

Specificity of lipoprotein lipase binding to endothelial cells

Monique F. Stins, Pillarisetti Sivaram, Atsuko Sasaki, and Ira J. Goldberg¹

Department of Medicine, Columbia University College of Physicians & Surgeons, 630 West 168th Street, New York, NY 10032

Abstract Lipoprotein lipase (LPL) hydrolyzes circulating lipoprotein triglyceride molecules while it is associated with the luminal surface of capillary endothelial cells. The precise molecular mechanism by which LPL attaches to these cells is unknown. LPL and a number of other molecules, including growth factors and clotting factors, bind to heparin-affinity gels and are eluted using high concentrations of salt. Of these molecules, antithrombin III and basic fibroblast growth factor have been shown to bind to specific cell surface heparan sulfate proteoglycans. Recent data from our laboratory (Sivaram et al. 1992. *J. Biol. Chem.* 267: 16517-16522) have shown that a heparin-sensitive, non-proteoglycan 116-kDa LPL-binding protein is present on cultured bovine aortic endothelial cells (BAEC). A series of experiments was performed to study the specificity of LPL binding to BAEC and to this 116-kDa protein. At low amounts of LPL (1 μ g) ¹²⁵I-labeled LPL binding to the cells was inhibited up to 82% by the addition of a 20-fold excess of unlabeled LPL. LPL binding to the BAEC was not decreased by the addition of similar amounts of either antithrombin or thrombin. Specific LPL binding was eliminated by incubating the BAEC at 4°C with heparin containing buffer prior to the addition of LPL. Although cellular internalization of ¹²⁵I-labeled LPL at 37°C was decreased when an excess of each of the three proteins was added to the culture medium, LPL was most effective. Furthermore, when LPL interaction with the 116-kDa binding protein was studied using ligand blots, ¹²⁵I-labeled LPL binding was blocked only by unlabeled LPL. Low concentrations of heparin released LPL bound to endothelial surfaces and also decreased the number of LPL binding sites on the cells. Therefore, heparin might dissociate both LPL and its binding protein from the cells. To determine whether a heparin-sensitive LPL binding site was also present on aorta, LPL binding to control and heparin-treated pieces of dog aorta was assessed; 45% less LPL bound to the heparin-treated aorta. ■ Thus our data support the hypothesis that LPL binds to two different types of endothelial cell surface proteins, heparan sulfate proteoglycans and a specific 116-kDa, heparin-sensitive binding protein. —Stins, M. F., P. Sivaram, A. Sasaki, and I. J. Goldberg. Specificity of lipoprotein lipase binding to endothelial cells. *J. Lipid Res.* 1993. 34: 1853-1861.

Supplementary key words proteoglycans • glycosaminoglycans • heparin • triglyceride • capillaries • chylomicrons • antithrombin • thrombin

Lipoprotein lipase (LPL) is widely believed to interact with circulating lipoproteins while it is bound to the lumi-

nal surface of capillary endothelial cells (1, 2). The LPL molecule appears to have separate domains that bind to lipid/lipoproteins and the endothelial cell surface (3). Several lines of evidence have suggested that LPL binds to endothelial cells via interaction with heparan sulfate proteoglycans (HSPG). In 1943, Hahn (4) first noted that injection of heparin into dogs led to the disappearance of circulating chylomicrons and he postulated that heparin released a chylomicron "clearing factor" into the bloodstream. Subsequent work by a number of different laboratories showed that inclusion of heparin in cell culture medium or treatment of cells with heparinase/heparinitase decreased LPL binding to endothelial cells (5-7). Because the interaction between LPL and HSPG is greater when the proteoglycans contain a greater amount of sulfate residues and therefore a greater charge density (2), LPL has been assumed to bind to HSPG via electrostatic interactions (8). Unlike antithrombin III, which requires a specific five-unit glycosaminoglycan chain for high affinity interactions with HSPG (9, 10), LPL binding has not been shown to require a specific class of HSPG.

Although a number of molecules of biological importance bind to HSPG and heparin affinity gels, most of these heparin-binding proteins also have non-proteoglycan cell surface binding sites. Specific receptor molecules have been identified for several heparin-binding growth factors and coagulation factors. Although Shimada and Ozawa (11) showed that thrombin binds to endothelial cell surface HSPG, cellular effects of thrombin are due to its interaction with the specific thrombin receptor thrombomodulin (12). Although apolipoprotein E-containing lipoproteins interact with heparin and can be isolated using heparin-affinity chromatography, much of their cellular uptake is via interaction with specific lipoprotein receptors (13).

Abbreviations: LPL, lipoprotein lipase; BAEC, bovine aortic endothelial cells; HSPG, heparan sulfate proteoglycans; PMSF, *p*-methyl sulfonyl fluoride; PBS, phosphate-buffered saline; hrp-116, heparin-releasable protein of 116 kDa; FGF, fibroblast growth factor.

¹To whom correspondence should be addressed.

Cellular actions of basic fibroblast growth factor (FGF) require cell surface HSPG, but rather than acting as the specific receptors for this growth factor, the HSPG facilitate binding of basic FGF to other non-HSPG receptors (14, 15). Therefore, cell surface HSPG may be responsible for nonspecific binding of proteins or they may assist in recognition of ligands by other more specific binding proteins on the cell surfaces.

LPL might also have both relatively nonspecific interactions with endothelial cell HSPG and more specific interactions with a second LPL binding protein. In this regard, Sivaram, Klein, and Goldberg (16), using ligand blotting, identified a non-HSPG LPL binding protein of 116 kDa. This 116-kDa LPL binding protein was released from endothelial cells by heparin treatment. Therefore, it was named heparin-releasable protein 116 (hrp-116). Treatment of endothelial cells with heparin not only released hrp-116, but also reduced subsequent binding and internalization of LPL. This suggested that at least some LPL binding to endothelial cells was due to a heparin-sensitive binding site that was not an integral membrane protein.

In this report, we present studies to further characterize the interaction of LPL with endothelial cells. The specificity of LPL binding to hrp-116 was investigated and compared to that of other HSPG binding proteins. Our results suggest that hrp-116 is a good candidate to be a specific LPL binding protein.

METHODS

Materials

Na¹²⁵I was obtained from Amersham (Arlington Heights, IL); 6-well, 35 mm tissue culture plates were from Falcon (Oxnard, CA). Bovine serum albumin (BSA), antithrombin, and thrombin were from Sigma (St. Louis, MO). Heparin was from Elkins-Sinn (Cherry Hill, NJ). Dulbecco's Modified Eagle's Medium (DMEM), serum, penicillin, and streptomycin for cell culture were purchased from Gibco (Grand Island, New York). Prepacked PD-10 Sepharose columns were obtained from Pharmacia (Piscataway, NJ). Heparin agarose, avidin-horseradish peroxidase and horseradish peroxidase-staining reagent were from Bio-Rad (Richmond, CA). Iodobeads were from Pierce (Rockford, IL) and sodium chlorate was from Aldrich (Milwaukee, WI). All other chemicals and solvents were of reagent grade.

Purification and labeling of LPL, thrombin, and antithrombin

LPL was purified from fresh unpasteurized milk (17), protein concentration was determined by the method of Lowry et al. (18), and LPL was stored at -70°C . LPL

was radiolabeled with ¹²⁵I using the glucose oxidase method as described previously (17). Labeled LPL had a specific radioactivity of 500–1250 cpm per ng of protein. To remove any degradation products and salt prior to each experiment, ¹²⁵I-labeled LPL or native LPL was gel-filtered over a PD-10 column in DMEM containing 3% BSA. LPL was biotinylated as described by Cole, Ashman, and Ey (19) and LPL was repurified over heparin agarose as described for ¹²⁵I-labeled LPL. Using similar preparations we have shown that labeled proteins associated with the cells are LPL and not other heparin-binding proteins of a different molecular weight (20, 21).

Thrombin was labeled with ¹²⁵I as described by Bar-Shavit, Eldor, and Vlodavsky (22), with the exception that iodobeads were used. Antithrombin was labeled as described by Marcum et al. (23) using the lactoperoxidase method.

Cultures of bovine aortic endothelial cells

Primary cultures of bovine aortic endothelial cells (BAEC) were established and subcultured as described previously (24). Cells were grown in DMEM containing 10% calf serum, 100 units/ml of penicillin, 100 $\mu\text{g}/\text{ml}$ of streptomycin, and 1% glutamine. Approximately 0.5×10^5 cells per well were seeded into 6-well dishes. Experiments were conducted 5–6 days after seeding the cells when a confluent monolayer was observed.

Determination of LPL internalized and associated with the cell surface

LPL binding to the cells was assessed by allowing ¹²⁵I-labeled LPL (1–5 $\mu\text{g}/\text{ml}$) in DMEM–3% BSA to bind to the monolayers for 2 h at 4°C . To determine internalization of LPL, ¹²⁵I-labeled LPL in DMEM–3% BSA was incubated with the BAEC monolayers at 37°C for 1 h. After each incubation, the cells were put on ice and all further steps were performed in ice-cold DMEM–0.3% BSA. After the cells were washed, LPL bound to the cell surfaces was released with 100 units heparin/ml in DMEM–0.3% BSA for 30 min at 4°C . Intracellular LPL was assessed by washing the cells with DMEM–0.3% BSA and with Hanks buffer without calcium and magnesium. The cells were scraped free of the dishes, pelleted 5 min at 10,000 rpm in a model 5415C centrifuge (Eppendorf, Hamburg, Germany), and intracellular ¹²⁵I-labeled LPL was determined.

Competition studies using LPL and other heparin binding proteins

When indicated, in addition to ¹²⁵I-labeled LPL, a 20-fold protein excess of unlabeled LPL, thrombin, or antithrombin was present during the incubation for 2 h at 4°C . Thereafter, the cells were washed and the amount of LPL bound to the surface was determined.

Treatment of cells with heparin or chlorate

To test whether removal of the 116-kDa heparin-releasable protein affected the cellular association of LPL, cells were incubated in DMEM-3% BSA containing 0.001–100 units/ml heparin for 30 min at 4°C. The medium was removed and the cells were washed 5 times with DMEM to remove any residual heparin. LPL binding to the heparin-treated cells was then assessed at 4°C.

In some experiments proteoglycan metabolism was perturbed by culturing the cells overnight in medium containing 10–50 μ M sodium chlorate (25).

Heparin-agarose chromatography of medium

Heparin-agarose chromatography was used to partially purify and concentrate hrp-116 so that it could be detected by the ligand blotting technique. Frozen medium was thawed in the presence of *p*-methyl sulfonyl fluoride (PMSF) (final concentration 1 mM); heparin-agarose was added and the medium was incubated for 4 h at 4°C. Then the heparin-agarose was packed in a column and washed with 0.15 M NaCl in 20 mM 4-(2-hydroxyethyl)-piperazine ethane sulfonic acid (HEPES) buffer, pH 7.4, 0.1 mM PMSF. The column was eluted with 0.4 M NaCl in the same buffer. The peak fractions were concentrated using a Centricon filter and immediately prepared for gel electrophoresis and blotting.

Ligand blotting of BAEC plasma membranes

Plasma membranes from BAEC were prepared according to the method of Lin et al. (26), as modified by Sivaram et al. (16). Samples were analyzed by SDS-PAGE using 7.5% polyacrylamide gels and then transferred to nitrocellulose. The membranes were incubated overnight in phosphate-buffered saline (PBS) containing 3% BSA (PBS-3% BSA) at 4°C, washed in PBS-3% BSA and 0.05% Tween-20 (PBS-3% BSA-T), incubated for 2 h at 4°C with biotinylated LPL in PBS-3% BSA, and washed again in PBS-3% BSA-T. Biotinylated LPL bound to cellular proteins was reacted with avidin-conjugated horseradish peroxidase (1:1000 dilution, Bio-Rad) in PBS containing 3% BSA. Bands were visualized by incubation in a solution containing 40 ml PBS, 25 mg 4-chloro-1-naphthol (Bio-Rad) dissolved in 7.5 ml methanol, and 20 μ l 50% hydrogen peroxide.

Incubation of dog aortic segments with LPL

Dog aorta was obtained from purpose-bred animals that were killed for the harvesting of myocardium. The aortas were removed from the animals and placed in 4°C PBS. Within 15 min, the vessel was divided into approximately 10-mm² pieces that were cut to avoid the intercostal vessels. An inverted pipette tip was placed over each section and uniform 7-mm circular wells containing the luminal surfaces of aorta were produced by surrounding

the pipette tip with 2% agar. Care was given to assure that the exposed aorta was covered by DMEM during this procedure. After solidification of the agar, the segments were washed with DMEM. Some segments were treated with 100 U/ml heparin for 10 min. All segments were then washed 5 times with 4°C DMEM and then medium containing 5 μ g/ml ¹²⁵I-labeled LPL was added and allowed to remain on the aortas for 30 min at 4°C. The segments were then washed, the inverted pipette tips were removed (to eliminate any ¹²⁵I-labeled LPL nonspecifically bound to the plastic) and the surface-bound ¹²⁵I-labeled LPL was released with 100 U/ml heparin for 10 min.

RESULTS

¹²⁵I-labeled LPL binding to BAEC: competition with unlabeled LPL and antithrombin III

In order to assess the specificity of the binding of LPL to the endothelial cell surface, the effect of addition of other proteoglycan binding proteins on LPL binding was tested. Two hundred ng ¹²⁵I-labeled LPL was allowed to bind to BAEC at 4°C for 2 h in DMEM-BSA and in DMEM-BSA containing increasing amounts of unlabeled LPL or antithrombin III. As little as 2 μ g of LPL (a dilution of 1/10) led to a 20% decrease in ¹²⁵I-labeled LPL binding to the cells; 10 μ g/ml of unlabeled LPL decreased ¹²⁵I-labeled LPL binding to the cells approximately 50%. In contrast, even at the highest concentration used (10 μ g/ml) antithrombin had no effect (Fig. 1). Thus, these two proteins that have similar affinity for heparin do not compete for binding to the endothelial surface. This suggests that another protein may have been involved in LPL binding to cells, or that heparan sulfate chains with different sequences bind LPL and antithrombin.

LPL binding to control and heparin-treated BAEC

Previous studies from this laboratory showed that treatment of BAEC with heparin markedly reduced LPL binding to the BAEC surface (16). To determine whether the heparin-sensitive and heparin-insensitive binding to cell were similar, we next tested whether ¹²⁵I-labeled LPL binding to control and heparin-treated cells would be completed in the same manner by unlabeled LPL, thrombin, and antithrombin. In these experiments, ¹²⁵I-labeled LPL (1 μ g/ml) was allowed to bind for 2 h at 4°C to the surface of BAEC in the absence or presence of an excess of unlabeled LPL, thrombin, or antithrombin. In the experiment shown in Fig. 2A, addition of a 20-fold excess of unlabeled LPL (20 μ g) decreased the ¹²⁵I-labeled LPL binding to <25% of control. Addition of the same amount (20 μ g) of thrombin or antithrombin led to <10% reduction in the binding of ¹²⁵I-labeled LPL. When LPL binding studies were performed using cells

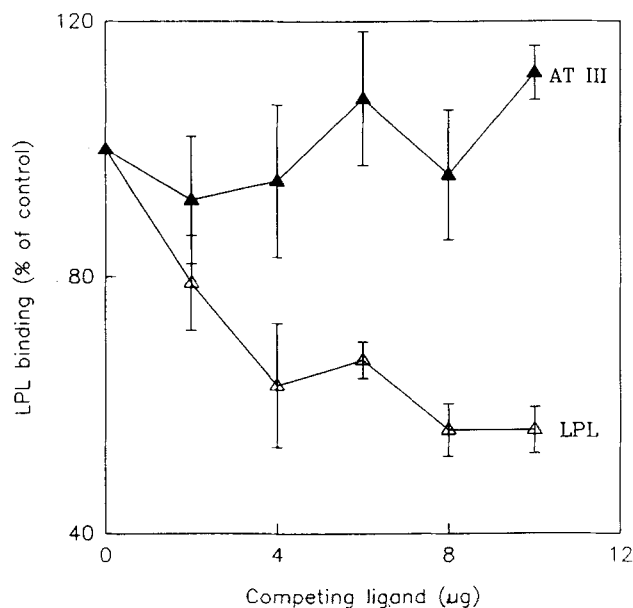


Fig. 1. LPL binding to BAEC: competition with different concentrations of LPL and antithrombin III (AT III). Confluent monolayers of BAEC were washed three times with DMEM-BSA and then cooled to 4°C. The cells were incubated with 250 ng of ^{125}I -labeled LPL alone or in the presence of the indicated amounts of unlabeled LPL or antithrombin III for 2 h at 4°C with gentle rocking. Unbound LPL was removed and cells were washed three times with DMEM-BSA, and cell surface ^{125}I -labeled LPL was released with 100 units/ml heparin in DMEM-BSA. Values given are the mean \pm SD of triplicate experiments.

that had been treated with heparin (Fig. 2B), binding of ^{125}I -labeled LPL to the heparin-treated cells was only 12% of its binding to control cells. In contrast to experiments using control cells, addition of unlabeled LPL no longer reduced the binding of ^{125}I -labeled LPL to the cells. Thrombin and antithrombin also failed to reduce the binding of the labeled LPL. Thus, heparin treatment appeared to primarily reduce the LPL binding sites that were previously competed for by unlabeled LPL. These data suggest that the heparin-sensitive binding sites mediate specific LPL binding to BAEC.

We postulated that heparin reduced LPL binding to BAEC by removal of the 116-kDa LPL-binding protein. Another explanation for our results is that residual heparin, either stuck to the cells or plastic, was released from the cell during a subsequent LPL binding experiments, and this, in turn, reduced LPL binding to the cells. Heparin will bind to cultured endothelial cells (27). One might expect that increasing the amount of BAEC cell surface heparin would increase LPL binding to the cells. Alternatively, during the incubation wherein LPL is allowed to bind to the cells at 4°C, one could imagine that if sufficient amounts of heparin were released from the cells back into the medium, it could affect the association of LPL-binding proteins with the cell surface.

We tested whether sufficient amounts of heparin had

been released into the medium from the heparin-treated cells to decrease the cell binding of LPL or other heparin-sensitive proteins. Heparin treatment did not reduce antithrombin binding; antithrombin binding in heparin-treated cells was 94.5% of control. In a second series of experiments, medium obtained from heparin-treated cells was applied to control cells to assess LPL binding. If residual heparin from the initial heparin wash was released into the media, it should have also affected LPL binding to control cells. Only if the cells were poorly washed and when high concentrations of heparin were applied to the cells (>100 units/ml) was there evidence of enough heparin in the medium to affect LPL binding to control cells. Thus, our data were most consistent with a postulated role for hrp-116 to facilitate LPL binding to BAEC.

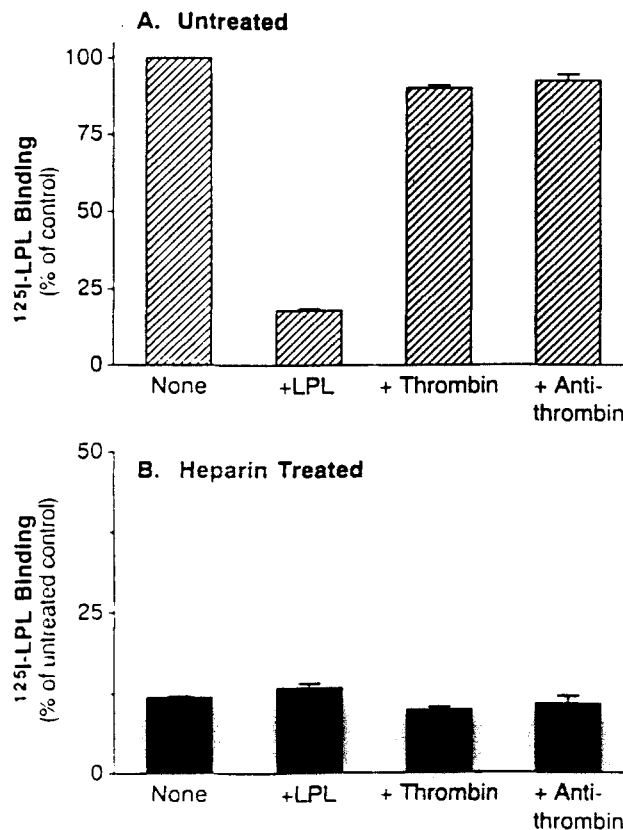


Fig. 2. LPL binding to control and heparin-treated BAEC: competition with LPL, thrombin and antithrombin III. A: Confluent monolayers of BAEC in 35-mm dishes were washed with DMEM-3% BSA and ^{125}I -labeled LPL (1 $\mu\text{g}/\text{ml}$) was allowed to associate with the cell surface during a 2-h, 4°C incubation. Binding of ^{125}I -labeled LPL to the cells was compared with its binding in the presence of a 20-fold excess (20 $\mu\text{g}/\text{ml}$) of unlabeled LPL, thrombin, or antithrombin III. B: Experiments similar to those in A were performed using cells (35-mm dishes) that were treated with 10 units/ml of heparin in DMEM for 30 min at 4°C. The dishes were washed 5 times with DMEM, and then the ^{125}I -labeled LPL binding was performed as described above. Shown are means \pm SD of experiments performed in triplicate.

Competition of heparin-binding proteins for ¹²⁵I-labeled LPL internalization

Cell surface LPL is internalized and recycled by cultured endothelial cells (21). To test whether internalization of ¹²⁵I-labeled LPL was competed for by an excess of unlabeled LPL, thrombin, or antithrombin, cells were incubated for 1 h at 37°C with medium containing 1 μg/ml of ¹²⁵I-labeled LPL in the presence or absence of 20 μg/ml of LPL, thrombin, or antithrombin. Addition of a 20-fold excess of unlabeled LPL decreased LPL internalization by over 60% (Fig. 3A). Thrombin reduced the internalization by 31% and antithrombin by 19%. Therefore, LPL was a more effective competitive ligand than the other heparin binding proteins. This suggests that much of the internalization of LPL by BAEC is via a specific mechanism.

Internalization of ¹²⁵I-labeled LPL by heparin-treated cells was also studied. Although at the beginning of the experiments these cells were presumably depleted of heparin-sensitive binding sites, during the 37°C incubation some heparin-binding sites reappeared on the cell surface (16). It should also be noted that the design of this experiment differed from previously reported experiments (28, 29) in which heparin-containing medium remained on the cells during the internalization period. Heparin treatment of BAEC cells reduced ¹²⁵I-labeled LPL internalization to 67% of control; addition of an excess of unlabeled LPL reduced the amount of internalized LPL further to 33%. Although excess unlabeled LPL reduced internalization in control cells by over 60%, the

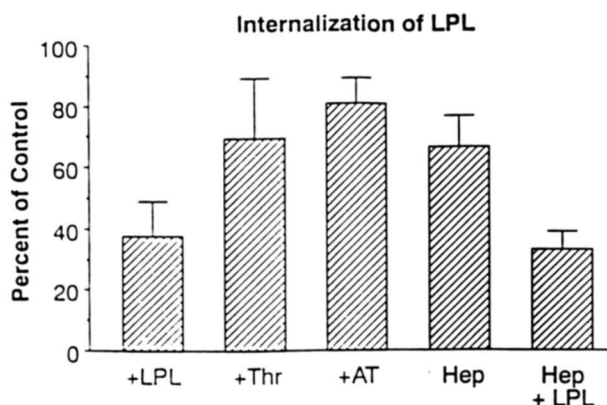


Fig. 3. Competition of LPL, thrombin, and antithrombin III for internalization of ¹²⁵I-labeled LPL. ¹²⁵I-labeled LPL (1 μg/ml) was incubated in DMEM-3% BSA at 37°C with BAEC. After 1 h, the cells were washed and the heparin-releasable, presumably cell surface, ¹²⁵I-labeled LPL was removed. The residual ¹²⁵I-labeled LPL, which represents LPL internalized by the cells, was then assessed. Competition for uptake between the labeled LPL and unlabeled LPL (+LPL), thrombin (+Thr), and antithrombin III (+AT) was performed by adding 20 μg of these proteins to the medium containing ¹²⁵I-labeled LPL. Similar experiments were performed using cells that had been treated with heparin (denoted hep). Shown are the means ± SD of experiments performed in triplicate.

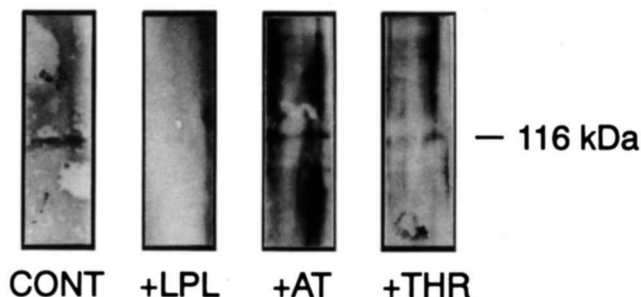


Fig. 4. Ligand blot showing interaction of biotinylated LPL with BAEC membranes. Plasma membranes were prepared from BAEC, analyzed by SDS-PAGE, and transferred to a nitrocellulose membrane. Strips of the membrane were then incubated in buffer containing biotinylated LPL alone (control) or in the presence of 20 μg/ml of unlabeled LPL (+LPL), antithrombin III (+AT), or thrombin (+THR). The membranes were then washed and developed using avidin-horseradish peroxidase as described in Methods.

amount of reduction was less using heparin-treated cells (34%, from 67 to 33%, versus >60%). This suggests that some of the specific LPL internalization was eliminated by first treating the cells with heparin. One could also interpret these data as showing that the heparin treatment effect on LPL internalization is abolished by addition of excess unlabeled LPL.

Ligand blots of LPL interaction with hrp-116

Ligand blots were performed to study the effects of heparin binding proteins on LPL interaction with hrp-116 directly. BAEC were grown to confluent monolayers, plasma membranes were isolated, subjected to SDS-PAGE, and transferred to nitrocellulose membranes. Biotinylated LPL was allowed to bind to the membrane in the presence or absence of an excess of unlabeled LPL, thrombin, or antithrombin and the adherent biotinylated LPL was detected by reaction with avidin-horseradish peroxidase. Biotinylated LPL bound to a band with a molecular mass of 116 kDa (Fig. 4). A 20-fold excess of unlabeled LPL decreased the binding of biotinylated LPL to the 116-kDa band. The two heparin-binding proteins, thrombin, and antithrombin reduced the intensity of band much less than did LPL.

Effect of chlorate on LPL binding

Growth of cells in chlorate-containing medium reduces the sulfation, and hence the charge, of newly synthesized glycosaminoglycan chains. To test whether perturbation of proteoglycan metabolism resulted in a release of the hrp-116 and a subsequent loss in specific binding of LPL, cells were grown overnight in medium containing 50 μM sodium chlorate. This treatment decreased LPL binding by 74% (Fig. 5). Heparin treatment did not decrease LPL binding further.

We next tested whether treatment with sodium chlorate

released hrp-116 into the media of the cells. In order to assess this, the media of control and treated cells were saved, subjected to heparin-agarose chromatography, gel electrophoresis, and ligand blotting. Chlorate treatment did not result in an appearance of the 116-kDa protein in the medium (data not shown). The 116-kDa band was found in the medium of heparin-treated, but not control, cells (16). Therefore chlorate treatment does not lead to release of hrp-116 from the cell surface.

Comparison of heparin concentrations required to release BAEC-associated LPL and to decrease heparin-sensitive LPL binding sites

Heparin treatment releases hrp-116 from cells and also results in a decrease in LPL binding to BAEC. In addition, it is well known that heparin releases LPL from the cell surface. We examined whether heparin would release hrp-116 and LPL together. As hrp-116 removal should decrease LPL binding, we tested whether the same concentrations of heparin were required both to decrease LPL binding to BAEC and to release cell surface-associated LPL (in a separate experiment). ^{125}I -labeled LPL was allowed to bind to the surface of BAEC for 2 h at 4°C and the bound ^{125}I -labeled LPL was released with varying concentrations of heparin. At concentrations >0.001 units/ml of heparin, LPL release was linear (Fig. 6, open circles). When concentrations equal to or greater than 1 unit/ml were included in the medium, all the bound LPL was removed. In parallel experiments performed at the same time, the amount of LPL binding to heparin-treated cells was assessed. Treatment of the cells with >1 units/ml of heparin led to the maximum decrease in ^{125}I -labeled LPL binding to the BAEC (solid circles). Although 0.01 units/ml of heparin released about 60% of the cell surface LPL (open circles), the same amount was not sufficient to decrease LPL binding to cells (closed circles),

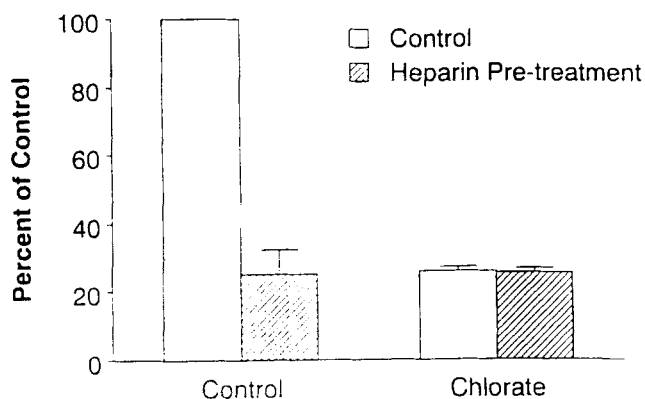


Fig. 5. Effect of chlorate on LPL binding to BAEC. Cells were grown overnight in chlorate-containing medium, which decreases the sulfation of glycosaminoglycans. ^{125}I -labeled LPL binding to control, heparin-treated, and chlorate-treated cells are shown. Data shown are the means \pm SD of experiments performed in triplicate.

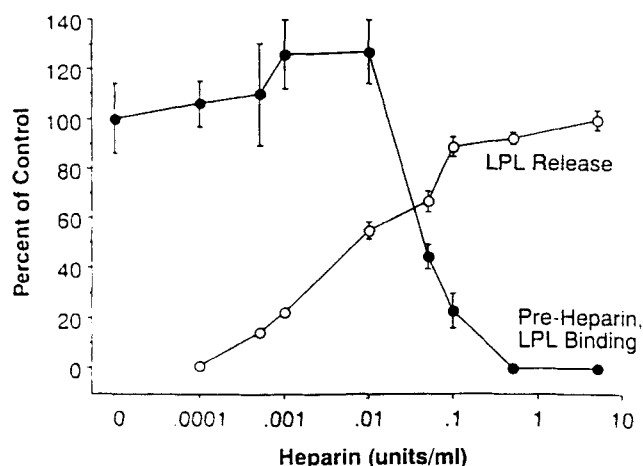


Fig. 6. Effects of heparin on LPL binding to and release from BAEC. ^{125}I -labeled LPL was allowed to associate with the surfaces of BAEC, as described in methods. The bound LPL was then released by incubating the cells for 30 min at 4°C in DMEM-3% BSA containing the indicated concentrations of heparin. The amounts of LPL released are displayed in the open circles as a percent of the total heparin-releasable radioactivity. Cells were also incubated using similar concentrations of heparin-containing DMEM prior to addition of the ^{125}I -labeled LPL. Shown in the solid circles is the reduction of LPL binding to cells that had been exposed to the indicated concentrations of heparin.

suggesting that hrp-116 was not released at this heparin concentration. Thus, the shape of the titration curves for LPL release and for decreasing LPL binding were dissimilar and low doses of heparin released cell surface LPL without affecting the hrp-116. This suggests that heparin does not affect the two conditions in an identical manner.

Binding of LPL to dog aorta segments

In order to assess whether the decrease in LPL binding to BAEC could also be found using intact blood vessels, we studied LPL binding to segments of dog aorta. The luminal side of control and heparin-treated dog aortic segments was incubated with ^{125}I -labeled LPL. After the binding and washing of the aorta, we removed the plastic sleeve that had enabled us to form the media-containing wells. This step was important because it greatly reduced the nonspecific binding of ^{125}I -labeled LPL to the plastic and the agar that separated the aorta into sections. Treatment of aorta with heparin decreased LPL binding by approximately 40% (Fig. 7). Therefore, dog aorta also appears to have heparin-sensitive LPL binding sites.

DISCUSSION

Our data suggest that LPL binding to BAEC may involve both nonspecific interactions with HSPG and specific binding, perhaps via hrp-116. Several experimental observations support the hypothesis that at least part of

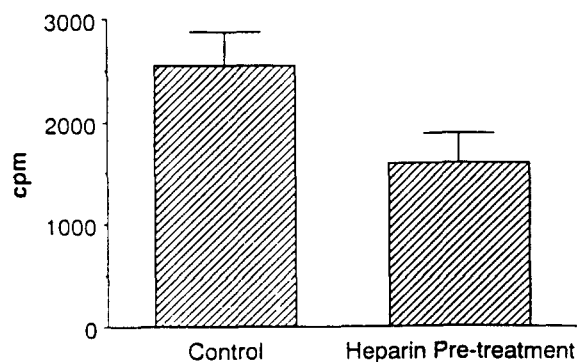


Fig. 7. ^{125}I -labeled LPL binding to dog aorta. Segments of fresh dog aorta (7 mm) were prepared as described in Methods. The luminal sides of the aorta were bathed in cold (4°C) DMEM or were treated for 10 min in DMEM containing 100 units/ml heparin. All aortic pieces were then rinsed 5 times with DMEM and ^{125}I -labeled LPL was allowed to bind to the aortic pieces for 30 min at 4°C . Shown are means \pm SEM ($n = 4$).

the interaction of LPL with endothelial cells is via a heparin-sensitive binding site with which LPL, but not other HSPG-binding proteins, interacts. First, unlabeled LPL decreased binding of ^{125}I -labeled LPL to the surface of BAEC by up to 80%. Second, addition of the same concentration of antithrombin III and thrombin, two other heparin-binding proteins, did not reduce LPL binding to the cells. Third, after heparin treatment, which results in a loss of hrp-116 from the cell surface, ^{125}I -labeled LPL binding to the cells was no longer reduced by the addition of a 20-fold excess of unlabeled LPL. This suggests that the specific LPL binding sites on the cell surface were eliminated by heparin treatment. In addition, because the binding of ^{125}I -labeled antithrombin III was not decreased after cells were treated with heparin, we are confident that the decrease in LPL binding to heparin-treated cells was not due to residual heparin that was not washed away from the cells after the initial 4°C -heparin treatment of the cells. It should be noted that the use of greater concentrations of ^{125}I -labeled LPL appeared to increase nonspecific LPL binding to cells and that the addition of unlabeled LPL did not reduce LPL binding to the same degree. Thus, as expected, when the specific binding sites are almost fully occupied, there is a greater amount of nonspecific ^{125}I -labeled LPL binding to the cells.

Although endothelial cells internalize LPL, most of the LPL is not degraded by the cells but is recycled back to the cell surface and into the medium (20). We therefore also studied whether internalization of ^{125}I -labeled LPL by the cells was via a "specific", LPL-competable process. Using $1\ \mu\text{g}/\text{ml}$ of LPL, most of LPL internalization by BAEC was via specific mechanisms. Thrombin and antithrombin III also decreased internalization of LPL but to a lesser degree. Thus, it appears that some of the LPL that bound to the cell surface via interactions to HSPG,

was also internalized by the cells. This might occur in conjunction with the turnover or recycling of the cell surface HSPG.

To assess how much of the ^{125}I -labeled LPL internalization was via heparin-sensitive cell surface proteins, cells were treated with heparin, which releases hrp-116 (16). Although LPL internalization decreased after heparin treatment of the cells, the decrease was less than that found when heparin was included in medium during the 37°C incubation (25). This latter condition affects LPL uptake via both HSPG and heparin-sensitive cell surface proteins. It should be noted, however, that in the current experiments some heparin-sensitive LPL binding sites probably reappeared on the cell surface during the 37°C incubation. Nonetheless, we conclude that some of the specific LPL uptake was via heparin-sensitive binding sites.

We have previously reported that LPL reacts on ligand blots with a 116-kDa protein that is released from the surface of endothelial cells when they are incubated with heparin (16). We now provide some additional evidence that this protein, hrp-116, is a specific LPL-binding protein. Ligand blotting showed that LPL specifically bound to hrp 116 and that the heparin-binding proteins, thrombin and antithrombin, did not influence this interaction.

Previous reports (25) showed that the LPL binding to avian adipocytes was reduced by chlorate which decreases sulfation of proteoglycans. Chlorate treatment markedly reduced LPL binding to the BAEC. Heparin treatment did not further reduce LPL binding to chlorate treated cells; therefore, chlorate appeared to decrease the heparin-sensitive LPL binding sites. Because hrp-116 was not found in the medium of chlorate-treated cells, we hypothesize that chlorate treatment prevents hrp-116 from accumulating on the outer plasma membrane.

It should be noted that in some experiments the effect of heparin treatment on LPL binding could not be demonstrated. Chlorate treatment of the same cells then also did not reduce the LPL binding. This usually occurred when we used cells that had been passaged a number of times. Cell surface proteoglycans sometimes decrease in cells that are old (30). Perhaps "aging" of the BAEC affects the proteoglycans important for binding of hrp-116 and LPL.

We studied whether the same concentrations of heparin were required to release LPL from the BAEC and decrease LPL binding sites on the cell surface. Although we cannot be absolutely certain that there are not small differences in the heparin sensitivity of these two processes, our data suggest that a dose of heparin of >0.1 units/ml is sufficient for both effects. Thus, in some situations, dissociation of LPL from endothelial cells may involve release of both LPL and hrp-116 from the cell surface. Because detection of hrp-116 requires large numbers of cells, we were unable to perform ligand blots using

these media. Thus, we assume but cannot prove that hrp-116 was released with the low concentrations of heparin.

To attempt to relate our tissue culture observations to the in vivo situation, we studied whether LPL binding to intact endothelial cells on the luminal surface of dog aorta was sensitive to heparin. The methods that we developed to assess LPL binding to aorta involved forming wells containing 7-mm pieces of aorta and exposing them to ¹²⁵I-labeled LPL. Using this method, we showed that binding of ¹²⁵I-labeled LPL to the aorta was sensitive to heparin treatment and confirmed that intact, noncultured endothelium also have heparin-sensitive LPL binding sites.

In summary, our data suggest that LPL binds to specific and nonspecific sites on the surface of BAEC. A possible mechanism for these interactions is shown in Fig. 8. Some LPL probably interacts with cell surface HSPG. As in vivo a number of other plasma heparin-binding proteins are found at concentrations that are orders of magnitude greater than that of LPL, we postulate that these nonspecific LPL binding sites play a relatively minor role in LPL binding to endothelial cells. We hypothesize that much of the LPL binding to endothelial cells is mediated by the LPL specific binding protein, hrp-116. Because this protein is released from cells with heparin treatment, it may be associated with HSPG on the cell surface. Alternatively, it may have ionic interactions with other BAEC surface proteins that are disrupted when the cells are treated with heparin. This scenario of LPL binding to a specific binding protein is similar to that of a number of other heparin-binding endothelial cell ligands. An alternative hypothesis is that a specific sequence of heparan sulfate mediates LPL, but not antithrombin, binding. This hypothesis is also compatible with our data which shows that unlabeled LPL, but not antithrombin, competes for LPL binding to the surface of BAEC. The proof

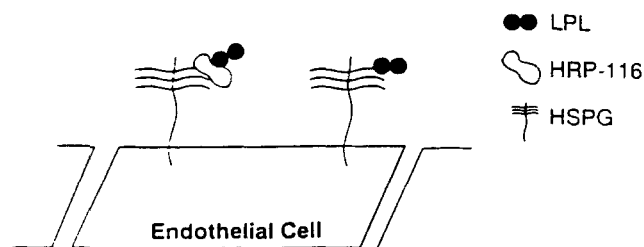


Fig. 8. Models of LPL binding to endothelial cells: role of hrp-116. Although LPL can bind to HSPG, this may represent a nonspecific interaction. In contrast, LPL interaction with hrp-116 may allow for specific interactions between LPL and the cell surface. Because hrp-116 is released from the surface of cultured endothelial cells with heparin, hrp-116 may bind to HSPG. Our data are also compatible with a hypothesis that HSPG and hrp-116 cooperate to anchor LPL to the cell surface.

of our hypothesis will require a more complete understanding of the structure of hrp-116 and the demonstration that specific immunological reagents against this protein do, indeed, affect the specific component of LPL binding to BAEC. ■

The studies were supported by grants HL 45095 (IJG and PS) and HL 21000 SCOR (IJG, AS, and MFS) from the NHLBI. Dr. Goldberg is the recipient of an Established Fellowship for Research from the American Heart Association, New York City Affiliate.

Manuscript received 30 December 1992, in revised form 9 April 1993, and in re-revised form 7 June 1993.

REFERENCES

- Eckel, R. H. 1989. Lipoprotein lipase: a multifunctional enzyme relevant to common metabolic diseases. *N. Engl. J. Med.* **320**: 1060-1068.
- Olivecrona, T., and G. Bengtsson-Olivecrona. 1987. Lipoprotein lipase from milk, the model enzyme in lipoprotein lipase research. In *Lipoprotein Lipase*. J. Borenstajn editor. Evener, Chicago. 15-58.
- Lalouel, J. M., D. E. Wilson, and P.H. Iverius. 1992. Lipoprotein lipase and hepatic triglyceride lipase: molecular and genetic aspects. *Curr. Opin. Lipidol.* **3**: 86-95.
- Hahn, P. F. 1943. Abolishment of alimentary lipemia following injection of heparin. *Science.* **98**: 19-20.
- Cheng, C. F., G. M. Oosta, A. Bensadoun, and R. D. Rosenberg. 1981. Binding of lipoprotein lipase to endothelial cells in culture. *J. Biol. Chem.* **256**: 12893-12896.
- Shimada, K., P. J. Gill, J. E. Silbert, W. H. J. Douglas, and B. L. Fanburg. 1981. Involvement of cell surface heparin sulfate in the binding of lipoprotein lipase to cultured bovine endothelial cells. *J. Clin. Invest.* **68**: 995-1002.
- Wang-Iverson, P., E. A. Jaffe, and W. V. Brown. 1980. Triglyceride hydrolysis by lipoprotein lipase bound to endothelial cells in culture. *Atherosclerosis.* **5**: 375-378.
- Bengtsson-Olivecrona, G., and T. Olivecrona. 1985. Binding of active and inactive forms of lipoprotein lipase to heparin. *Biochem. J.* **226**: 409-413.
- Atha, D. H., A. W. Stephens, and R. D. Rosenberg. 1984. Evaluation of critical groups required for the binding of heparin to anti-thrombin. *Proc. Natl. Acad. Sci. USA.* **81**: 1030-1034.
- Lindahl, U., L. Thunberg, G. Backstrom, J. Riesenfeld, K. Nordling, and I. Bjork. 1984. Extension and structural variability of the antithrombin-binding sequence in heparin. *J. Biol. Chem.* **259**: 12368-12376.
- Shimada, K., and T. Ozawa. 1985. Evidence that cell surface heparin sulfate is involved in the high affinity thrombin binding to cultured porcine aortic endothelial cells. *J. Clin. Invest.* **75**: 1308-1316.
- Esmon, C. T., N. L. Esmon, S. Kurosawa, and A. E. Johnson. 1986. Interaction of thrombin with thrombomodulin. *Ann. N.Y. Acad. Sci.* **485**: 215-222.
- Mahley, R. W. 1988. Apolipoprotein E: cholesterol transport protein with an expanding role in cell biology. *Science.* **240**: 622-630.
- Rapraeger, A. C., A. Krufka, and B. B. Olwin. 1991. Requirement of heparan sulfate for bFGF-mediated fibroblast growth and myoblast differentiation. *Science.* **252**: 1705-1708.

15. Yayon, A., M. Klagsbrun, J. D. Esko, P. Leder, and D. Ornitz. 1991. T1 cell surface heparin-like molecules are required for binding of basic fibroblast growth factors to its high affinity receptor. *Cell*. **64**: 841-848.
16. Sivaram, P., M. G. Klein, and I. J. Goldberg. 1992. Identification of a heparin releasable lipoprotein lipase binding protein from endothelial cells. *J. Biol. Chem.* **267**: 16517-16522.
17. Saxena, U., L. D. Witte, and I. J. Goldberg. 1989. Release of endothelial cell lipoprotein lipase by plasma lipoproteins and free fatty acids. *J. Biol. Chem.* **264**: 4349-4355.
18. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**: 265-275.
19. Cole, S. R., L. K. Ashman, and P. L. Ey. 1987. Biotinylation: an alternative to radioiodination for the identification of cell surface antigens in immunoprecipitates. *Mol. Immunol.* **24**: 699-705.
20. Saxena, U., M. G. Klein, and I. J. Goldberg. 1990. Metabolism of endothelial cell-bound lipoprotein lipase. Evidence for heparan sulfate proteoglycan-mediated internalization and recycling. *J. Biol. Chem.* **265**: 12880-12886.
21. Sasaki, A., and I. J. Goldberg. 1992. Lipoprotein lipase release from BFC-1 β adipocytes: effects of triglyceride-rich lipoproteins and lipolysis products. *J. Biol. Chem.* **267**: 15198-15204.
22. Bar-Shavit, R. A., M. Eldor, and I. Vlodavsky. 1989. Binding of thrombin to subendothelial extracellular matrix: protection and expression of functional properties. *J. Clin. Invest.* **84**: 1096-1104.
23. Marcum, J. A., D. H. Atha, L. M. S. Fritze, P. Nawroth, D. Stern, and R. D. Rosenberg. 1986. Cloned bovine aortic endothelial cells synthesize anticoagulant active heparin sulfate proteoglycan. *J. Biol. Chem.* **261**: 7507-7517.
24. Saxena, U., M. G. Klein, and I. J. Goldberg. 1991. Identification and characterization of the endothelial cell proteoglycan binding site for lipoprotein lipase. *J. Biol. Chem.* **266**: 17516-17521.
25. Hoogewerf, A., L. A. Cisar, D. C. Evans, and A. Bensadoun. 1991. Effect of chlorate on the sulfation of lipoprotein lipase and heparin sulfate proteoglycans. *J. Biol. Chem.* **266**: 16564-16571.
26. Lin, P. H., R. H. Selinfreun, E. Wakschull, and W. Warton. 1988. Rapid isolation of plasmalemma from cultured a431 cells: characterization of epidermal growth factor receptors. *Anal. Biochem.* **168**: 300-305.
27. Barzu, T., J. L. M. N. van Rijn, M. Petitou, G. Tobelem, and J. P. Caen. 1987. Heparin degradation in the endothelial cells. *Thromb. Res.* **47**: 601-609.
28. Cupp, M., A. Bensadoun, and K. Melford. 1987. Heparin decreases the degradation rate of lipoprotein lipase in adipocytes. *J. Biol. Chem.* **262**: 6383-6388.
29. Cisar, L. A., A. J. Hoogewerf, M. Cupp, C. A. Rapport, and A. Bensadoun. 1989. Secretion and degradation of lipoprotein lipase in cultured adipocytes. *J. Biol. Chem.* **264**: 1767-1774.
30. Kent, W. M., F. M. Funderberg, and L. A. Culp. 1986. Proteoglycans in the substratum of adhesion sites of human papillary or retinal dermal fibroblast: aging in vivo and in vitro. *Mech. Ageing Dev.* **33**: 115-137.