Tumor Necrosis Factor Induced Release of Endothelial Cell Lipoprotein Lipase

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The major functional pool of lipoprotein lipase (LPL) that hydrolyzes triglycerides in circulating lipoproteins is located on the vascular endothelium. The macrophage-secreted cytokine tumor necrosis factor (TNF), a molecule known to affect endothelial cell functions, was used to test the hypothesis that alterations of endothelial cell metabolism regulate the binding of LPL to these cells. TNF addition induced rapid (maximum release at 45 minutes) dissociation of LPL protein and activity from its binding sites on cultured porcine aortic endothelial cells. LPL release by TNF required endothelial cell metabolic event(s) which involved cell secretion. In addition, LPL release was inhibited by pertussis toxin, suggesting the involvement of guanine nucleotide regulatory protein(s). Addition of arachidonic acid, a molecule known to be released by endothelial cells due to phospholipase A2 activation by TNF treatment, released LPL from the cell surface. Furthermore, direct modulation of cellular phospholipase A2 activity also led to changes in the release of LPL. Our studies demonstrate that alterations in the cellular metabolism of endothelial cells, for example, by TNF, may release functional pools of LPL from the vascular endothelium. This decrease in LPL on endothelial cell surfaces might be involved in the development of hypertriglyceridemia and redirection of energy flow during infections and inflammation.


Several metabolic and physiologic alterations characteristically follow parasitic and bacterial infections.1 These changes include mobilization of energy from peripheral muscle and fat sources and increased nutrient uptake by liver for protein synthesis. A large body of research has shown that many of the observed biological responses to infection are mediated by host-secreted cytokines.2 One such cytokine, tumor necrosis factor (TNF), also termed cachectin, is believed to be responsible for the pathological state of cachexia associated with chronic infections.3,4 Among the several host changes that frequently follow infection5–9 or TNF administration10 is hypertriglyceridemia due to marked elevations of plasma very low density lipoproteins (VLDL). Decreased VLDL catabolism during infection is due, at least in part, to a loss of activity of lipoprotein lipase (LPL) in peripheral tissues.5

LPL, which is bound to glycosaminoglycans on the vascular endothelial surface,11,12 is responsible for hydrolysis of triglycerides in plasma lipoproteins. The importance of endothelial cell-bound LPL in the hydrolysis of triglyceride-rich lipoproteins is supported by clinical observations. Patients with auto-antibodies to heparan sulfate molecules have marked hypertriglyceridemia and reduced levels of LPL activity in their postheparin plasma.13 This is presumably due to antibody inhibition of the attachment of LPL to endothelial cells. Thus, events that reduce LPL interaction with endothelial cells can regulate its activity in vivo.

Several studies have shown that TNF dramatically alters a number of properties of endothelial cells. These changes include modulation of the hemostatic and coagulant properties of the cell surface,14,15 increased secretion of platelet-derived growth factor (PDGF),16 and rapid release of arachidonic acid and eicosanoid products.17 In addition to these effects on endothelial cells, TNF administration to animals10 and humans18 affects LPL activity in various tissues and produces hypertriglyceridemia. Injection of TNF into rats, mice, and guinea pigs10 decreases adipose tissue LPL synthesis and activity. However, TNF increases LPL activity in heart, lung, liver, and plasma.10 Thus, TNF redistributes LPL activity among various tissues. This may direct the flow of fatty acids away from storage sites during infection or inflammation. Because of the known actions of TNF on endothelial cells and the role of endothelial cell bound LPL in triglyceride clearance, we explored whether the binding of LPL to the endothelial cell surface is altered by TNF treatment.

**Methods**

**Endothelial Cell Cultures**

Endothelial cells were isolated from porcine aorta and cultured as described previously.19 At confluence, endothelial cells were subcultured with 0.125% trypsin and 0.02% ethylenediaminetetraacetic acid. Endothelial cells were plated in dishes (35 mm×10 mm, Corning Glass Works, Corning, NY), and experiments were performed...
1 day after the cells reached confluency. The cells were cultured at 37°C in a humidified atmosphere of 5% CO₂ in air in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, antibiotics [100 units/ml penicillin and 100 μg/ml streptomycin (Hazelton Research Products, Lenexa, KS)], and glutamine (2 mM). The medium was replaced every other day. For some experiments, the cells were incubated for 18 hours with pertussis toxin (List Biological, Inc, CA), 100 ng/ml, or for 3 hours with colchicine, 200 μg/ml.

Purification of Bovine Milk Lipoprotein Lipase and Measurement of Enzyme Activity

LPL was purified from fresh bovine milk as described previously20 with the method of Soccoro et al.21 Unpasteurized milk was adjusted to 0.4 M NaCl by the addition of solid NaCl and was centrifuged at 3000 g at 4°C to remove the cream. Heparin-agarose gel (Bio-Rad, Richmond, CA) (80 ml gel) was added to the skim milk (3.5) and incubated at 4°C for 18 hours on a platform rocker. The gel was washed consecutively with 20 ml of Tris-HCl buffer (pH 7.4) containing 0.4 M NaCl and then with 0.75 M NaCl, and was transferred to a column (2.5x20 cm). The enzyme was eluted with 20 mM of Tris-HCl buffer (pH 7.4) containing 1.5 M of NaCl. Protein was determined by the method of Lowry et al.22 by using bovine serum albumin (BSA) as a standard. The purified enzyme had a specific activity of about 500 cpm/ng LPL, and the radioactivity in 1 ml of the aqueous phase was counted by using 5 ml of scintillation fluid (Hydroflour, National Diagnostics, Manville, NJ).

Recovery of Lipoprotein Lipase by TNF, PDGF, Quinacrine, Melittin, Indomethacin, or Fatty Acid:BSA Solutions

The release of 125I-LPL was studied by using cells to which LPL had been bound. The cells were incubated with 1 ml of media containing either recombinant human TNFα (Genentech, South San Francisco, CA), quinacrine, melittin, indomethacin (all from Sigma), purified PDGF, or fatty acid:BSA solutions at 37°C for 1 hour, unless otherwise stated. In some experiments, TNF was added together with either quinacrine or indomethacin. Fatty acid:BSA solutions were prepared by first dissolving the fatty acid (oleic or arachidonic acid) in isopropanol and then adding the solution to DMEM containing 0.44 mM BSA25 After the incubation, the media with the released 125I-LPL were collected. The cells were then treated with 100 units of heparin (The Upjohn Co., Kalamazoo, MI) for 15 minutes at 25°C to estimate the radioactive LPL protein remaining on the cells. Cell viability was routinely checked by trypan blue exclusion and was found to be unchanged under all conditions used.

Release of Radiolabeled Gycosaminoglycans

Endothelial cells were grown as described above and were labeled by incubation for 3 days in DMEM containing 36SO₄ (2.2 mCi/ml) and D-6-3H-glucosamine (1 mCi/ml). At the end of the labeling period, the medium was removed, and the cells were rinsed three times with 2 ml of DMEM. The cells were then incubated with medium alone or with medium containing TNF or insulin (100 ng/ml) for 1 hour at 37°C. The media were collected, and the glycosaminoglycans and proteoglycans were precipitated with 95% ethanol containing 1.3% potassium acetate or cetylpyridinium chloride. The radioactivity was measured.

Mitogenic Assay

Stimulation of 3H-thymidine incorporation into the deoxyribonucleic acid of cultured BALB/c 3T3 cells was measured by using media from TNF-treated or control endothelial cells. The 3T3 cells were pulse-labeled with 3H-thymidine for 2 hours after stimulation for 15 to
Figure 1. Release of lipoprotein lipase (LPL) from porcine aortic endothelial cells by tumor necrosis factor (TNF). Endothelial cells were cultured, and $^{125}$I-LPL was bound to the surface as described in the Methods section. The cells were then washed three times with 2 ml of Dulbecco's modified Eagle's medium and were incubated with 1 ml of medium containing various concentrations of recombinant human TNF at 37°C for 1 hour (A). The amount of $^{125}$I-LPL in the media and bound to the cells was then determined. A time course of the release of endothelial cell-associated LPL with either TNF (100 ng/ml) or oleic acid:bovine serum albumin (BSA) (6:1 molar ratio) is shown in B. The data are the average of two separate experiments performed in duplicate. The 100% (maximum) release of LPL into the media with TNF was 62 ng, and 73 ng with oleic acid:BSA.

17 hours. TNF was also added directly to 3T3 cells to determine whether it contained mitogenic activity.

Results

Cells with bound $^{125}$I-LPL were incubated with increasing concentrations of TNF, and the release of $^{125}$I-LPL into the media is shown in Figure 1A. TNF addition resulted in a concentration-dependent release of $^{125}$I-LPL, with a corresponding decrease in the amount of LPL remaining bound to the cells. The amount released reached a plateau at 100 ng of TNF per ml. Using 100 ng of TNF, the medium contained a threefold greater amount of $^{125}$I-LPL released than did control medium. Similarly, TNF released LPL catalytic activity bound to endothelial cells and decreased the amount of cell-associated activity (results not shown).

The time course of the release of $^{125}$I-LPL from the endothelial cells by TNF is shown in Figure 1B. The maximum release occurred at 45 minutes with no further increase. We have previously demonstrated that incubation with oleic acid can release LPL bound to the endothelial cells. The mechanism of release by oleic acid was postulated to be independent of any cellular metabolic event and due to direct interaction between oleic acid and LPL molecules. A comparison of the kinetics of $^{125}$I-LPL release induced by TNF and oleic acid (Figure 1B) shows that oleic acid-induced release was more rapid and peaked by 20 minutes. These results suggested that TNF-induced release of LPL may require additional events other than a direct interaction between TNF and LPL molecules.

A requirement for endothelial cell metabolic event(s) in the TNF-induced release of cell-associated $^{125}$I-LPL was suggested by the results of experiments in which release of LPL by TNF was studied at 4°C. Endothelial cells incubated at 4°C for 60 minutes with various concentrations of TNF (10 to 200 ng/ml) did not increase the release of $^{125}$I-LPL, compared to a control incubation (only 100 ng/ml TNF data point is shown) (Figure 2). TNF affects the secretion of a number of proteins and lipid molecules by endothelial cells which may, in turn, affect the endothelial cell membrane. To examine whether a cell secretory event was required for LPL release, cells were pretreated with colchicine, a cytoskeletal disrupting agent, that blocks cell secretion. The effect of pretreatment with pertussis toxin on LPL release was also studied. This agent inactivates guanine nucleotide regulatory proteins and has previously been shown to block the TNF-mediated increase in eicosanoid production by endothelial cells. Both colchicine and pertussis toxin abrogated the TNF-induced release of $^{125}$I-LPL (Figure 2).
The binding of LPL to the cell surface. TNF-induced secretion of molecule(s) from the endothelial cells. The cells were incubated with TNF (100 ng/ml) at 37°C for 1 hour, and this incubation media and media to which TNF was directly added were then tested for stimulation of 3H-thymidine incorporation into BALB/C 3T3 cells' nucleic acids. The results presented are the differences in the incorporation of 3H-thymidine between experimental incubations and control incubations (DMEM alone) in which 3000 cpm were incorporated. Each data point is the average of triplicate determinations.

Figure 3. The release of mitogenic stimulation activity from endothelial cells treated with tumor necrosis factor (TNF). Unlabeled lipoprotein lipase (LPL) was bound to endothelial cells. The cell surface-bound LPL was blocked by colchicine, LPL release may require the secretion of molecule(s) from the endothelial cells. One molecule secreted within short periods of time by vascular endothelial cells in response to TNF treatment is PDGF.16 PDGF is a cationic protein containing several basic amino acids capable of interaction with negatively charged endothelial cell surface glycosaminoglycans.26 In the above experiments, endothelial cells incubated with TNF did not release PDGF-like mitogenic activity (Figure 3). The maximum release was estimated to be less than 5 ng/ml. To determine if TNF-mediated release of cell surface-bound LPL was due to a competition between LPL and PDGF (secreted in response to TNF by endothelial cells) for the same binding sites on the cell surface, cells with 125I-LPL bound were incubated with increasing concentrations of purified PDGF. As shown in Figure 4, the addition of PDGF (up to 100 ng/ml) failed to release 125I-LPL bound to the endothelial cells. Thus, PDGF was unable to displace LPL from glycosaminoglycans on the endothelial cell surface.

Increasing amounts of arachidonic acid generated during TNF incubation with the cells may have affected the binding of LPL to the cell surface. TNF-induced release of arachidonic acid from bovine endothelial cells peaked at 15 minutes,17 while release of LPL by TNF was maximal at 45 minutes (Figure 1). This time course and our previous studies which demonstrated LPL release from endothelial cells by some types of fatty acids20,27 support this hypothetical role for arachidonic acid. Therefore, the effects of incubation of endothelial cells with increasing molar ratios of arachidonic acid:BSA on membrane-associated LPL were investigated. Figure 4 shows that addition of increasing arachidonic acid:BSA molar ratios decreased the amount of LPL bound to the cells; there was a corresponding increase in the amount of LPL in the media.

The generation of arachidonic acid by endothelial cells in response to TNF is mediated by the activation of phospholipase A2,17 in other cell types, phospholipase activation has been implicated in the actions of TNF.28 Because addition of arachidonic acid:BSA solutions were able to release LPL from the cells, we directly examined the role of phospholipase A2 in release of LPL. The addition of melittin (3 µg/ml), a protein which has been shown to stimulate phospholipase A2 activity,29,30 resulted in the release of LPL from the cells (65% increase over control incubation, Figure 5). In another set of experiments, the effect of quinacrine (3 µM), a phospholipase A2 inhibitor,31 on TNF-induced release of LPL was examined. Incubation of cells bound with 125I-LPL with quinacrine alone showed a slight decrease (15%) in the dissociation of LPL relative to control incubation (absence of quinacrine, Figure 5). This decrease may be due to the inhibition of some basal phospholipase A2 activity by quinacrine. The addition of quinacrine (3 µM) together with TNF (25 ng) completely abolished the ability of TNF to release LPL bound to the cells (Figure 5). The inhibition of TNF-induced release of LPL by quinacrine and the augmentation of this release by melittin suggest that phospholipase A2 activation is an important event in TNF-mediated release of LPL.

Since the TNF-stimulated release of arachidonic acid also leads to eicosanoid generation,17 eicosanoids may mediate TNF-induced release of LPL. To test this hypothesis, we added TNF (100 ng) to the culture medium...
Figure 5. The effect of melittin and quinacrine on the release of lipoprotein lipase (LPL) from endothelial cells. Endothelial cells with $^{125}$I-LPL bound were incubated at 37°C for 1 hour with melittin (3 µg/ml) or quinacrine (3 µM). Quinacrine was also added in the presence of tumor necrosis factor (TNF, 25 ng/ml). The results are expressed as percents of control incubation in the presence of DMEM alone. The amount of $^{125}$I-LPL (20 ng) released during this control incubation was assigned as 100%. Each bar represents the mean of three experiments±SE.

Together with 50 µM indomethacin, a cyclooxygenase inhibitor, which blocks the synthesis of eicosanoids derived from arachidonic acid. This addition only slightly decreased TNF release of LPL (<20% decrease) suggesting that eicosanoid generation from arachidonic acid is not important in TNF induced release of LPL.

LPL binds to endothelial cells via surface heparan sulfate molecules. We therefore examined whether the release of LPL in response to TNF was secondary to the release of glycosaminoglycans. Endothelial cell glycosaminoglycans were metabolically labeled with $^{35}$S-sulfate and $^3$H-glucosamine. The labeled cells with LPL bound were then incubated with different concentrations of TNF (up to 200 ng/ml) at 37°C for 60 minutes. At each concentration of TNF tested, there was no increased release over a control incubation (no TNF) in the amount of glycosaminoglycan-associated radioactivity in the media (Figure 6 shows the release at 100 ng/ml TNF). Cell-associated radioactivity also remained unchanged (data not shown). Since insulin and insulin-like growth factors have been shown to stimulate proteoglycan synthesis and secretion in vascular and connective tissues, insulin treatment was studied as a control. As expected, insulin (100 ng/ml) increased the release of glycosaminoglycans into the media (Figure 6).

Discussion

We demonstrated that TNF treatment caused the release of LPL activity and protein bound to endothelial cells. Such an effect in vivo would be expected to decrease LPL actions. Our studies directly show for the first time that perturbations of endothelial cell metabolism may modulate the amount of LPL on endothelial cell surfaces, an effect which may regulate lipolysis at the vascular endothelium. Thus, an important new pathogenesis for defective triglyceride hydrolysis and hypertriglyceridemia may be illustrated by our results.

Regulation of the activity of LPL is in many ways more complex than that of most secretory proteins. The steps involved in regulating LPL functions in vivo include not only synthesis and secretion by adipose and muscle cells but also transport to an appropriate binding site on the endothelial cell. In addition, activation by a serum cofactor (apolipoprotein C-II) and inactivation and removal of the enzyme from the endothelial cells may modulate LPL activity. Once released from endothelial cells, LPL is thought to be transported to the liver, where it is slowly inactivated and degraded. Our previous studies and those in this report illustrate that the endothelial cell binding of LPL may be an important regulatory site for LPL actions.

Vascular endothelial cells possess cell surface receptors for TNF. Our results demonstrate that TNF causes the release of LPL protein and activity bound to endothelial cells. Colchicine blocks the effect of TNF, suggesting the involvement of a secretory event. Pertussis toxin, which ADP-ribosylates and inactivates certain G proteins, also inhibits TNF-induced release of LPL, suggesting that the release may be coupled through a guanine nucleotide regulatory protein. It is also possible that pertussis toxin may have an unidentified effect on endothelial cells, which blocks the ability of TNF to release LPL. TNF treatment does not alter the amount or secretion of glycosaminoglycans by endothelial cells, suggesting that TNF does not decrease the total number of LPL binding sites.

A reasonable mechanism for TNF-induced release of LPL that is compatible with all of the above observations relates to the release of LPL by arachidonic acid (Fig-
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LPL molecules have a fatty acid binding site,12 and our previous studies20,27 have shown that some types of free fatty acids appear to specifically release this protein from endothelial cells. It is conceivable that arachidonic acid secreted in response to TNF, a step which is pertussis toxin-sensitive17 and also fulfills the requirement of a colchicine inhibited secretory event, may interact with and release some of the LPL bound to the endothelial cells. Furthermore, a requirement for the activation of phospholipase A2 and subsequent arachidonic acid generation in TNF-induced release of LPL also was demonstrated in the experiments using melittin and quinacrine. The generation of even small amounts of arachidonic acid at or close to the cell membrane site where LPL is bound may be sufficient for the release of endothelial cell-bound LPL.

The in vivo effects of TNF on triglyceride metabolism may be complex. Increases in triglyceride synthesis in humans and diabetic animals have been reported after TNF administration.18,19 TNF also decreases LPL activity in fat tissue.10,46,47 In addition to effects on adipose tissue, TNF administration rapidly increases plasma and liver LPL activity in several animal species.10 These results, together with the data presented here, support the hypothesis that TNF releases LPL from endothelium, and the enzyme is then transported via the plasma to the liver. The increased LPL in the liver and decreased LPL activity at its functional location on the endothelial surface may cause a greater flow of free fatty acids (energy) to the liver to meet the acute metabolic demands of inflammatory responses. In this regard, Chajek-Shaul et al.48 showed that TNF treatment of rats resulted in the uptake of triglyceride-enriched chylomycin remnants by the liver, suggesting a decrease in peripheral triglyceride lipolysis. TNF-mediated rapid release of LPL from the endothelium may be involved in generation of the hypertriglyceridermia, which occurs during infection and in response to administration of endotoxin, both of which trigger the synthesis of TNF (in the host).

Moderate levels of TNF may confer survival advantage with respect to bacterial or viral infection by providing a useful mobilization of energy reserves for the increased metabolic demand. Thus, while one acute effect of TNF is to induce hypertriglyceridermia by loss of endothelial cell-bound LPL, prolonged exposure to TNF may induce cachexia by depleting and depriving peripheral adipose tissue and muscle of free fatty acids.

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References

38. Darville C, Mason RN. Effects of serum and insulin on hyaluronate synthesis by cultures of chondrocytes from swarf rat chondrosarcoma. Biochim Biophys Acta 1983;760:53-60
47. Price SR, Olvecrona T, Pekala PH. Regulation of lipoprotein synthesis by recombinant tumor necrosis factor. The primary role of the hormone in 3T3 L1 adipocytes. Arch Biochem Biophys 1986;251:738-746

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