type I hyperlipoproteinemia) (5, 6). This disorder is found in approximately 1 in 1 million in the population (5–8), is autosomal recessive in inheritance, and typically presents in childhood with chylomicronemia, pancreatitis, and eruptive xanthomata. Although the type I hyperlipoproteinemia phenotype occasionally occurs due to apoC-II deficiency (9) or a circulating inhibitor of LPL (10, 11), the majority of patients with the type I phenotype have no measurable LPL activity in adipose tissue and/or postheparin plasma (7, 12, 13). Whether this decreased enzyme activity results from a decreased mass of normal

Abbreviations: LPL, lipoprotein lipase; ELISA, enzyme-linked immunosorbant assay; BSA, bovine serum albumin; PBSAT, phosphate-buffered saline, 0.1 % BSA, 0.1 % Triton X-100; FFA, free fatty acids.

enzyme or the production of a defective LPL protein could not be answered by measuring only the catalytically active LPL protein.

We report herein the characterization of plasma LPL with a polyclonal antibody that specifically recognizes several molecular forms of LPL in plasma. In addition to studying LPL immunoreactive mass in pre- and postheparin plasma, the plasmas from five patients with type I hyperlipoproteinemia were analyzed.

## METHODS

### Patients and plasma preparation

The procedure for obtaining postheparin plasma was approved by the respective Institutional Review Boards. Plasma was collected both before and 10–15 min after the injection of 60 units per kg of heparin, and immediately placed on ice. The plasmas were then frozen until subsequent analysis of LPL activity or mass. Pre- and postheparin plasma was obtained from eight normal subjects, and five patients with classic type I hyperlipoproteinemia who were deficient in LPL activity (postheparin plasma LPL activity <10% of normal). All samples from the type I patients were assayed by several different methods. Most samples were initially assayed using a gum arabic-based assay in which hepatic lipase was inhibited with an anti-hepatic lipase antibody (2).

### Assay for LPL catalytic activity

LPL activity was determined by measuring the difference between total lipase activity and the activity remaining after inhibition of LPL activity with an inhibitory monoclonal antibody (2). Total lipase activity was determined essentially as described previously (14). Plasma (10  $\mu$ l) was diluted in 140  $\mu$ l of PBS and exposed to an emulsified [ $^{14}$ C]triolein-containing substrate. The liberated  $^{14}$ C-labeled FFA was separated from glycerides according to the method of Belfrage and Vaughn (15). Lipase activity was expressed as nEq FFA released/h per ml of plasma. To differentiate LPL from hepatic lipase activity, LPL activity was inhibited with a monoclonal antibody raised against human milk LPL, as described previously (2). A dilution of ascitic fluid of 1:200 yielded maximal inhibition of postheparin lipase. The remaining lipase activity in postheparin plasma has previously been shown to be >90% inhibited by an antibody to hepatic lipase (2). Thus, plasma (10  $\mu$ l) was preincubated with 140  $\mu$ l of the indicated dilution of either specific antibody or nonspecific ascitic fluid for 15 min at 25°C, followed by the addition of lipase substrate. The difference between total lipase activity and activity remaining after LPL inhibition was taken as LPL activity.

### Enzyme-linked immunosorbant assay (ELISA) for LPL

LPL immunoreactive mass was measured by ELISA as described previously (16). In brief, affinity-purified anti-bovine LPL antibodies were bound to microtiter wells, and sample or bovine LPL standards were then added in buffer containing 1 M NaCl, 0.01% Triton X-100, 0.1% bovine serum albumin (BSA), and protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 1 mM EDTA, and 0.05 mM aprotinin). The wells were then washed, and biotinylated; affinity-purified antibody was then added, followed by streptavidin-peroxidase (Bethesda Research Labs, Bethesda, MD). The peroxidase reaction was developed, and read in an ELISA plate reader.

### Western blotting and immunoprecipitation

To identify forms of LPL that differed in molecular weight, immunoprecipitation and Western blotting were performed. To 0.2 ml of pre- or postheparin plasma (0.6 ml of plasma from type I patients), protease inhibitors (as described above) were added, and the plasma was incubated with 1  $\mu$ g of biotinylated, affinity-purified anti-LPL overnight at 4°C. Streptavidin-agarose (60  $\mu$ l, Bethesda Research Labs) was then added for 2 h at 25°C with constant mixing and the mixture was then centrifuged. The pellet was then washed 3  $\times$  with PBS containing 0.1% Triton X-100, and was then boiled for 5 min in 100  $\mu$ l cracking buffer (5% betamercaptoethanol, 2% sodium dodecyl sulfate, 10% glycerol, and 0.5 M Tris, pH 6.8).

The immunoprecipitates were analyzed on a 7.5–15% linear gradient SDS-polyacrylamide gel with a 3% stack, followed by electrophoretic transfer to nitrocellulose for Western blotting. Nonspecific binding was blocked by incubating the nitrocellulose overnight with PBS containing 1% Triton X-100 and 5% BSA. The blot was then incubated with anti-LPL antiserum diluted 1:6000 in PBS containing 0.1% Triton X-100 and 0.1% BSA (PBSAT), followed by four washes in PBSAT. After incubating the nitrocellulose with rabbit anti-chicken IgG (1:25,000 in PBSAT) for 2 h, the blot was washed and incubated with  $^{125}$ I-labeled protein A (Amersham, Arlington Heights, IL, approximately 200,000 cpm/ml). The nitrocellulose was again washed, dried, and then exposed to X-ray film at  $-70^{\circ}\text{C}$ . To demonstrate which bands on the blot were LPL, some samples were blotted with the same dilution of “flow-through” from the affinity purification step (16). This “flow-through” represents antiserum that was stripped of the specific anti-LPL antibodies by passage through a bovine-LPL affinity column. To determine whether any of the LPL forms were due to LPL degradation that occurred after plasma collection, pre- and postheparin plasma was collected into tubes containing heparin, as

well as protease inhibitors. No difference in LPL immunoreactive mass was found, and the profile on Western blotting was identical with plasma collected as described above. In addition, LPL from freshly collected postheparin plasma was immunoprecipitated and Western blotted without prior freezing. There was no difference in migration of the LPL species compared to previously frozen plasma.

Human adipose tissue was extracted in a NP-40/deoxycholate buffer, as described previously (17), and Western blotted. Bovine LPL standards for blotting and ELISA were purified from bovine milk (18), and hepatic lipase was purified from rat liver as described previously (19).

To determine whether LPL aggregates were responsible for some of the observed LPL forms on the gels, gels containing 4 M urea were used. LPL was immunoprecipitated as described above, but the immunoprecipitated material was boiled in cracking buffer containing 4 M urea, and separated on a 10% acrylamide, 4 M urea gel. Western blotting was then performed as described above. To assess the effect of N-linked glycosylation on the different LPL species, LPL in plasma was immunoprecipitated, and the immunoprecipitates were then treated with peptide:N-glycosidase-F (N-Glycanase, Genzyme Corp., Boston MA), as described previously (20), followed by SDS-PAGE and Western blotting.

To identify radiolabeled LPL in plasma, a 200-g Sprague-Dawley rat was injected with 5 mCi of [<sup>35</sup>S]methionine through the tail vein. After 90 min, the rat was administered 100 units/kg heparin IV, and 15 min later plasma was collected into heparinized tubes. The plasma LPL was then immunoprecipitated as described previously (21), using affinity-purified anti-LPL antibody, followed by rabbit anti-chicken antiserum (Sigma) and immobilized protein A (RepliGen, Cambridge, MA). To demonstrate that the immunoprecipitated bands were LPL, competition for the immunoprecipitation was carried out using 1 µg of bovine LPL, as described previously (16, 22).

### Protein mapping

To demonstrate that the different immunoprecipitated species of LPL were structurally related, protein mapping was carried out using a modification of the method of Cleveland (23). Pre- and postheparin plasma was immunoprecipitated and separated by SDS-PAGE, as described above. The bands corresponding to each LPL species were then cut out of the gel and placed into a well of a 15% acrylamide gel with a 5-cm (3% acrylamide) stacking gel. Each band was localized according to its anticipated molecular weight based on prestained molecular weight markers (Bethesda Research Labs), and the first gel, from which the bands were cut, was then Western blotted to confirm that the correct band was indeed cut

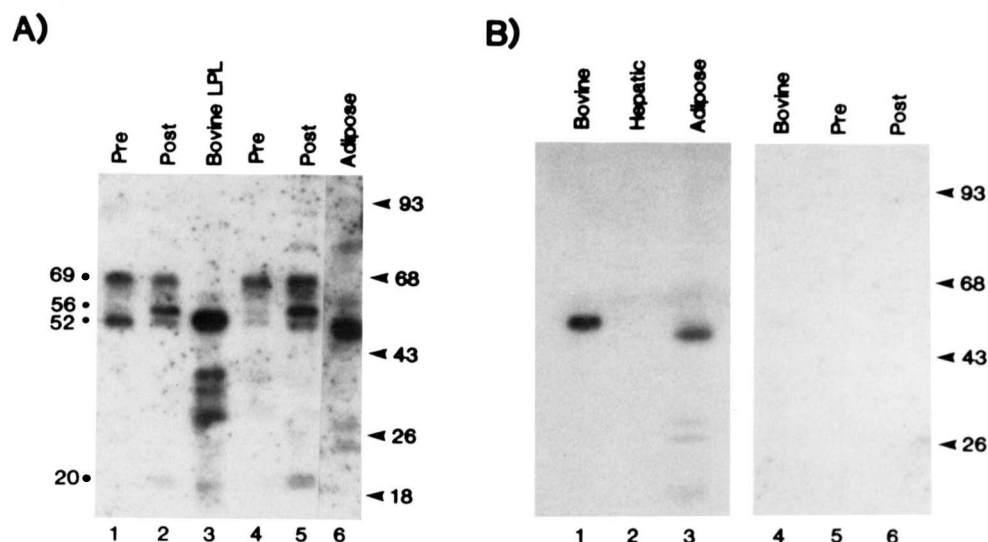
out. To the wells of the second gel, containing the excised bands, were added 10 µg of Staph V8 protease (Boehringer Mannheim, Indianapolis, IN). The proteins were electrophoresed into the stacking gel and power was then turned off for 1 h to allow proteolysis. Electrophoresis then proceeded to completion, and the separated peptides were transferred to nitrocellulose and blotted with anti-LPL antibody. To increase sensitivity for the protease-derived LPL fragments, the nitrocellulose was blotted with the biotinylated affinity-purified anti-LPL, followed by <sup>125</sup>I-labeled streptavidin (Amersham).

## RESULTS

To identify the forms of LPL in pre- and postheparin plasma, Western blotting was performed. Because of interference with the Western blotting technique by immunoglobulins, BSA, and perhaps other plasma proteins, some form of partial purification of LPL was necessary. Therefore, LPL in plasma was immunoprecipitated, and the immunoprecipitates were analyzed by SDS-PAGE and Western blotting (see Methods). As shown in Fig. 1, and demonstrated previously (22), adipose tissue LPL is identified as a somewhat broad band at a molecular weight of 53 kD. The blot of pre- and postheparin plasma, however, identified four different proteins. Two distinct bands were noted at 56 kD and 52 kD that migrated close to the broad band produced by human adipose LPL and bovine LPL. The 56 kD form of LPL was only weakly present in preheparin plasma, and increased considerably after heparin injection. In contrast, the band at 52 kD did not respond as predictably as the 56 kD band. The 52 kD band was present in preheparin plasma, and either increased, decreased, or remained essentially the same after heparin injection. A band at 20 kD was present in postheparin plasma (Fig. 1), and occasionally in preheparin plasma, and comigrated with a commonly observed LPL breakdown product (22, 24). A protein that migrated more slowly than adipose tissue LPL or purified bovine LPL was also detected by blotting. This protein, which had an apparent molecular weight of 69 kD, was present in both pre- and postheparin plasma.

The specificity of the antibodies for LPL from rat and human adipose tissue has been illustrated previously (16, 22). One potential problem with the application of this antibody to plasma is the potential cross-reactivity of the antibody with hepatic lipase, which is structurally similar to LPL (25). To assess antibody cross-reactivity with hepatic lipase, both purified bovine LPL and rat hepatic lipase were blotted with the anti-LPL antiserum. As shown in Fig. 1B (lanes 1 and 2), 5 ng of purified bovine LPL yielded a dense autoradiographic band, whereas 50 ng of rat hepatic lipase yielded no signal.





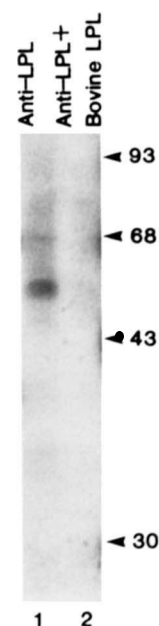
**Fig. 1.** A. Western blot of LPL from normal subjects. LPL in pre- and postheparin plasma from two normal subjects was immunoprecipitated and blotted as described in Methods. The locations of the 69 kD, and 56 kD, 52 kD, and 20 kD bands are indicated on the left of the figure. Bovine LPL and an extract of human adipose tissue are shown in the indicated lanes. B. Lanes 1–3 were blotted with anti-LPL antiserum. Five ng of bovine LPL and 50 ng of hepatic lipase were loaded into lanes 1 and 2, respectively, and human adipose tissue extract was loaded into lane 3. Lanes 4–6 represent the same samples as in Fig. 1A lanes 3–5 except that blotting was performed with the “flow-through” from the affinity purification step (see text).

To determine whether any of these bands were artifacts of the immunoprecipitation, and not related to LPL, the same samples as those depicted in Fig. 1A were blotted with the “flow-through” from the antiserum affinity-purification step (see Methods). This “flow-through” represented antiserum that had been specifically stripped of anti-LPL antibody. As shown in Fig. 1B (lanes 4–6), all of the bands present after blotting with specific antibody were not present when blotting was performed with the “flow-through.” Thus, these bands were all recognized by the antibody in a specific manner.

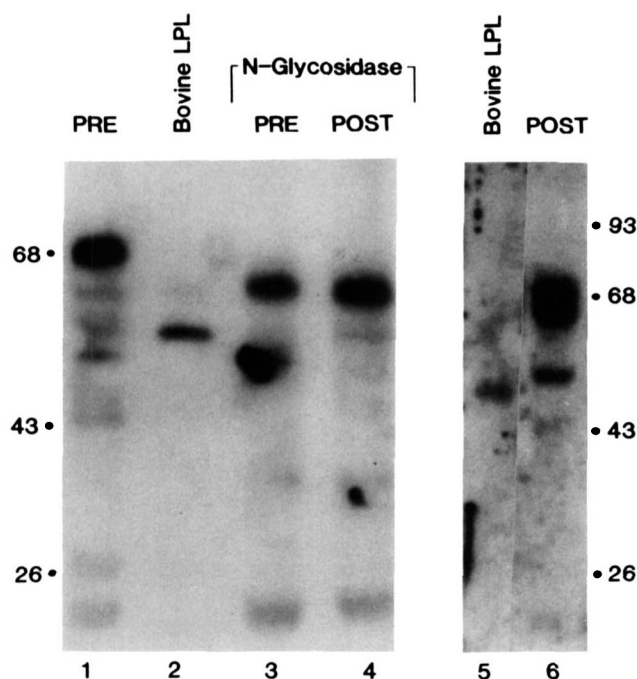
To further study different forms of LPL in plasma, a rat was injected intravenously with [ $^{35}$ S]methionine to radiolabel tissue proteins. After injection of heparin, plasma was collected and LPL was immunoprecipitated and analyzed by SDS-PAGE (see Methods). As shown in Fig. 2, a predominant band was immunoprecipitated; the band was not evident when the immunoprecipitation was carried out in the presence of bovine LPL. However, a protein of higher molecular weight was also immunoprecipitated and was also displaced by the addition of bovine LPL. Thus, these data suggested that the higher molecular weight protein on the gel was a form of LPL. Alternatively, the addition of bovine LPL may have altered the immunoprecipitation reaction in such a manner that the precipitation of a contaminant was prevented.

Additional experiments were performed to determine whether the 69 kD form of LPL could be a highly glycosylated form of LPL or could have resulted from aggrega-

tion of other forms of LPL. To assess glycosylation, immunoprecipitates were treated with peptide:N-glycosidase-F prior to Western blotting. As shown in Fig. 3 (lanes 3 and 4), the 69 kD form of LPL was reduced in molecular weight by the glycosidase treatment, but only to approximately 65 kD. To determine whether aggregation of LPL proteins was occurring, gels containing 4 M



**Fig. 2.** Immunoprecipitation of LPL from plasma from a radio-labeled rat. A rat was injected with [ $^{35}$ S]methionine, and lipases were then released with heparin (see Methods). The LPL in plasma was then immunoprecipitated with anti-LPL antibodies in the absence (lane 1) and presence (lane 2) of 1  $\mu$ g of bovine LPL.

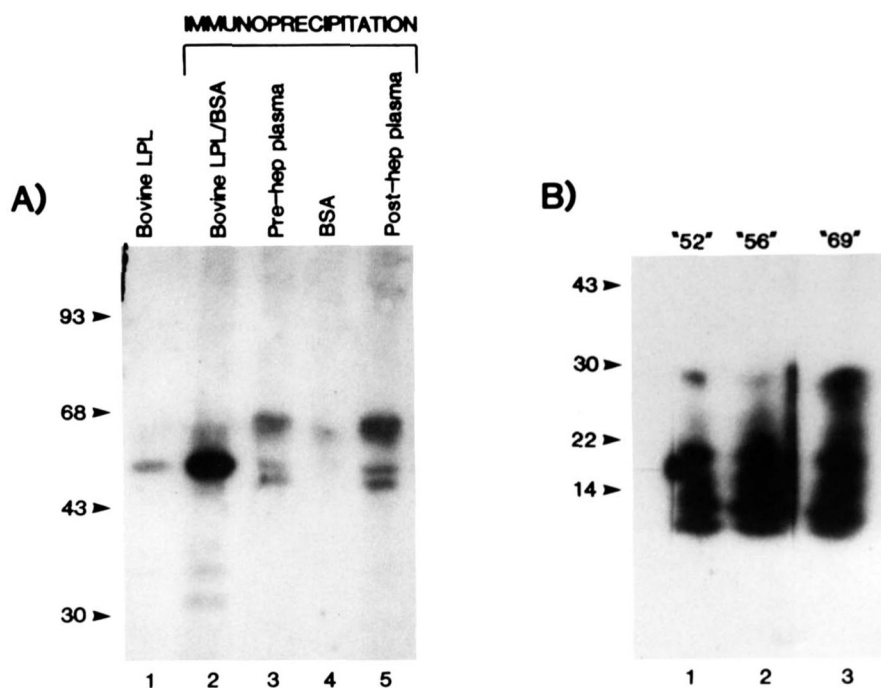


**Fig. 3.** Characterization of plasma LPL. LPL in pre- and post-heparin plasma was immunoprecipitated and then treated with peptide:N-glycosidase-F to remove N-linked oligosaccharides. In lanes 3 and 4 are the Western blots of glycanase-treated LPL. In lanes 5 and 6, bovine LPL and postheparin plasma immunoprecipitates were analyzed on a 4 M urea gel prior to transfer to nitrocellulose and immunoblotting.

urea were used to analyze the LPL immunoprecipitates, as described in Methods. As shown in Fig. 3 (lanes 5 and 6), the 69 kD form of LPL was even more diffuse on the urea gel, and bovine LPL ran faster than the 56 kD form of plasma LPL. However, multiple forms of LPL were still present in this different gel system, suggesting that the high molecular weight form of LPL was not due to aggregation.

Further experiments were carried out to determine whether the 69 kD band on the Western blots was a form of plasma LPL. Because the molecular weight of albumin is approximately 69 kD, we wondered whether this 69 kD band resulted from a small degree of nonspecific adherence of the antibody to albumin. To explore this possibility, BSA (40 mg/ml) was added to PBS in the presence and absence of bovine LPL (150 ng/ml). These solutions were then immunoprecipitated and Western blotted as described previously for plasma (see Methods). As shown in Fig. 4A (lane 2), immunoprecipitation and Western blotting of PBS-BSA-LPL yielded a single band corresponding to the LPL. In addition, when the same procedure was carried out on PBS-BSA without added LPL (Fig. 4A, lane 4), no bands were immunoprecipitated. Thus, the 69 kD band could not be reproduced on the Western blot by the addition of BSA to a solution containing LPL.

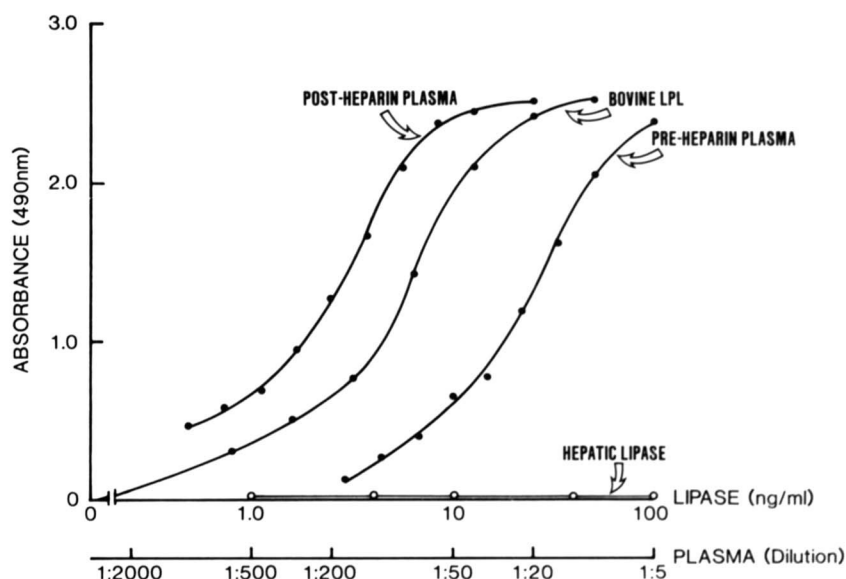
To further demonstrate that the 69 kD band on the gel was a form of LPL, peptide mapping was performed as



**Fig. 4.** Identification of the 69 kD band as LPL-related protein. A. The samples in lanes 1–5 were blotted with anti-LPL antiserum. Lane 1: 5 ng of bovine LPL; lane 2: a solution of BSA/bovine LPL was immunoprecipitated as described previously for plasma; lanes 3 and 5: immunoprecipitation of LPL in pre- and postheparin plasma; lane 4: immunoprecipitation of BSA solution without added LPL. B. Peptide mapping of the bands cut out at 52 kD, 56 kD, and 69 kD. The bands were cut out of a gel, digested with Staph V8 protease, and the peptides were separated on another gel and Western-blotted with anti-LPL antibody (see Methods).



**Fig. 5.** Standard curves for pre- and postheparin plasma in the ELISA. The indicated dilutions of pre- and postheparin plasma were assayed in the ELISA, and compared to the curve for bovine LPL and rat hepatic lipase in the same assay.

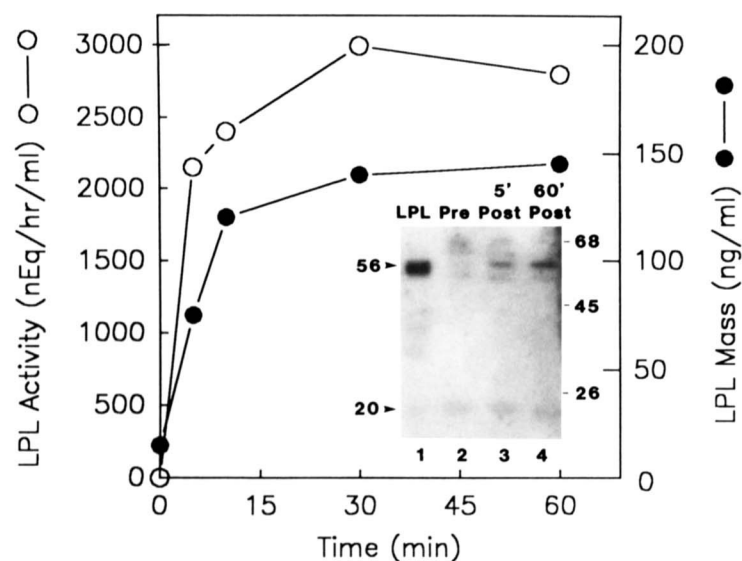


described in Methods. The 69, 56, and 52 kD bands were cut out of a gel, digested with Staph V8 protease, and the peptide fragments were separated on another gel. These peptide fragments were then identified by Western blotting. As shown in Fig. 4B, the peptide map of the 69 kD protein demonstrated several bands that comigrated with the bands from the 56 and 52 kD LPL proteins. These data strongly suggest that there is considerable sequence homology between these three different forms of LPL.

To accurately quantitate total LPL immunoreactive mass in plasma, a sandwich-type ELISA was established using affinity-purified anti-LPL antibodies (see Methods). In Fig. 5, the standard curve for bovine LPL is shown alongside the curve generated by serial dilutions of pre- and postheparin plasma. As can be seen, the curves for

bovine LPL, pre-, and postheparin plasma were all parallel. In addition, hepatic lipase did not react with the antibodies to any significant degree. Thus, the bovine LPL standard curve was used to reflect LPL immunoreactive mass in plasma.

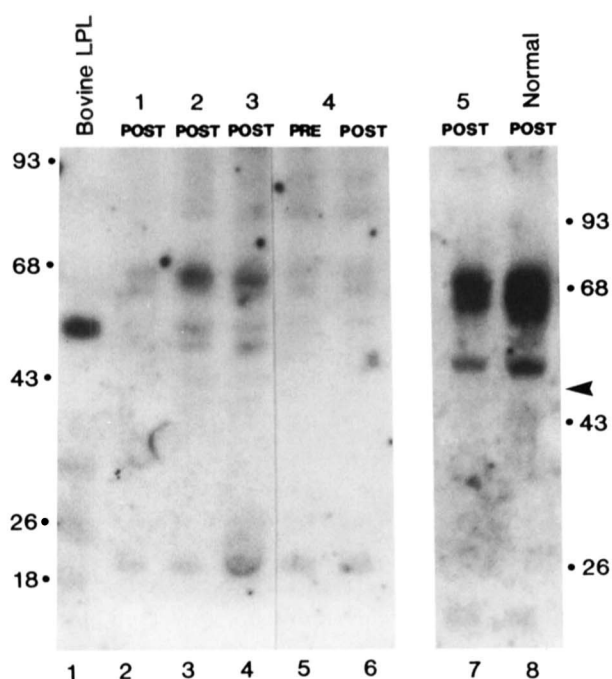
The time-course of release of LPL by heparin was studied to assess the impact of these different forms of the enzyme on LPL activity and immunoreactive mass. Plasma was collected from a normal subject at time intervals after an intravenous heparin bolus of 60 U/kg. LPL activity and immunoreactive mass were measured at various times after heparin injection, and the samples were immunoprecipitated and blotted to identify the different LPL forms (Fig. 6). LPL activity and mass were initially low, and essentially none of the 56 kD LPL species was



**Fig. 6.** Release of LPL by heparin. Plasma was obtained before and at the indicated intervals after the injection of 60 U/kg heparin into a normal subject, and LPL activity and immunoreactive mass were measured at each time point. As shown in the insert, the LPL in preheparin plasma, as well as the 5-min and 60-min postheparin plasma (lanes 2-4, respectively), were immunoprecipitated and Western-blotted along with bovine LPL (lane 1).

present in preheparin plasma. Thus, the preheparin LPL forms that were present at 69, 52, and 20 kD were mostly inactive. After the heparin injection, the 56 kD band appeared, coincident with an increase in active LPL protein, which reached a maximum at 30 min.

Pre- and postheparin plasma were obtained from five patients with type I hyperlipoproteinemia. To determine whether there were any differences in the molecular weight of the plasma LPL species, LPL in pre- and postheparin plasma was immunoprecipitated and Western blotted, as described previously (Fig. 7). Patient number 1 (lane 2) had little detectable LPL by Western blotting, and the small amount of material present was the higher molecular weight form. Patients 2 and 3, on the other hand, had distinct LPL bands at 69 kD, 56 kD, 52 kD, and 20 kD. More plasma was used for the original immunoprecipitation because of the low levels of LPL protein present in these patients. Both pre- and postheparin plasma were analyzed for patient number 4 (lanes 5 and 6). Although only a small amount of LPL protein was identifiable in preheparin plasma, postheparin plasma contained a faint 56 kD band. The postheparin plasma from patient number 5 is shown on a 4 M urea gel in lane 7, alongside the postheparin plasma of a normal subject (threefold more plasma was used to obtain the image of



**Fig. 7.** Western blots of plasma from five type I patients. LPL in plasma from five type I patients (numbered 1–5) were immunoprecipitated and Western-blotted as described in Methods. LPL in both pre- and postheparin plasma were immunoprecipitated for patient number 4 (lanes 5 and 6). Lanes 1–6 are from a conventional gradient gel, and lanes 7 and 8 are from a 4 M urea gel. The arrowhead outside of lane 8 indicates the position of bovine LPL on the urea gel. Lane 1 depicts bovine LPL, and lane 8 depicts the immunoprecipitation and Western blot of a normal subject for comparison.

**TABLE 1.** Plasma LPL data from patients and controls

Subjects	Preheparin Plasma Mass  ng/ml	Postheparin Plasma		
		Activity  nEq/h/ml	Mass  ng/ml	Specific Activity  nEq/h/ng
Type I patients				
1	4.9	94	19.8	4.75
2	ND	*	11.8	<3
3	3.8	251	11.6	21.6
4	8.3	*	25.8	<3
5	ND	*	19.0	<3
Normal subjects				
6	19.5	2432	113	21.5
7	8.0	1930	94	20.5
8	13.0	1262	75	16.8
9	8.5	2124	91	23.3
10	25.0	1968	145	16.6
11	15.0	1222	114	10.7
12	ND	1402	86	16.3
13	ND	1903	86	22.1

ND, not determined; \*, undetectable.

the type I patient). As with patients number 2 and 3, the electrophoretic pattern of LPL peptides was the same as in the normal subjects, although reduced in quantity.

LPL immunoreactive mass and activity were measured in the plasma from patients and control subjects, and the data are shown in Table 1. Low or absent levels of LPL activity were detected in the type I patients, and all had detectable immunoreactive mass. Among the type I patients, patient number 3 had LPL activity which was low, but clearly measurable. In addition, this patient had the least LPL immunoreactive mass, such that her specific activity was similar to that of the normal subjects. All subjects had immunoreactive LPL in preheparin plasma. Immunoreactive mass in preheparin plasma was  $13.2 \pm 1.3$  ng/ml (mean  $\pm$  SE) in 16 normal subjects, and  $5.7 \pm 1.4$  ng/ml in the three type I patients from whom preheparin plasma was available ( $P < 0.05$  vs normal subjects). LPL activity in preheparin plasma was less than 50 nEq/h per ml (the limit of detectability of this assay), suggesting that the specific activity of LPL in preheparin plasma was generally less than 3.6 nEq/h per ng.

## DISCUSSION

The measurement of LPL in human postheparin plasma has been a useful and convenient approach to the study of this enzyme for some time. Although hepatic lipase is released into plasma by heparin along with LPL, antibodies have been developed to both hepatic lipase and LPL (2) which selectively inhibit the activity of each enzyme and therefore permit an accurate differentiation of the different lipase activities. One long-standing problem,

however, has been the inability to conveniently measure plasma LPL immunoreactive mass. Antisera to LPL have been reported previously, but in only a few instances has cross-reactivity with human LPL been demonstrated (26–29). Goldberg et al. (2) reported an ELISA to postheparin plasma LPL using a monoclonal antibody raised against human milk LPL. However, samples were first partially purified by passage over heparin-Sepharose. Thus, this assay may not detect LPL that is defective in the heparin-binding site. Scheibel et al. (3) reported an ELISA to pre- and postheparin plasma LPL using a single monoclonal antibody in an antibody-LPL-antibody sandwich. Using this assay, LPL immunoreactive mass has recently been measured in patients with type I hyperlipoproteinemia and in type I heterozygotes (4). A requirement for this ELISA, however, is a dimerized form of LPL with two intact epitopes for the monoclonal antibodies. If some circulating LPL is monomeric or lacking in a certain epitope, such an ELISA will likely not recognize the protein. Thus, an immunoassay is needed that can detect low levels of LPL protein in plasma without introducing steps that may alter LPL recovery.

For the antibody used in this study, the specificity for LPL has previously been demonstrated in adipose tissue from both rats and humans (16, 22). Because hepatic lipase is structurally similar to LPL (25) and is present in plasma in high levels, it was important to demonstrate that this antibody does not cross-react with hepatic lipase. As demonstrated herein, hepatic lipase was not detected by Western blotting, and yielded no signal in the ELISA, suggesting that any cross-reactivity of this antibody with hepatic lipase is very low.

To identify different forms of LPL in plasma, Western blotting was performed. Because LPL represented only a small fraction of plasma protein, other major plasma proteins (albumin and immunoglobulins) interfere with LPL in this procedure. Therefore, some form of purification step was necessary to demonstrate LPL on a Western blot. Thus, the immunoprecipitation step described herein was used to separate LPL from other plasma proteins, and the immunoprecipitates were then Western-blotted. By blotting samples with antiserum that had been stripped of specific anti-LPL antibody, the LPL bands could be distinguished from any proteins that may have been artifacts. As shown in Fig. 1B, all the bands identified by specific antiserum were not present when the immunoprecipitated samples were blotted with the “flow-through” from the affinity purification step. Thus, these bands were all immunologically related to LPL.

Further studies were performed to clearly demonstrate that each band identified by the antibody was indeed LPL. The immunoprecipitation of plasma from a radiolabeled rat yielded a pattern similar to that in human plasma, and each radiolabeled form of LPL in rat plasma was not identified when the immunoprecipitation was car-

ried out in the presence of unlabeled bovine LPL. When the immunoprecipitates were treated with peptide:N-glycosidase-F, the 69 kD band fell in molecular weight to 64 kD. Therefore, this protein was not simply a highly glycosylated form of the 56 kD LPL. The 69 kD form of LPL ran as a somewhat diffuse band, and was even more diffuse on a 4 M urea gel, suggesting that this “band” may be made up of more than one discrete protein. The addition of BSA to purified LPL verified that these methods did not yield co-immunoprecipitation of albumin. Finally, when the bands at 52 kD, 56 kD, and 69 kD were all cut from the gel and subjected to peptide mapping, there were a number of bands that comigrated, strongly suggesting that all three proteins were forms of LPL. In a study on the synthesis of LPL in newborn rat liver (30), a number of bands were immunoprecipitated by anti-LPL antibody, including bands at approximately 69 and 52 kDa. Whether these other forms of LPL are due to enzyme degradation or alternate processing in nonadipose tissues is not clear.

Previous studies by us (21, 22) and others (31–34) have demonstrated that LPL is synthesized in adipose tissue and adipocytes as a protein with a subunit molecular weight of approximately 55–60 kD. This molecular weight of the mature protein agrees with the molecular weight of the unglycosylated protein, as predicted from the cDNA sequence (35), and the known carbohydrate content (36). Because the 56 kD band was increased after heparin, this protein probably represents predominantly fully processed, active enzyme.

The 52 kD protein was present in preheparin plasma, and either decreased, increased, or stayed the same in postheparin plasma. Because preheparin plasma contained low LPL activity, the 52 kD band was likely of a very low specific activity. This 52 kD form of LPL may have been a partially glycosylated LPL that was secreted or released from tissues, or perhaps a breakdown product of the mature 56 kD LPL. The 20 kD protein was likely a breakdown product, and was variably present in preheparin plasma, and always present in postheparin plasma. The nature and origin of the 69 kD form of LPL is not clear. Because the 69 kD form was present under reducing conditions including a 4 M urea gel, it is unlikely that this LPL species represents an aggregate of lower molecular weight forms of LPL. Nevertheless, it is possible that this 69 kD species represents some kind of *in vitro* artifact, or a nonLPL plasma protein that cross-reacts with the antibody. Alternatively, LPL may be secreted as a 56 kD protein, and then taken up by cells and covalently modified.

The ELISA detected LPL immunoreactive material in both pre- and postheparin plasma. The standard curves covered a wide range and yielded an accurate working range for the estimation of LPL mass. Because human LPL was unavailable to use as a standard and the slopes



of the curves for bovine LPL, pre-, and postheparin plasma were all parallel, LPL immunoreactive mass in the plasma samples was taken from the bovine LPL curve. Nevertheless, it is possible that the antibodies reacted with bovine LPL in a different manner than they reacted with human LPL, and thus that the true mass of LPL in human plasma may be different.

LPL activity and mass were released in parallel after heparin injection and levels of the enzyme were sustained for 1 h. The 56 kD protein appeared immediately after heparin injection, and therefore likely represented the active form of LPL. Although it is likely that the 20 kD protein on the Western blots represented an LPL breakdown product, the samples from 5 and 60 min postheparin showed no detectable increase in the 20 kD fraction. In addition, the 20 kD protein was sometimes seen in preheparin plasma and was always present immediately after heparin injection. Therefore, this protein may be partially derived from degradation of plasma LPL, but more importantly may be the product of intracellular LPL degradation, and thus may be stored and released from tissues along with intact LPL.

Most previous studies of patients with type I hyperlipoproteinemia have been limited to the study of LPL catalytic activity. Although all patients have severe chylomicronemia syndrome, some have a small amount of LPL activity (5-7). Thus, type I hyperlipoproteinemia may be a heterogeneous disorder, with some patients producing defective enzyme, and others producing deficient enzyme. In this study, pre- and postheparin plasma from five type I patients were Western-blotted using the same technique that was successful for normal subjects. As shown in Fig. 7, some form of LPL immunoreactive protein was found in the plasma of each type I patient, although some patients' plasma contained virtually no 56 kD LPL, which is likely the active form of LPL. Quantitation of LPL immunoreactive mass with the ELISA confirmed that the type I patients' postheparin plasma contained approximately 20% of the LPL immunoreactive protein contained in normal postheparin plasma. Similar data were reported recently by Babirak et al. (4). When LPL activity and mass were measured in the postheparin plasma, LPL specific activity was low in four patients, but was normal in one of the patients (patient number 3). The patient with normal specific activity had normal-appearing LPL forms by Western blotting, suggesting that the little LPL protein that was produced was bioactive. However, other patients' postheparin plasma contained some identifiable 56 kD LPL. In these patients, it is possible that there was a small structural mutation, leading to an inactive protein that migrated on the gel like normal LPL. Thus, these studies agree with others (37) and suggest that there is considerable heterogeneity in the LPL protein manifested by these five patients with type I hyperlipoproteinemia.

In summary, we have reported herein the identification of different forms of the LPL protein in pre- and postheparin plasma. In addition, LPL immunoreactive mass has been quantitated in plasma from normal subjects, and patients with type I hyperlipoproteinemia. The pre- and postheparin plasma of five patients with type I hyperlipoproteinemia contained reduced levels of LPL immunoreactive protein that was of variable specific activity. These methods of characterization of plasma LPL will prove useful for further studies on humans with a variety of lipoprotein disorders. ■

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