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Lipoprotein lipase isoelectric point isoforms in humans

Míriam Badia-Villanueva [a], Pere Carulla [a], Montserrat Carrascal [b], Joaquín Abián [b], Miquel Llobera [a,*], Albert Casanovas [c], M. Dolores López-Tejero [a]

[a] Department of Biochemistry and Molecular Biology, Faculty of Biology, University of Barcelona, Barcelona, Spain
[b] CSIC/UAB Proteomics Laboratory, IIBB-CSIC-IDIBAPS, Barcelona, Spain
[c] Department of Biochemistry and Molecular Biology, University of Southern Denmark, Odense, Denmark

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ABSTRACT

Lipoprotein lipase (LPL) hydrolyzes circulating triacylglycerols (TAG) into free fatty acids and glycerol. It is present in almost all tissues and its tissue-specific regulation directs the flow of circulating TAG in the body. We demonstrated in a previous study that, in rat heart and post-heparin plasma (PHP), LPL consists of a pattern of more than 8 forms of the same apparent molecular weight, but different isoelectric point (pI). In the present study we describe, for the first time, the existence of at least nine LPL pI isoforms in human PHP, with apparent pI between 6.8 and 8.6. Separation and characterization of these forms was carried out by 2DE combined with Western blotting and mass spectrometry (MALDI-TOF/MS and LC–MS/MS). Further studies are needed to discover their molecular origin, the pattern of pI isoforms in human tissues, their possible physiological functions and possible modifications of their pattern in different pathologies.

1. Introduction

Lipoprotein lipase (LPL EC.3.1.1.34) is a glycoprotein enzyme with a central role in lipid metabolism [1,2] that hydrolyzes circulating triacylglycerols (TAG) from chylomicra and VLDL into free fatty acids and monoacylglycerol. LPL is present in almost all extrahepatic tissues (the highest activities are found in adipose tissue, heart and mammary gland) and its tissue-specific regulation, which remains incompletely understood, channels the flow of circulating TAG in the body [2]. LPL is synthesized and secreted by the parenchymal cells of each tissue and is transported across the endothelium to the vessel lumen by glycosylphosphatidylinositol-anchored high-density lipoprotein-binding protein 1 (GPIHBP1) [3,4], but the cellular mechanisms of this transport remain unclear [4,5]. On the luminal surface of the endothelium, LPL is present in its mature and active form as a dimer bound to endothelial heparan and dermatan sulphate chains through electrostatic interactions [1]. After a short half life [6], LPL is inactivated and released by a poorly characterized process from the endothelium to the bloodstream, through which it travels before being taken up and degraded by the liver [7]. Our previous results suggest that the release of LPL to the bloodstream may constitute a post-translational mechanism for tissue-specific LPL activity regulation in physiological conditions like stress [8,9].

Despite progress in our understanding of LPL regulation [4], the multiple aspects of LPL biology that remain unclear indicate that we are still left with an incomplete characterization of the enzyme. In recent years, the use of proteomic tools (i.e., two-dimensional electroporesis (2DE) and mass spectrometry (MS)) in LPL research (i) revealed the existence of more than 8 LPL isoforms of the same apparent molecular weight but different pI in the rat heart [10] uncovering a level of diversity previously unknown, (ii) enabled the identification of a novel post-translational modification of the enzyme [11], and (iii) yielded the first (partial) sequence to be described from direct study of rat LPL protein [10].

In humans, the central role of LPL in the homeostasis of blood lipids is evidenced by the disease outcomes of LPL dysfunction [12], featured by hypertriglyceridemia, and an increased risk of pancreatitis [13], and associated to pathological conditions like atherosclerosis, obesity, Alzheimer's disease and diabetes [1]. Over 100 mutations in the LPL gene resulting in total or partial loss of LPL function have been described in humans [13]. Likewise, the deficiency in LPL activity can also be derived from mutations in

Abbreviations: GPIHPB1, glycosylphosphatidylinositol-anchored high-density lipoprotein-binding protein 1; HL, hepatic lipase; LPL, lipoprotein lipase; PHP, post-heparin plasma; TAG, triacylglycerol.

* Corresponding author. Address: Department de Bioquímica i Biologia Molecular, Facultat de Biologia, Universitat de Barcelona, Av. Diagonal 643, E-08028 Barcelona, Spain. Fax: +34 93 402 15 59.
E-mail address: millobera@ub.edu (M. Llobera).

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proteins that interact with LPL, such as the activating cofactor apolipoprotein C-II or GPIHBP1 [14]. Heparin administration induces the release of mature LPL from the endothelium to the bloodstream and LPL activity in post-heparin plasma (PHP) is determined in the diagnostic of LPL deficiency [12]. The recent development of gene therapy to treat LPL deficiency in patients with familial chylomicronemia underscores the efforts directed to combat LPL-related disorders [14–16].

Given the importance of LPL in human health and disease, we aimed to explore the existence of LPL isoforms in humans. Remarkably, most of the information about the LPL sequence is derived from the LPL gene or transcript while studies on the human LPL protein are scarce and based on LPL derived from milk [17,18], whose function remains an open question [19]. Here, we analyzed LPL in PHP from human healthy volunteers. Due to the complexity and dynamic range of protein concentrations in human plasma [20], we combined affinity-based purification strategies, 2DE and MS to uncover the presence of LPL pI isoforms in humans.

2. Materials and methods

2.1. Subjects

PHP was obtained from four healthy adult (between 20 and 30 years) men volunteers, with a body mass index between 18.5 and 25, through the Centre for Drug Research (CIM), Sant Pau Hospital, Barcelona, Spain. The study was approved by the Ethics Committee on Research Involving Human Subjects of the Sant Pau-CIM and the "Agencia Española del Medicamento y Productos Sanitarios" (EUDRA CT2007-007194-23). Informed consent was obtained from all individuals.

2.2. PHP samples

After overnight fasting, post-heparin blood (240 mL) was obtained 15 min after an intravenous injection of 50 U/kg heparin (5% w/v sodium heparin, Rovi). Blood samples were kept on ice until centrifugation (1620*g*, 10 min, 4 °C). The separated plasma was immediately frozen and stored at −80 °C until analyzed. This method has been widely used for over 50 years [21–23].

2.3. Heparin-Sepharose affinity chromatography

LPL was partially purified using heparin-Sepharose affinity chromatography as described elsewhere [10,24]. Partially purified LPL was precipitated using trichloroacetic acid followed by acetone washing [25].

2.4. LPL activity assay

The assay of LPL activity has been described in detail elsewhere [24,26]. One unit of lipase activity corresponds to the release of 1 μmol of oleate per minute at pH 8.5 and 25 °C. As hepatic lipase in plasma cross-reacts with LPL lipolytic assay, PHP samples were incubated 1:1 v/v with anti-HL serum for 120 min at 4 °C prior to the assay. LPL activity was also determined in fractions of the heparin-Sepharose chromatography. In this case, the incubation with anti-hepatic lipase (HL) serum was omitted because HL and LPL elute separately on the heparin-Sepharose chromatography [27,28].

2.5. IgG depletion

Partially purified LPL from human PHP contained IgG that was depleted from the sample using a column packed with 1 mL of Protein G Sepharose (HiTrap Protein G HP, GE Healthcare) before 2DE. 7 mL of partially purified LPL from PHP were loaded to the column at a flow rate of 0.7 mL/min. The IgG was retained in the column and the flow through was collected.

2.6. One-dimensional electrophoresis

Chromatographic fractions of PHP were mixed with sample buffer (250 mM Tris–HCl, pH 6.8, 40% v/v glycerol, 8% w/v SDS, 600 mM DTT and bromophenol blue) 3:1 v/v, boiled for 10 min and applied to a 9% w/v polyacrylamide gel.

2.7. Two-dimensional electrophoresis

The sample, before or after IgG depletion, was processed as we described elsewhere [10] and applied to rehydrated IPG strips. Isoelectrofocusing was performed at 20 °C on IPGphor (GE Healthcare) according to the following schedule: linear ramp to 500 V in 1 h, linear ramp to 1000 V in 1 h, linear ramp to 5000 V in 1 h and 5000 V up to 25 KVh. After that IPG strips were cut at 7.5 cm from anode (pH 6) and processed as we described before [10]. SDS–PAGE was performed using 9% w/v polyacrylamide gel, the equilibrated strips were loaded and sealed using a solution containing 0.5% w/v agarose, 15 mM Tris, 0.1% w/v SDS, 192 mM glycine and bromophenol blue using the Mini-Protean II Electrophoresis Cell (Bio-Rad). The second dimension was carried out at 50 V for 30 min and at 150 V until the end of electrophoresis.

2.8. Silver staining of proteins in gel

Proteins were silver-stained using a staining procedure compatible with mass spectrometry, as previously described [10].

2.9. LPL Western blot assay and quantification

We followed the method described by Casanovas et al. [10,29] using monoclonal antibody 5D2 (1:2000 v/v, the kind gift of Dr. J.D. Brunzell, University of Washington, Seattle, WA, USA).

The quantification was done on images of LPL 2DE Western blot. To this end, the density of all spots in each film was measured by densitometry (Multi Gauge Fujifilm). The relative abundance of each spot/isoform was calculated relative to the total LPL content in the sample. For each individual, three measurements from three different membranes were averaged. In turn, each measurement was calculated as the average of at least three different exposition times (data not shown). In addition, we calculated the apparent pI of each spot. Based on the linear pH gradient of the IPG strips, the pI of each spot was inferred from the distance between the spot and the anodic (pH 6) edge of the gel.

2.10. In-gel digestion

The spots of interest were excised from silver-stained gels and subjected to in-gel digestion with trypsin (Promega), using a Digest ProMS (Intavis Bioanalytical Instruments AG) and following standard procedures [30,31]. The process includes washes with ammonium bicarbonate pH 7.8, reduction with DTT, alkylation with IAA and enzyme digestion with trypsin (37 °C, 8 h). Tryptic peptides were extracted with ACN/water (1/1, v/v) and 0.25% v/v TFA. Extracts were dried in a Speed-Vac and redissolved in 5 μL of ACN/water (1/1, v/v) containing 0.1% v/v TFA.

2.11. Mass spectrometric protein identification

Proteins were identified with a MALDI-TOF/TOF mass spectrometer (AB Sciex 4800) by peptide mass fingerprinting (PMF), as we

described elsewhere [10]. MASCOT engine was used for searches. All identifications were manually validated. The database used for identification was UniprotKB/SwissProt restricted to human proteins. The parameters used for the searches were trypsin digestion, 2 missed cleavages, carbamidomethylation of cysteine, oxidation of methionine and 25 ppm maximum error tolerance.

Some spots were also analysed by LC–MS/MS, using an LTQ-Orbitrap high resolution mass spectrometer (ThermoFisher, San Jose, CA). The instrument was set to perform a high resolution (60,000 FWHM) MS scan in the range of m/z 400–1800 followed by an MS/MS scan on each of the 8 most intense signals detected on the MS scan. Proteome Discoverer 1.3 software was used for protein identification.

3. Results

LPL activity in plasma after heparin administration (PHP) was similar in all individuals (111 ± 11.2 mU/mL) and dramatically higher than before heparin administration (0.7 ± 0.5 mU/mL). Despite the increased levels of LPL activity in PHP, the enzyme was not detected by Western blot in crude samples (Fig. 1C). For this reason, we partially purified LPL from human PHP by heparin-Sepharose affinity chromatography. The result of a representative purification is shown in Fig. 1A. Fractions collected upon elution with 1.5 M NaCl have high levels of LPL activity and a low amount of total protein. The peak of lipolytic activity observed upon elution with 0.75 M NaCl corresponds to HL activity, which has lower affinity for heparin than LPL [27,28]. Importantly, the partial purification enabled LPL detection by Western blot, which further substantiated the presence of the enzyme exclusively in the 1.5 M NaCl eluate (Fig. 1C). The fractions collected at this peak of 1.5 M NaCl were pooled and used as the source of LPL to analyze the possible presence of pI isoforms in human PHP.

The analysis of partially purified LPL by 2DE Western blot (Fig. 2) revealed the existence of nine isoforms with an apparent pI between 6.8 and 8.6. The pattern of LPL pI isoforms was remarkably similar between the four individuals studied. We speculate that the stain observed on the left in Fig. 2 (individual 1) may be LPL not focused due to the high amount of sample loaded to facilitate the detection of minor spots.

Despite the presumed high specificity of the antibody used in the Western blot (5D2) [32], the nonspecificity of other antibodies against LPL detected in previous studies [29] prompted us to identify the spots detected in the Western blot analysis using MS. To this end, proteins were separated by 2DE and silver-stained, and the spots of interest were excised for protein identification by PMF (Fig. 3A). IgG was initially identified in all spots (Table S1). Notably, IgG is one of the most abundant proteins in human plasma [33] and, despite partial LPL purification with heparin-Sepharose affinity chromatography, IgG was present in the 1.5 M NaCl eluate fractions. The heavy chain of this immunoglobulin has a similar molecular weight [34] and pI to that of LPL [33]. For this

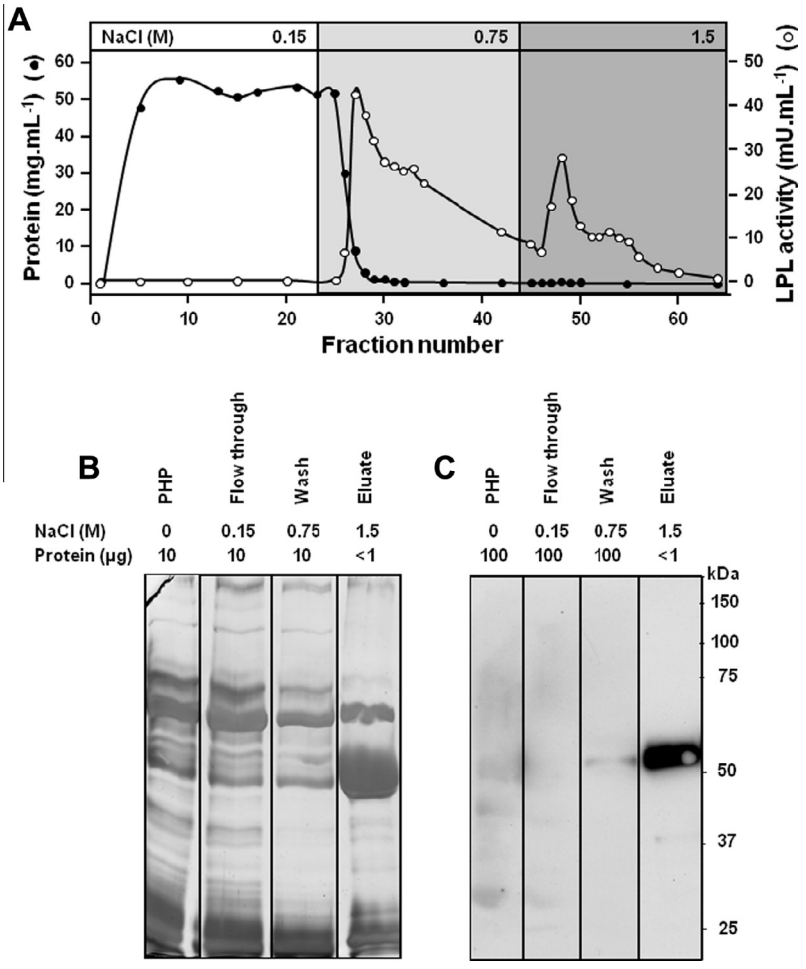


Fig. 1. Partial LPL purification from human PHP using heparin-Sepharose affinity chromatography. (A) Fractions were collected after a stepwise increase in NaCl concentration, as indicated at the top. Total protein content (filled circles) and LPL activity (open circles) in fractions are given. SDS–PAGE and silver staining (B) and Western blot against LPL (C) of fractions at different steps of the purification process are shown. The step and the amount of sample loaded are indicated above each lane.

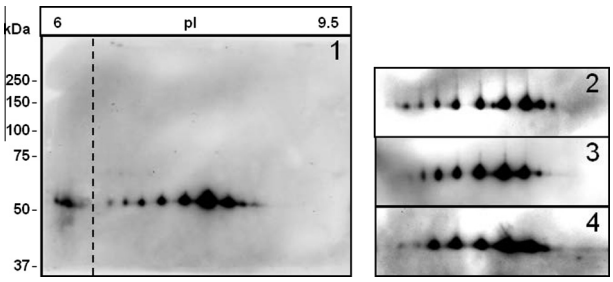


Fig. 2. LPL pI isoforms in human PHP. 2DE Western blot against LPL of partially purified LPL from human PHP. 2DE was performed using pH 6–11, 11 cm IPG strips in the first dimension. Western blots belong to the four individuals studied (1–4).

reason, both proteins overlap in the gel and the high abundance of IgG hampers the identification of low-abundance underlying proteins, including LPL. Therefore, prior to 2DE analysis, we depleted the samples of this IgG by using HiTrap Protein G HP (Fig. 3B), which enabled the identification of LPL isoforms by PMF and MS/MS (Table S2 and Table S3).

Finally, to compare the pattern of LPL pI isoforms found in the four individuals studied, we quantified the density of each spot in each 2DE Western blot (Fig. 4). The variability found between individuals (SEM in Fig. 4) was of the same order of magnitude as in the three technical replicates from each individual (data not shown), indicating that the variability observed between subjects can mainly be attributed to the methodology used. Therefore, the pattern of LPL isoforms (pI and relative amount) found in the four individuals studied is very similar. The most abundant isoform (33% of total LPL content) has an alkaline pI (8.01) whereas the next two most abundant isoforms have neighbouring pIs (7.71 and 8.31). The theoretical pI calculated for mature human LPL based on the amino acid sequence (http://web.expasy.org/compute_pi/) is within this range (8.23).

4. Discussion

In the present study we describe for the first time the existence of at least nine LPL pI isoforms in human PHP, with apparent pI between 6.8 and 8.6. The pattern of LPL isoforms (number and distribution) is consistent across the individuals studied and similar to that observed in other mammals [10], which suggests conserved features in the molecular origin of this heterogeneity likely related to the potential specific function and/or regulation of individual isoforms.

The origin of LPL isoforms in humans is unknown. Human LPL is encoded by a single gene [35]. In general terms, protein diversity derived from a single gene can be originated at the DNA, RNA, or protein level, due to allelic variations, alternative mRNA splicing or differential PTMs, respectively [36]. The multiple number of human LPL isoforms observed in single individuals rules out the possibility that such heterogeneity is only due to allelic variants and the conserved pattern between all individuals points towards an alternative origin of these differences. Two LPL mRNA of different sizes have been described in humans due to the alternative use of 2 possible polyadenylation sites [35]. These 2 transcripts differ in their translational efficiency but not in the final protein sequence encoded [37]. This scenario points towards the PTMs as the most likely origin of LPL pI isoforms.

The glycoprotein nature of LPL suggests that the diversity of the attached glycan structures may account for the pI heterogeneity observed. However, deglycosylation only partially reduced the number of LPL pI isoforms in the rat [10] indicating that other PTM may also be involved. Notably, human and rat LPL have 2 and 3 predicted

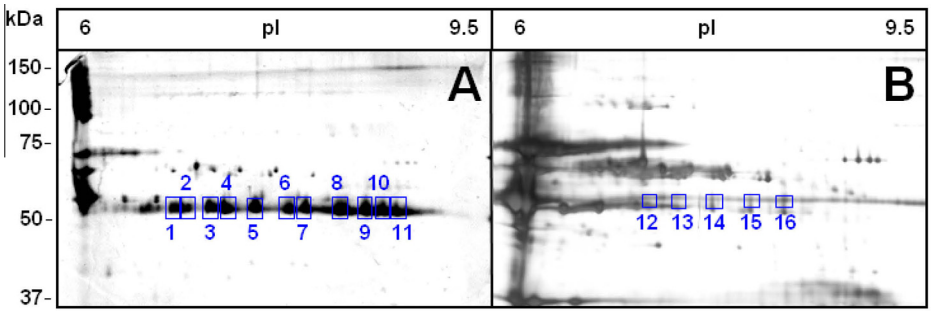
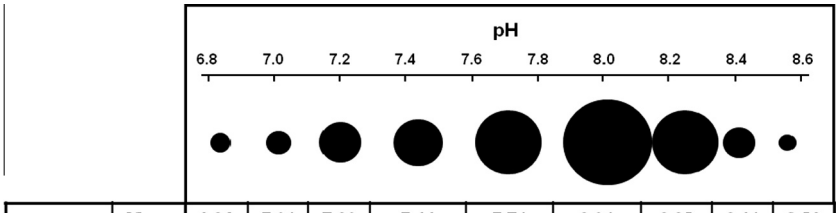


Fig. 3. IgG depletion enables the identification of LPL isoforms using MS. Two-dimensional analysis and silver staining of partially purified LPL from human PHP before (A) and after (B) IgG depletion. 2DE was performed using pH 6–11, 11 cm IPG strips in the first dimension. Spots excised and identified as LPL by PMF or LC–MS/MS are numbered (Tables S1, S2 and S3 in Supplementary data).



pI	Mean	6.84	7.01	7.20	7.44	7.71	8.01	8.25	8.41	8.56
	SEM	0.01	0.01	0.02	0.01	0.01	0.01	0.00	0.01	0.01
Relative amount (%)	Mean	1.73	3.00	7.76	10.39	18.95	33.00	18.86	4.94	1.32
	SEM	0.77	0.62	0.53	0.91	1.65	1.81	0.81	0.97	0.81

Fig. 4. pI and relative abundance of the nine LPL isoforms found in human PHP. The bubble size represents the amount of each isoform relative to the total LPL content. The location is according to their pI. SEM, standard error of the mean of $n = 4$.

glycosylation sites, respectively (predicted using NetNGlyc 1.0, Centre for Biological Sequence Analysis, Technical University of Denmark, Kongens Lyngby, Denmark), excluding a potential site, present in both species, with the Asn-Pro-Ser sequence that is rarely glycosylated in eukaryotes [1]. Hence, an increased number of LPL pI isoforms could be expected in the rat as the result of the higher number of combinations of glycan structures. By contrast, the number of LPL isoforms in human PHP is similar to that of rat heart LPL and higher than that of rat PHP, supporting the notion that other PTMs may contribute to LPL pI heterogeneity. Protein phosphorylation is a PTM previously associated to pI isoforms of other proteins [38]. However, we demonstrated that phosphorylation does not contribute to LPL pI heterogeneity in the rat [10]. In a recent study, we identified nitrated tyrosine residues in rat LPL in response to LPS challenge [11], demonstrating that LPL can undergo other PTMs *in vivo*. Hence, tyrosine nitration or other not yet identified PTMs can potentially contribute to LPL pI heterogeneity, although further research will be required to elucidate the molecular differences between LPL isoforms. Since PTMs induce a pI shift, the pI difference between LPL isoforms can provide the first small clue about the possible molecular origin of this heterogeneity. Finally, the presence of LPL isoforms in a single tissue in the rat [10] suggests that the multiorgan origin of LPL in PHP is not the source of LPL pI heterogeneity observed in human PHP.

At the current time, the potential functional and/or regulatory differences between LPL isoforms remain unexplored. However, since LPL in PHP is derived from the massive release to the bloodstream of mature LPL anchored to the endothelial lumen [7,39], the presence of LPL pI isoforms in PHP indicates that a population of mature LPL molecules with different net charges coexists at certain pH. Importantly, the molecular binding between LPL and the heparan sulfates in the endothelium is mediated by electrostatic interactions [40], between basic residues in the LPL molecule and the negative charge of sulfate groups in the proteoglycans. Hence, the differential net charge between LPL isoforms may impinge their heparin-binding affinity and play a role in the poorly understood release of LPL from its binding site at the endothelium. Notably, the release of LPL from the endothelium has been proposed as a physiological mechanism of tissue-specific LPL regulation [8,9] underscoring the need to advance our understanding of this process.

In conclusion, our results reveal the existence of LPL isoforms in humans raising a new dimension in LPL biology. In light of these results, elucidating the molecular nature and function of LPL pI isoforms emerges as a formidable task for future research with the potential to contribute to our understanding of aspects of LPL biology in physiological or pathological situations.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.02.028.

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