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Characterization of Lipid Binding Region of Lipoprotein Lipase

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Abstract

Lipoprotein lipase (LPL) is an enzyme that catalyzes the hydrolysis of triacylglycerols of chylomicrons and VLDL to produce 2-acylglycerols and fatty acids. The enzyme, LPL, is localized on the surface of the capillary endothelium and is widely distributed in extrahepatic tissues including heart, skeletal muscle and adipose tissue. LPL has been isolated from bovine milk by affinity chromatography on heparin-sepharose in 2 M NaCl, 5 mM barbital buffer, pH 7.4. To elucidate the lipid-binding region, LPL was digested with trypsin and then separated by gel filtration. Lipid-binding region of LPL has been investigated by recombining LPL peptides with DMPC vesicles. Proteolytic LPL fragments with DMPC were reassembled and stabilized by cholate. Lipid-binding region of LPL was identified by a PTH-automated protein sequencer, as AQQHYPVSAGYTK. The analysis of the secondary structure of the lipid-binding peptides revealed a higher probability of α-helix structure compared to the whole LPL protein. The prediction of hydrophobicity of lipid-binding region was highly hydrophobic (-1.1) compared to LPL polypeptide (-0.4).

Key words: lipoprotein lipase (LPL), lipid binding region, protein sequence

INTRODUCTION

Chylomicron and very low density lipoprotein (VLDL) triglyceride (TG) hydrolysis require lipoprotein lipase (LPL) (1-3). This enzyme is synthesized in adipose, muscle, and severial other tissues and is transported to the luminal surface of capillary endothelial cells (2,3). The function of LPL is to direct the influx of plasma TG in the form of fatty acids into the peripheral tissues for storage and to provide fuel for energy requirement. For this reason, the control of LPL is an important regulatory step for directing traffic of TG-fatty acids to fulfill the energy requirements of peripheral tissues in a tissue specific manner.

In addition to lipoprotein lipids, LPL also catalyzes the hydrolysis of water-soluble short chain fatty acyl ester substrates such as P-nitrophenyl acetate and P-nitrophenyl butyrate (4,5). For maximal hydrolysis of long chain fatty acyl esters, the enzyme requires apo C-II (6,7), a protein constituent of triglyceride-rich lipoproteins and high density lipoprotein (8); apo C-II does not enhance the hydrolysis of water-soluble substrate or of short chain triacylglycerols such as tributyrylglycerol. The deficiency or dysfunction of LPL has been found and it is associated with the pathogenesis of hypertriglyceridemia (1,3).

From the previous finding (9), the use of heparin affinity chromatography showed that there is a direct interaction between LPL and heparin. In addition, the release of LPL into the circulation by intravenous administration of heparin implies that this treatment causes detachment of LPL from the endothelium due to competition of heparin with cell-surface glycosaminoglycans (6). There is a general agreement that the interaction of LPL and heparin leads to the stabilization of LPL (10,11). This stabilizing effect may be the ground for the early suggestion about the role of heparin as an activator of LPL. In addition to the stabilizing of LPL, the interaction of heparin and LPL has also been seen to retard the hepatic clearance of the enzyme in circulation (2,5). Also, this enzyme is bound to the vascular endothelium by affinity for heparin sulfate (6,9,11), and only low levels of LPL are found in the circulation. The binding of LPL to the endothelium is thought to be weakened by local fatty acid accumulation that may result from lipolysis of plasma triglycerides (12-14).

At present, the lipid binding site of LPL has not been identified. Although monoclonal antibodies to bovine LPL that block apo C-Ⅱ binding have been obtained (14), the exact apo C-Ⅱ binding site has yet to be determined. But one of the candidates is the high positive charged N-termlnal 50 residues of LPL. Enerback et al. (2) have identified a putative heparin binding region with the sequence of KVRAKRSSK. The deficiency or dysfuntion of LPL has been found and it is associated with the pathogenesis of hypertriglyceridemia (15,16).

The purpose of this study was to investigate the binding region between LPL and lipoprotein.

MATERIALS AND METHODS

Materials

Bovine fresh milk was obtained from dairy farm (Kangwon National University). Heparin sepharose, heparin, p-nitrophenyl butyrate (PNPB) were purchased from Sigma (St. Louis, USA). Acetonitrile, methanol, and HPLC grade water were obtained from J.T Baker (USA). Triglyceride GII kit was purchased from Wako chemicals (Japan). Other chemicals used

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were the highest reagent grade available.

Isolation of lipoprotein lipase from bovine milk

Unpasteurized raw skim milk was obtained from the dairy farm (KangWon Natl. Univ.). Isolation of LPL (Fig. 1) was followed by the previous method (9). In a cold room, the milk was stirred with 200 ml of heparin sepharose per 4 L of milk. After overnight, filtration was begun using 500 ml coarse sintered glass funnel. The heparin sepharose containing the bound lipase was then washed as described (9,17); 2 L of 5 mM barbital, 0.2% triton X-100, pH 7.4; 2 L of 5 mM barbital, pH 7.4; and 2 L of 0.75 M NaCl, 5 mM barbital, pH 7.4 (Fig. 1). The lipase bound resin was suspended in 0.75 M NaCl, 25% glycerol, 5 mM barbital (pH 7.4) and packed into a column, 2.8×20 cm. After washed with at least 500 ml of the same buffer, lipase was collected overnight by step-wise elution with a solution of 2.0 M NaCl, 25% glycerol, and 5 mM barbital, pH 7.4, at a flow rate of 16 ml/hr. Tubes were read at 280 nm, and purity was analyzed by 10% SDS- PAGE. The protein concentration was measured by Biuret method.

Measurement of lipase activity

The reaction mixtures contained PNPB (50 µl), LPL (24 µg), and heparin (20 ng) in a final volume of 1.0 ml of 0.1 M sodium phosphate, pH 7.2, containing 0.9% NaCl. The hydrolysis of PNPB was determined by monitoring the increase of absorbance at 400 nm continuously using a no-enzyme contained mixture as a blank or by measuring the absorbance after extracting p-nitrophenol from the reaction mixture (4,9,14,18).

Enzyme digestion

Enzymatic digestion of LPL was performed with trypsin. The enzyme/substrate ratio was 1:100 (w/w). The mixture was stirred at room temperature for 6 hrs and hydrochloride was then added to the final concentration of 0.1 M to stop further enzyme reaction.

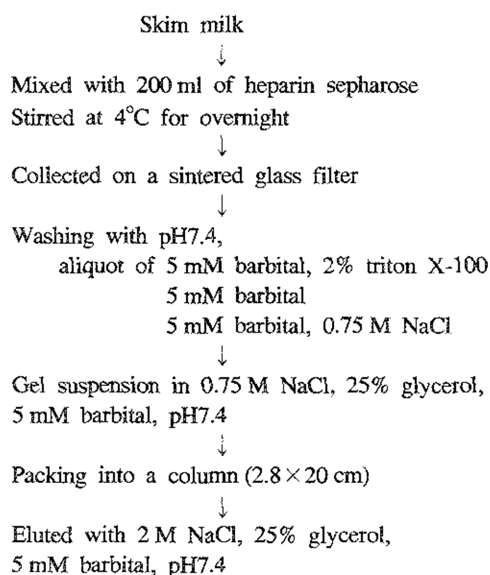

Fig. 1. Preparation of lipoprotein lipase.

Isolation of LPL lipid-binding peptides

A single bilayer vesicles of DMPC (dimyristoyl phosphartidylcholine) were prepared by sonication (13,14). 60 mg of DMPC in chloroform was placed in pyrex tube and the organic solvent was removed with a stream of nitrogen gas. The tube was placed in a lyophilized flask and placed under a vacuum for 10 min to remove the trace of solvent. Phospholipid dispersions were prepared by adding 5 ml of 0.9% NaCl, 0.01 M Tris-HCl, pH 7.2. The lipid dispersion was sonicated at 42°C for 15 min using a cell disrupter (Fisher, Inc. Dismembrator. Model 300). The sonicated vesicles were subjected to ultracentrifugation at 15°C in a Beckman type 70.1 Ti rotor for 1 hr at 40,000 rpm. After ultracentrifugation, the supernatant was used in the experiments. The digested LPL (17.6 mg in 20 ml) was mixed with sonicated single bilayer vesicles of DMPC (52.8 mg in 5 ml) at 1 hrs, at 24°C, the transition temperature of the lipid. The lipid protein complex was monitored using UV-spectrophotometer at 220 nm. The mixtures were subjected to ultracentrifugation at 15°C in a Beckman type 70.1 Ti rotor for 18 hrs at 30,000 rpm to separate the peptides that bind to DMPC from the rest of the peptides. The top 1.0 ml was then removed by pipette and discarded. The middle 5.0 ml was then collected and dissolved in 10% sodium cholate. The sample was applied to Sephadex G-50 (2.8×40 cm) column in 0.1 M ammonium bicarbonate buffer, pH 8.0, for desalting. The fractions that eluted in the void volume were lyophilized and subjected to additional purification by HPLC.

Peptide sequencing

The peptide separation was carried out by a Waters Associates HPLC system (9). Separation was achieved with a Vydac C_{18} column, (Buffer A: 0.1% TFA in water, Buffer B : 0.08% TFA in 95% acetonitrile and 5% water), at the rate of 1% B per min and a flow rate of 1.5 ml/min. Peptides were detected by their absorbance at 220 nm. Individual peaks were collected by the manual method and freeze dried. The dried peptides were detected by Biuret method and were analyzed for amino acid sequence using a gas-phase automated sequencer (Applied Biosystems). Phenylthiohydantoin (PTH) amino acids were identified by on-line analytical HPLC system equipped with a Brownlee C_{18} column.

Computer analysis of the determined lipid-binding peptide

The prediction of the secondary structure of the lipid binding peptide was obtained from the probability method of Fasman (17). Other predictions were based on the hydrophobicity profiles of Kyte and Doolittle (19).

RESULTS AND DISCUSSION

LPL was isolated from unpasteurized bovine milk by affinity chromatography on heparin-sepharose in 2 M NaCl, 5 mM barbital buffer, pH 7.4. and purity was identified by 10% SDS-PAGE.

Molecular weight of LPL from previous result (9) was

55KD on SDS-PAGE, and LPL activity was improved approximately 5 times when heparin was added. The regulation of LPL is an important regulatory step for directing metabolism of TG-fatty acids to fulfill the energy requirements of peripheral tissues.

Separation of trypsin digested LPL peptide

To elucidate the lipid-binding region, LPL was digested with trypsin. And, lipid-binding region of LPL has been investigated by recombining LPL peptides with DMPC vesicles. Proteolytic LPL fragments with DMPC were reassembled and stabilized by cholate, and then separated by gel filtration (Fig. 2) and followed by HPLC (Fig. 3). And, lipid-binding region of LPL was identified by a PTH-automated protein sequencer.

To find the lipid binding region, LPL was digested with trypsin first, and bound to single bilayered DMPC vesicle, and then separated into tryptic digested mixtures with DMPC vesicles by gel filtration (Fig. 2) to ensure that the lipid associating peptides were derived from a mixture of small, soluble species and were not representative of only those peptides that did not aggregate.

The gel filtration chromatogram shows that the major peaks consisted of two parts, designated as fraction I and II. The two fractions were pooled and lyophilized, then, separated with HPLC system. Fig. 3 shows the HPLC chromatogram of fraction I and II. In fraction I, the major peaks of isolated peptide were 3, such as retention times of 11.6, 11.8 and 15.7 min, respectively. From separated small-sized peptides, 15 peaks, were isolated and lyophilized for identification. These peaks were tested with Biuret test to see if they contained peptides. The result of this test was that retention times (min) of 7.4 (1), 8.5 (2), 10.3 (3), 11.6 (4), 15.7 (5) and 17.1 (6) showed positive in the test. But, peaks from retention

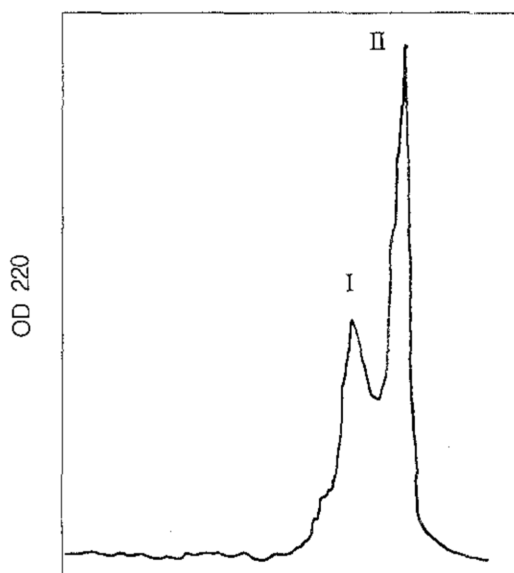

Fig. 2. Separation of mixtures of trypsin-digested LPL peptides and DMPC vesicle with Sephadex G-50. LPL was digested with trypsin, and mixed with DMPC vesicle. After centrifugation, the middle 5 ml was collected and was applied to Sephadex G-50 column.

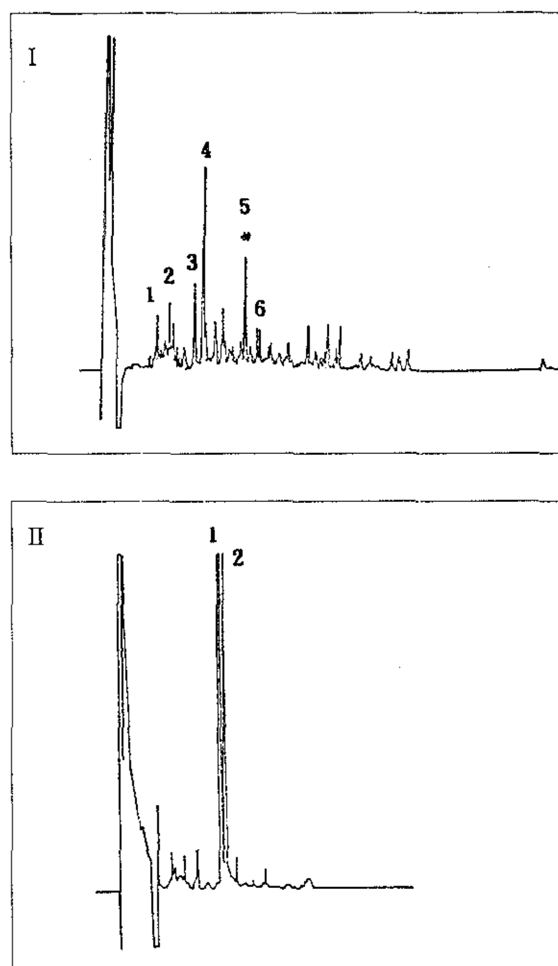

Fig. 3. HPLC separation of trypsin-digested LPL fractions I and II.

times (min) of 7.4 and 8.5 were putatively considered as dipeptide or free amino acids, because the earlier elution peaks at reverse phase column are small molecular size and highly polar. Peaks of retention times (min) of 10.3, 11.6 and 15.7 were lyophilized and analyzed with a gas-phase automated protein sequencer.

Amino acid sequence of lipid binding region

Results from PTH-amino acid sequence analysis, only one peak (retention time, 15.7 min) showed the peptide sequence. It was considered as the lipid-binding peptide, and was identified as AQQHYPVS.

But, in fraction II, two major peaks appeared on the HPLC chromatogram, and collected and analyzed with protein sequencer. However, two peaks did not show any amino acid sequence. And, compared with the fraction I, the sequence of first peak from two peaks in fraction II was conformed as same as the 4th peak (retention time, 11.6 min) of fraction I.

Trypsinized LPL, which contains 34 lysine and 21 arginine residues, potential sites of tryptic cleavage, should give rise to over 50 peptides on this separation. The primary structure of each cycle of the lipid-associating peptide was determined by automated techniques and the results are presented in Fig.

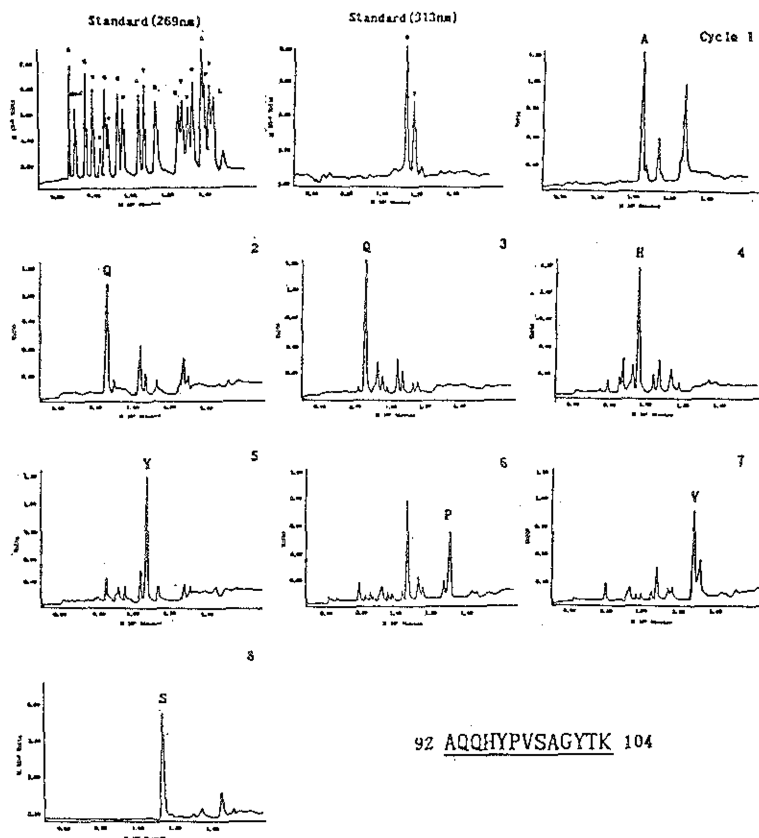

Fig. 4. Sequence analysis of lipid-binding region of LPL.

4. The selective purification of peptides suggests that the interaction of these peptides with lipid is specific and is a function of structural determinants that are not distributed evenly through the entire lipase polypeptide chain.

Computer prediction

Also, an analysis of the secondary structure of the lipid-binding tryptic peptides of LPL (AQQHYPVS) with the modified algorithm of Chou and Fasman (17) revealed a higher probability of α-structure in these fragments compared to the whole LPL protein. As a consequence, the obtained peptide was close to the region that corresponds to enzyme-active site and lied on the surface of the enzyme, and, also, β-turn was shown on the secondary prediction of lipid binding region (Figs. 5 and 6).

Prediction of hydrophobicity of lipid binding region by Kyte and Doolittle method (19) showed high nonpolar (-1.1) property which means less polar than total LPL polypeptide (-0.4). The present computer analysis data showed that the lipid binding regions of LPL liberated by trypsinolysis are highly hydrophobic.

Lipoprotein lipase (LPL) is acylglycerol hydrolase (EC 3.1.1.34) and its presence in plasma was known as 'Clearing factor'. The observed phenomenon of plasma clearance of turbidity by 'Clearing factor' was the result of action of a lipolytic enzyme. LPL is synthesized in adipose, muscle, and several other tissues and is transported to the luminal surface of capillary endothelial cells where it can interact with cir-

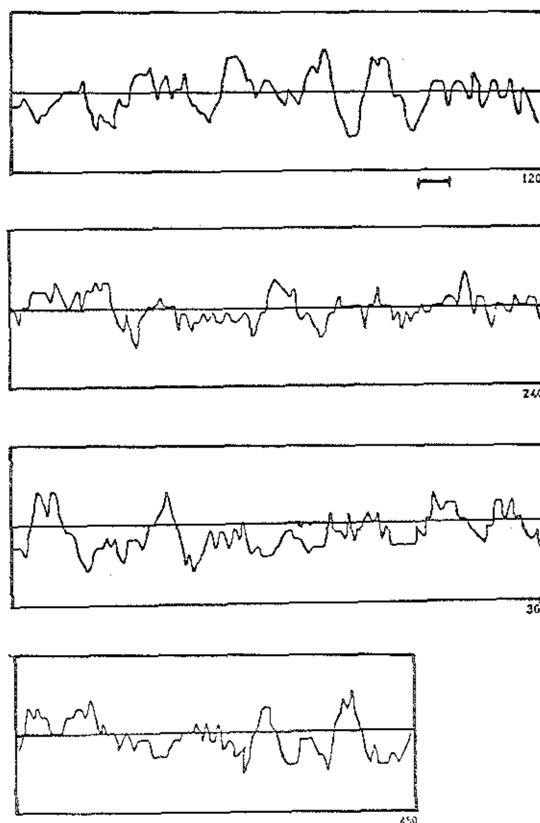

Fig. 5. Hydrophobicity profile of LPL by Kyte and Doolittle method hydrophobicity of LPL were predicted by computer analysis.

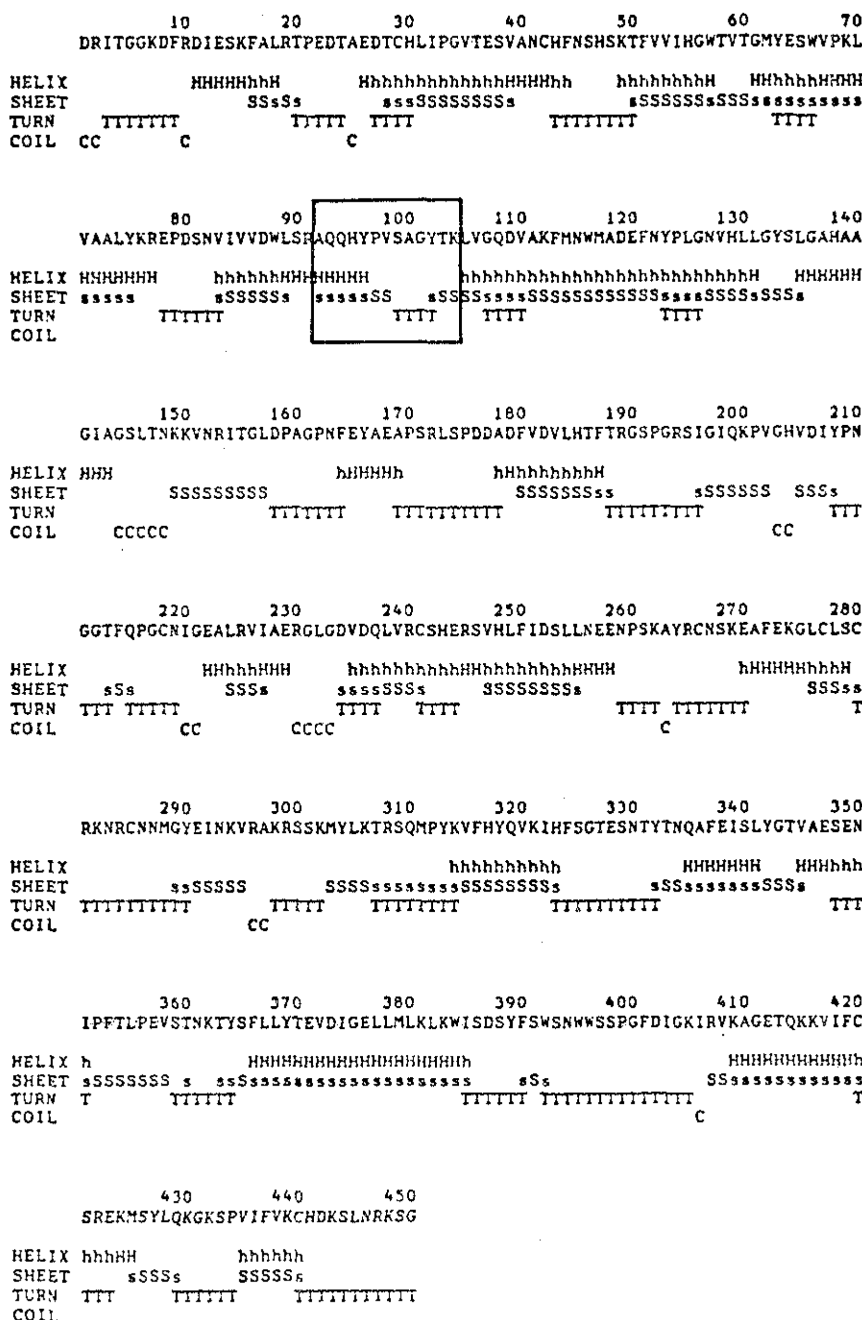

Fig. 6. Secondary structural prediction of LPL by Fasman method. Fasman computer prediction method was used to predict the secondary structure of LPL.

culating lipoproteins.

The function of LPL is to direct the influx of plasma TG in the form of fatty acids into the peripheral tissues for storage and to provide fule for energy requirement. For this reason, the control of LPL is an important regulatory step for directing traffic of TG-fatty acids to fulfill the energy requirement of peripheral tissues in a tissue specific manner.

There is a general agreement that the interaction of LPL and heparin leads to the stabilization of LPL (11). The binding of LPL to the endothelium is thought to be weakened by local fatty acid accumulation that may result from lipolysis of plasma triglycerides (3,8).

High concentration of either short chain or saturated FA, or both showed only weak capacity to dissociate LPL from the endothelial cells *in vitro* whereas specifically cis-monounsaturated as well as polyunsaturated FA, were highly active in that respect (6,12). Some degree of parallelism was also seen between plasma triglycerides and LPL activity. This could imply lipoprotein binding of LPL.

LPL is released from its endothelial binding by a highly local free fatty acid concentration induced by active lipolysis and subsequently binds to lipoprotein remnants, serving as a signal that the particle is ready to be taken up by the liver (15,16). Plasma LPL may thus reflect the presence of remnant

lipoprotein particles. A fatty acid feed-back control system has been proposed to regulate LPL at the vascular endothelium. The rate of lipolysis of triglyceride-rich lipoproteins can be partly regulated by accumulated free fatty acid that dissociates LPL from its endothelial binding sites.

The binding, uptake, and degradation of LPL by various cell types have been compared (20,21). Cells which synthesize LPL, i.e, mesenchymal heart cells and adipocytes showed at least 10-fold higher ratio of degradation to binding than endothelial cells and fibroblasts which do not synthesize LPL. An alternative route of clearance for LPL is via release of it from the endotheloal cells into the circulation and eventual degradation in the liver and other tissues (12,15). However the mechanism responsible for release of LPL from endothelial cells has not been delineated. By contrast, the inhibition of LPL activity due to fatty acids is widely believed to be mediated by a specific binding site for fatty acids on the LPL molecules (11,22). The regulation of LPL activity involves a number of intra- and extracellular events. Hormonal and nutritional factors have been shown to regulate LPL synthesis and activity in adipose tissue *in vitro* (3,10).

The LPL contains 10 cystein residues all of which exist in disulfide linkages. One highly conserved sequence (Gly-X_{aa}-Ser-X_{aa}-Gly) contains the active site serine (4,16). And, Ser-Asp-His, are essential to the catalytic activities of LPL (16).

At present, the location of the cofactor binding site of LPL has not been identified. Although monoclonal antibodies to bovine LPL that block apo C-II binding have been obtained (14), the exact location of the apoC-II binding site has yet to be determined. But one of the candidates is the high positive charged N-terminal 50 residues of LPL. However, some investigators (16) have described 9 residue sequence containing Gly-X-Ser-X-Gly as part of a lipid binding segment.

LPL is an important enzyme in lipid metabolism, thus, the deficiency or dysfunction of LPL has been reported that it is associated with the pathogenesis of hypertriglyceridemia (13,15).

Further studies should be continued on the effects of LPL activity on lipid metabolism.

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