Insulin-Stimulated Release of Lipoprotein Lipase by Metabolism of Its Phosphatidylinositol Anchor

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Lipoprotein lipase (LPL) plays a critical role in the metabolism of plasma lipoproteins. In 3T3-L1 adipocytes, insulin elicits the rapid release of LPL through mechanisms that are independent of energy metabolism and protein synthesis. Some of the metabolic actions of insulin may be mediated by the activation of a specific phospholipase that hydrolyzes a glycosyl phosphatidylinositol (PI) molecule. The insulin-sensitive glycosyl-PI is structurally similar to the glycolipid membrane anchor of a number of proteins. LPL appears to be anchored to the 3T3-L1 cell surface by glycosyl-PI, and its rapid release by insulin may be due to activation of a glycosyl-PI–specific phospholipase C.

LIPOPROTEIN LIPASE (LPL) IS SYNthesized and released by the parenchymal cells of various tissues. The enzyme is then transferred through the vascular endothelium and binds to the capillary luminal surface. At this site, the enzyme hydrolyzes tri- and diglycerides from circulating chylomicrons and lipoproteins. Although LPL was previously thought to be synthesized as a cytosolic enzyme, recent studies have revealed that in some cells LPL is associated with several membrane structures (*1*). Little is known about the precise structure of the enzyme, its functional requirements for activity, or the molecular mechanism involved in its release from cells.

Insulin appears to be the predominant hormonal regulator of LPL in adipose tissue. Severe uncontrolled diabetes is always associated with hypertriglyceridemia and low plasma LPL activity, both of which revert to normal levels upon insulin treatment (*2*). The biochemical events involved in the hormonal regulation of LPL activity have been most thoroughly studied in mouse 3T3-L1 cells, embryonic fibroblast cells that differentiate to adipocytes. Insulin appears to stimulate both the transcription and translation of the enzyme. In addition, insulin induces rapid release (in less than 30 min) of LPL from 3T3-L1 cells; this action of insulin is independent of energy metabolism and protein synthesis (*3*).

Because insulin induces rapid release of LPL from 3T3-L1 cells with a time course and concentration range that are similar to those observed in regulation of intermediary metabolism, related signal transduction mechanisms could be involved. We proposed earlier (*4*) that some of the actions of insulin are mediated by the activation of a specific phospholipase that hydrolyzes a glycosyl phosphatidylinositol (PI) molecule. As a result, diacylglycerol and an inositol phosphate glycan that modulates the activities of certain insulin-sensitive enzymes are generated (*4*). The insulin-sensitive glycosyl-PI is structurally similar to a glycolipid membrane anchor of various proteins (*5*). The anchor contains a covalent linkage from the protein to an oligosaccharide, which is in turn glycosidically linked to PI. Although the precise physiological role of the glycosyl-PI protein anchor is unknown, it may represent a mechanism to permit hormonal regulation of the concentration of a protein at the cell surface or regulation of its rate of release. We therefore explored the possibility that the activation of a glycosyl-PI–specific phospholipase by insulin might represent the means by which LPL is rapidly released by the hormone.

3T3-L1 cells were exposed to insulin or to a PI-specific phospholipase C (PLC) isolated from *Bacillus thuringiensis*, and the release of LPL activity into the medium was monitored (Fig. 1). The bacillus PLC has no detectable protease activity and can hydrolyze PI and its glycosyl derivatives, but no other phospholipids (*6*). Both insulin and PI-PLC stimulated the release of LPL activity into the medium. In both cases the release was maximal within 10 min and declined to basal levels by 30 min (Fig. 1A). This rapid release of LPL by insulin and PI-PLC was not inhibited by cycloheximide (*7*), an indication that the process is independent of protein synthesis. The kinetics of release of

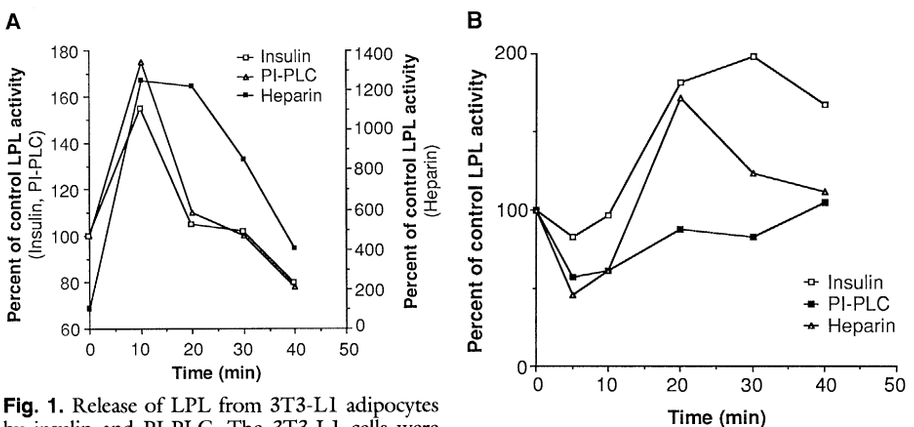

Fig. 1. Release of LPL from 3T3-L1 adipocytes by insulin and PI-PLC. The 3T3-L1 cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (BSA) (Gibco, 19P1266). Plates (100 mm) of confluent cells were differentiated with 1 μ*M* dexamethasone, insulin (1 μg/ml), and 0.5 m*M* isobutylmethylxanthine for 2 days. The medium was then replaced with DMEM plus 10% bovine serum albumin (BSA), which was changed every second day. The culture was used 7 to 14 days after attaining confluence. (**A**) On the day of the experiment, the cells were rinsed three times with 2 ml of Hanks buffer, *p*H 7.4, with 0.1% BSA added. Each plate was then incubated at 37°C with either buffer alone (control), insulin (100 ng/ml), PI-PLC (4 U/ml) from *B. thuringiensis*, or heparin (50 U/ml) in 3 ml of Hanks buffer, *p*H 7.4, with 0.1% BSA added. At each time point indicated, a portion of medium was removed and the cells were returned to the incubator. The medium was centrifuged for 30 s in a Beckman microfuge (at 10,000*g*), and assayed for LPL activity in triplicate (*27*). Incubations were carried out at 37°C for 30 min in a total volume of 0.2 ml. The assay substrate contained a concentrated anhydrous emulsion of [^{3}H]triolean in glycerol stabilized by lecithin. The final concentrations were triolean, 5.66 μmol/ml; lecithin, 0.35 μmol/ml; albumin, 1% (w/v); and serum, 8.5% (v/v) in tris-HCl, 0.07*M* (*p*H 8.0), containing 8.5% glycerol. Variation between triplicates in each assay was less than 4%. (**B**) Plates (100 mm) of confluent 3T3-L1 cells were incubated with heparin (50 U/ml) for 60 min at 37°C in DMEM with 10% FBS. After incubation, the cells were washed three times with 2 ml of Hanks buffer, *p*H 7.4, with 0.2% BSA added, and then incubated with buffer alone, insulin, PI-PLC, or heparin, as described in (A). The results of several independent experiments consistently showed that LPL activity was maximally released within 10 to 20 min of exposure to insulin or PI-PLC. For insulin there was a three- to sevenfold increase over control and for PI-PLC a two- to threefold. One-hundred percent activity represents 11 mU per plate of cells, where 1 mU is the release of 1 nm of free fatty acid per minute.

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LPL induced by insulin was indistinguishable from that induced by PI-PLC but was quite different from that induced by heparin, which peaked by 20 min and declined to basal levels by 40 min. Since released LPL activity is rapidly lost at 37°C in the presence of 3T3-L1 cells (*8, 9*), the activity measured in the medium reflects the difference between the release and inactivation of the enzyme by the cells.

There is evidence that released LPL is bound to heparan sulfate proteoglycan at the cell surface (*10*). This proteoglycan appears to be anchored to glycosyl-PI in a rat hepatocyte cell line (*11*), although there is no evidence of such a covalent association in any other cell type. It is conceivable, therefore, that the release of LPL in response to insulin and PI-PLC may be secondary to the release of a pool of heparan sulfate. To determine whether insulin, PI-PLC, and heparin cause the release of LPL from similar or distinct pools, we first treated cells extensively with heparin, washed them, and subsequently exposed them to insulin, PI-PLC, or again to heparin (Fig. 1B). In cells pretreated with heparin, PI-PLC and insulin were still effective in the release of LPL, although the time course was slower. When heparin was then again added to those cells first treated with heparin, no further release of LPL activity occurred, thus illustrating that the heparin-releasable pool had been completely depleted. These results suggest that both insulin and PI-PLC stimulate the release of a pool of LPL that is probably covalently associated with cells, whereas the heparin-releasable pool [bound noncovalently to glycosaminoglycan at the cell surface (*10*)] consists of LPL that has most likely been previously released during ongoing cell culture conditions.

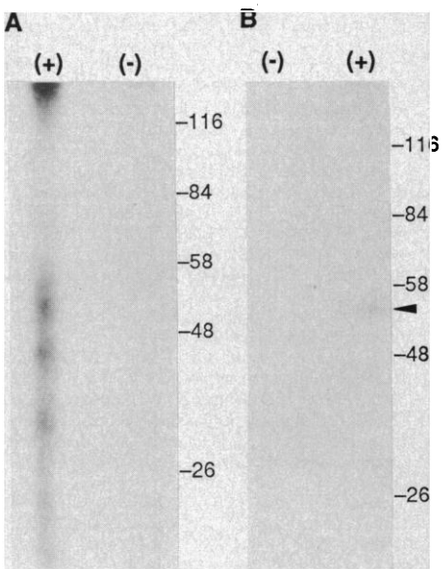

Fig. 2. PI-PLC–induced release of (**A**) [^{32}P]orthophosphate and (**B**) [^{3}H]glucosamine-labeled LPL. Differentiated 3T3-L1 cells were labeled with [^{32}P]orthophosphate (100 μCi/ml) (Du Pont, Biotechnology Systems) in 3 ml of phosphate-free DMEM with 5% dialyzed calf serum, or 20 μCi/ml of [^{3}H]glucosamine (Du Pont, Biotechnology Systems) in 3 ml of DMEM with 5% dialyzed calf serum for 16 hours. After labeling, the cells were washed three times with Hanks buffer, *p*H 7.4, with 0.2% BSA added, and treated with (+) or without (−) 4 PI-PLC (4 U/ml) in 2.5 ml of Hanks buffer at 37°C for 10 min. After incubation, the medium was removed and centrifuged for 30 s in a Beckman microfuge (at 10,000*g*). The supernatant was passed through a Millex GV 0.2-μm filter and then subjected to immunoprecipitation for 16 hours with a 1:100 dilution of rabbit antibody to human milk LPL. The specificity of this antibody for LPL has been established (*13*). After immunoprecipitation, the immune complex was sedimented by addition of Pansorbin cells (Calbiochem), washed three times before boiling in 100 μl of Laemmli sample buffer, and analyzed by electrophoresis in a 10% polyacrylamide gel (*28*). The dried gel was subjected to autoradiography at −80°C. The molecular size markers are β galactosidase, 116 kD; fructose 6-phosphate kinase, 84 kD; pyruvate kinase, 58 kD; fumarase, 48.5 kD; and triosephosphate isomerase, 26.6 kD.

Although the rapid release of LPL induced by PI-PLC and insulin was consistently observed in 3T3-L1 cells, the magnitude of the release was three to seven times that of the control for insulin, and two to three times that of the control for PI-PLC. The variation is probably due in part to the in vitro life span of the cells. Cells that have been cultured for more than six generations are less responsive to insulin, as also shown by reduced sensitivity of lipogenesis and glucose transport (*7*). The loss of insulin sensitivity correlated with the decreased release of LPL in response to insulin and PI-PLC, whereas the heparin-releasable component remained unchanged (*7*). Such variations in the release of LPL by insulin regarding the in vitro life span of 3T3-L1 cells have been previously observed (*12*).

To further explore the possibility that LPL is covalently linked to the cell surface by glycosyl-PI, we labeled cells with [^{32}P]orthophosphate and [^{3}H]glucosamine, both of which are components of the glycosyl-PI protein anchor. These labeled cells were treated with or without PI-PLC, and the proteins released into the media were immunoprecipitated with an antibody to human milk LPL that cross-reacts with the murine enzyme (*13*). The immunoprecipitates were then subjected to electrophoresis, followed by autoradiography (Fig. 2). The PI-PLC treatment of cells resulted in the liberation into the media of a specifically immunoprecipitated 55-kD protein faintly labeled with [^{3}H]glucosamine and more effectively labeled with [^{32}P]orthophosphate; the size of this protein corresponds to the known molecular size of LPL in 3T3-L1 cells (*8*). Two [^{32}P]orthophosphate-labeled bands of lower molecular size are probably proteolytic fragments of the 55-kD protein precipitated by the antibody. Because LPL is highly susceptible to proteolysis, such proteolytic fragments are frequently detected (*8, 14*).

Although the presence of [^{32}P]orthophosphate and [^{3}H]glucosamine in the immunoprecipitated LPL specifically released by PI-PLC supports the hypothesis that LPL is a glycosyl-PI–anchored protein, it is still possible that these radiolabeled precursors are incorporated into other sites on the protein. Therefore, experiments were also designed to detect the specific release of LPL that was surface labeled in intact cells. The 3T3-L1 cells were labeled with biotin. The labeled cells were then extracted with Triton X-114; the aqueous phase containing the water-soluble proteins was discarded, and the detergent phase was incubated with or without PI-PLC in the presence of protease inhibi-

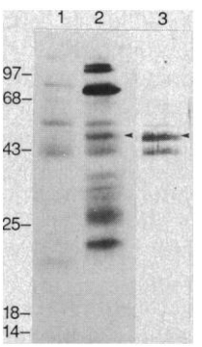

Fig. 3. Partitioning of Triton X-114–extracted LPL into the aqueous phase after treatment with PI-PLC. Plates (100 mm) of differentiated 3T3-L1 adipocytes were cell surface labeled with sulfo-*N*-hydroxysuccinimido biotin (Pierce Chemical) (0.5 mg/ml) at 4°C for 30 min (*29*). The cells were then lysed with 1 ml of 1% Triton X-114 in 10 m*M* tris, *p*H 7.4, 0.15*M* NaCl, and 1 m*M* EDTA containing leupeptin (10 μg/ml), pepstatin (10 μg/ml), and antipain (10 μg/ml) at 4°C for 60 min. After temperature-induced phase separation, the aqueous phase was discarded, and 100 μl of the washed detergent phase was diluted to 500 μl with 100 m*M* tris, *p*H 7.4, 50 m*M* NaCl, and 1 m*M* EDTA containing the same protease inhibitors and incubated at 37°C with continuous vortexing for 1 hour in the absence (−), lane 1, or presence (+), lane 2, of PI-PLC (6 U/ml) (*30*). Resulting aqueous phases were precipitated with sodium deoxycholate (125 μg/ml) and 6% trichloroacetic acid and resuspended in Laemmli sample buffer. Excess acid was neutralized with ammonium hydroxide vapors. After electrophoresis in a 10% polyacrylamide gel, proteins were transferred to nitrocellulose and incubated with ^{125}I-labeled streptavidin (25°C for 2 hours) under conditions that reduce nonspecific binding. Biotinylated protein was then visualized by autoradiography. The nitrocellulose filter (after autoradiography) was coated with polyclonal rabbit antibody to human milk LPL and visualized with alkaline phosphatase–conjugated goat antibody to rabbit immunoglobulin G as described by the manufacturer (Promega), lane 3. The molecular size markers are phosphorylase b, 97 kD; BSA, 68 kD; ovalalbumin, 43 kD; soybean trypsin inhibitor, 25 kD; and lysozyme, 18 kD.

tors. After this incubation, the solution was partitioned into detergent and aqueous phases to determine whether a putative detergent-binding domain of LPL had been removed by PI-PLC, as shown by the appearance of the protein into the aqueous phase. The aqueous phase material was precipitated with trichloroacetic acid and subjected to SDS–polyacrylamide gel electrophoresis (PAGE), followed by transblotting onto nitrocellulose, and visualization with ^{125}I-labeled streptavidin. An autoradiogram of the nitrocellulose blot indicates that PI-PLC caused the release of biotin-labeled proteins into the aqueous phase, including a 55-kD protein (Fig. 3). This 55-kD band was putatively identified as authentic LPL by coating the same nitrocellulose blot with polyclonal rabbit antibody to human milk LPL that was visualized with alkaline phosphatase–conjugated goat antibody to rabbit immunoglobulin G (Fig. 3, lane 3). The lower molecular size band is most likely a frequently detected proteolytic fragment of LPL (Fig. 3, lane 3). This experiment suggests that LPL is directly anchored to the cell surface by glycosyl-PI, since the high concentrations of Triton X-114 would have caused the release of the noncovalently associated proteins into the discarded aqueous phase of the first detergent extraction. However, we cannot conclusively exclude the possibility that the release of LPL from cells or its apparent conversion from a detergent to a water-soluble form by PI-PLC reflects the tight association of LPL with another glycosyl-PI–anchored protein.

All of the glycosyl-PI–anchored proteins analyzed thus far are attached to a glycolipid that contains an ethanolamine with a free amino group. This lipid-protein attachment is thought to occur in the lumen of the endoplasmic reticulum or Golgi apparatus (*15*). For at least two of the proteins studied thus far [Thy-1 and variant surface glycoprotein (VSG)], a proteolytic processing event near the carboxyl terminus must either precede attachment or occur at the same time (*16*). This proteolysis occurs at a predicted short hydrophobic region of these proteins, although no clear consensus sequence for the cleavage site has emerged (*5*). The predicted sequences of human adipose and mouse macrophage LPL reveal the presence of several short regions containing some degree of hydrophobicity, including one near the carboxyl terminus (*17*). The precise sequence of the 3T3-L1 enzyme has not been ascertained. Whether the amino acid sequence of the mature form of the murine fat cell–derived protein precisely matches that of the sequence predicted by complementary DNA cloning of the human adipose or mouse macrophage protein is unknown. Some processing event such as that which occurs in Thy-1 and VSG may be important for anchoring.

The similarity in the kinetics of LPL release by PI-PLC and insulin indicates that the release of LPL by PI-PLC occurs in a pool identical to that released by insulin and also suggests that the release of the enzyme by insulin may have resulted from activation of a glycosyl-PI–specific phospholipase. There is some evidence that insulin might also stimulate the release of other glycosyl-PI–anchored proteins. Insulin caused reduced levels of alkaline phosphatase in osteoblastoma cells (*18*). Observed changes in circulating alkaline phosphatase levels in fasting and refeeding in diabetes and in pregnancy (*19*) may be the result of hormonal regulation of the metabolism of the glycosyl-PI anchor. Insulin induces rapid release of this enzyme in BC_3H1 myocytes (*20*). Insulin also stimulates the release of PI-anchored heparan sulfate proteoglycan (*12*). In addition, the release of PI-anchored acetylcholinesterase from chromaffin cells occurs in response to certain stimuli (*21*), and PI-anchored 5′-nucleotidase levels in adipose tissue are increased in diabetic rats (*22*). Although a glycosyl-PI–specific PLC (*23*) and phospholipase D (PLD) (*24*) that can hydrolyze this anchor have been identified, there is thus far no evidence regarding the precise identity of a putative hormone-sensitive enzyme. Furthermore, it is not clear whether insulin will lead to the release of all accessible glycosyl-PI–anchored proteins or only a specific subset. Exploration of this issue may help to resolve whether there are distinct hormone-sensitive and insensitive "structural" pools of glycosyl-PI, as have been proposed for phosphoinositides (*25*).

This mode of anchoring for LPL in fat cells may ensure the proper orientation of the enzyme for access to plasma triglyceride, without allowing the hydrolysis of cell membrane lipids. This anchoring mechanism may also provide a means by which insulin, through activation of a specific phospholipase, can regulate the release, catalytic activity, and metabolism of the enzyme. Questions can now be raised concerning the topological orientation of the relevant phospholipases in the plasma membrane. The insulin-induced intracellular generation of the inositol phosphate glycan-enzyme modulators occurs presumably by hydrolyzing a cytoplasmically oriented glycophospholipid (*26*). Separate insulin-linked phospholipases may mediate hydrolysis reactions on different sides of the membrane. This issue can be resolved only when the structure of the isolated glycosyl-PI anchor for the insulin-released form of LPL has been analyzed.

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