Metabolism of Endothelial Cell-bound Lipoprotein Lipase

EVIDENCE FOR HEPARAN SULFATE PROTEOGLYCAN-MEDIATED INTERNALIZATION AND RECYCLING*

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Uday Saxena, Michael G. Klein, and Ira J. Goldberg‡
From the Department of Medicine and Specialized Center of Research in Arteriosclerosis, Columbia University, College of Physicians and Surgeons, New York, New York 10032

Lipoprotein lipase (LPL) hydrolyzes triglyceride in plasma lipoprotein primarily while bound to vascular endothelial cells. LPL metabolism by cultured endothelial cells was studied. Purified radioiodinated bovine LPL bound to porcine aortic endothelial cells at 4 °C with an association constant of 0.18 × 10^7 M^-1. Analysis of the time course of LPL dissociation from endothelial cells at 4 °C yielded a dissociation rate constant of 3.9 × 10^-4 s^-1. After 1 h at 37 °C, 28% of the LPL initially bound to the cell surface was no longer releasable by heparin or trypsin treatments, suggesting that LPL was internalized by the cells. Addition of heparin to the medium or pretreatment of the cells with heparinase markedly reduced the amount of LPL internalized, establishing a requirement for cell surface heparan sulfate proteoglycans in the process. When cells containing internalized LPL were incubated at 37 °C, a time-dependent increase in the amount of LPL in the medium and a corresponding decrease in LPL associated with the cells was found. This suggested that internalized LPL was released back into the medium. The catalytic activity, molecular size, and heparin-binding characteristics of the released LPL was similar to native LPL. Addition of either heparin, heparinase, or excess unlabeled LPL to prevent the rebinding of released [125I]-LPL to the cell surface increased the amount of [125I]-LPL present in the medium, suggesting that there is a process of recycling of [125I]-LPL bound to the cell surface. Studies examining the effect of pH on dissociation of LPL from its binding site showed less dissociation of cell surface bound LPL at pH 5.5 compared with pH 7.4 and 8.5. These results suggest that even at acidic pH as in endocytic vesicles, LPL remains bound to proteoglycans and this may facilitate the recycling of internalized LPL molecules.

Plasma triglycerides transported within chylomicrons and very low density lipoproteins are hydrolyzed by lipoprotein lipase (LPL). The hydrolysis is primarily mediated by LPL which is bound to the vascular endothelium (1). Several studies have investigated the nature of LPL-binding sites on the endothelial cell surface (2-4). In these studies, pretreatment of the cells with either heparinase or trypsin abolished the binding of LPL, thus providing evidence that heparan sulfate proteoglycans on the cell surface mediate the association of LPL with these cells. Cell surface heparan sulfate proteoglycans are either directly intercalated in the lipid bilayer of the cell membrane through hydrophobic regions in the protein moiety of the proteoglycans (5) or are associated with the cell membrane by interaction with other cell surface components (6). The extracellular location of these macromolecules makes them suitable for interaction with plasma components and vascular endothelial cell proteoglycans are implicated as binding sites or receptors for several plasma proteins (7-10).

In previous studies (11), we reported a potentially important role of plasma lipoproteins and free fatty acids in the regulation of LPL interaction with endothelial cell heparan sulfate proteoglycans. The interaction of free fatty acids with LPL dissociated enzyme from the endothelial cell surface. Mono-unsaturated fatty acids were more effective than either saturated or polyunsaturated fatty acids in release of LPL from endothelial cells (12). We have further demonstrated that perturbation of endothelial cell metabolism with tumor necrosis factor leads to the release of LPL bound to the cell surface (13). These data suggest that the amount of LPL participating in plasma triglyceride metabolism may be regulated by alterations of LPL binding to endothelial cell surfaces or by the metabolism of LPL by these cells.

Several studies have examined the synthesis and the metabolic fate of LPL in adipocytes (14-16), as well as the uptake of LPL in heart and liver cells (17, 18). Most of the LPL secreted or exogenously added to cultured adipocytes and heart cells is internalized and degraded in the lysosomal compartment. Similarly, when isolated rat liver was perfused with LPL-containing medium, the enzyme was taken up, inactivated, and then degraded. There has only been one study which directly examined the metabolism of LPL by cultured endothelial cells. In this study by Friedman et al. (19), the limited data presented with endothelial cells suggested that these cells catabolize LPL very slowly in comparison to the above mentioned cell types. However, these experiments did not directly address the metabolic fate of LPL associated with the endothelial cell surface heparan sulfate proteoglycans. To gain further insights into the fate of LPL bound to endothelium, we have specifically examined the process of binding and metabolism of LPL by endothelial cells in culture. In this report we provide evidence for the presence of an intracellular pool of LPL resulting from internalization of LPL bound to the endothelial cell surface. The fate of the intracellular LPL has been examined and the effect of pH, temperature, biochemical inhibitors of cellular processing and transport on the metabolism of LPL studied.
MATERIALS AND METHODS

Endothelial Cell Culture—Primary cultures of porcine aortic endothelial cells were established and subcultured as described before (11). The cells (4–12 passages) were cultured and maintained in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal calf serum. Confluent cultures were maintained overnight in DMEM alone and used the next day.

Purification of Bovine Milk LPL, Radioiodination, and Measurement of Enzyme Activity—LPL was purified from fresh bovine milk using heparin-agarose (Bio-Rad) gel chromatography following the method of Sorocoro et al. (20), essentially as described previously. The purified protein was stored at −70 °C and the protein content determined by the method of Lowry et al. (21) using bovine serum albumin (BSA) (Fraction V, Sigma) as a standard. The purity of the enzyme preparation was assayed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using 4–30% gradient gels (Pharmacia LKB Biotechnology Inc.). The enzyme activity of the purified protein was measured using the substrate emulsion described by Hietanen and Greenwood (22).

Purified LPL was radioiodinated using lactoperoxidase and glucose oxidase enzymes and reprimed using heparin-agarose affinity chromatography. Heparin-agarose (Bio-Rad) gel stored in the presence of 1% BSA at −70 °C. Radioactivity was measured using a model 1274 gamma counter (LKB Instruments, Gaithersburg, MD). The specific activity of labeled LPL was typically about 500 dpm/ng LPL and most of the counts (>90%) were precipitable by 10% trichloroacetic acid. On the day of the experiment, the stored LPL was chromatographed on Bio-Rad agarose-5F-D (Pharmacia) and the elution column equilibrated with DMEM containing 3% BSA and the labeled enzyme was separated from free 125I and degraded proteins.

Binding, Internalization, and Release of 125I-LPL by Endothelial Cells—The experiments were performed using confluent endothelial cell monolayers in 35-mm tissue culture dishes. For binding of 125I-LPL to the cells, the monolayers were rinsed three times with DMEM containing 3% BSA (DMEM BSA), 1 ml of DMEM BSA containing 125I-LPL was then added and the cells were incubated for 2 h at 4 °C. At the end of the incubation period, the unbound 125I-LPL was removed and the cells were washed three times with DMEM-BSA. The cells were then incubated for 30 min at 4 °C with 100 units of heparin (The Upjohn Co.) in 1 ml of DMEM to release cell surface bound 125I-LPL (11). The non-heparin releasable radioactivity associated with the cells during the incubation at 4 °C was then estimated by washing the cells three times with DMEM-BSA, detaching the cells with a rubber policeman, and measuring the radioactivity.

The dissociation rate constant and the half-life of the dissociation of 125I-LPL bound to the endothelial cell surface were determined by first incubating endothelial cells with 10 μg of 125I-LPL at 4 °C for 2 h. After this incubation, the medium was removed and cells washed three times with DMEM-BSA and then medium containing 10 μg of unlabeled LPL was added to the cells. The cells were then incubated at 4 °C for up to 5 h. 125I-LPL present in the medium, on the cell surface, and associated with the cells was then measured.

To study the internalization of LPL by the endothelial cells, after binding LPL to the endothelial cells using 6 μg of LPL/ml as described above, the cells were incubated at 37 °C for 1 h. After this incubation 125I-LPL was measured in three compartments: 1) medium, 2) cell surface, obtained by washing the cells with DMEM-BSA and treating the cells with 100 units of heparin for 30 min at 4 °C, and 3) cell-associated, estimated by removing the cells with a rubber policeman and measuring the radioactivity associated with the cells.

To examine the fate of the internalized 125I-LPL, the enzyme was internalized, all surface LPL removed by heparin treatment, and cells washed as described above. The monolayers were then incubated for various periods of time at 37 °C. After the required time period, the cells were transferred to 4 °C, the medium collected, and the radioactivity present in the three compartments: medium, cell surface, and cell-associated, was performed by addition of concentrated acid to give a final concentration of 10% (v/v) to each of the above fractions.

Characterization of LPL Present in the Medium—LPL in the medium was characterized by triglyceride hydrolytic activity, molecular weight, and binding to heparin-agarose gel. The triglyceride hydrolytic activity of the enzyme present in the medium was measured using 100 μl of a high specific activity substrate emulsion as described previously (23). The molecular size of the released LPL was examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using a 12.5% acrylamide gel. Because of the presence of 5% BSA in the medium, the amount of LPL present in the medium to which the antibody would be applied to the gel was insufficient for autoradiography. Therefore, the gel was sliced into 10-mm sections and radioactivity counted. If the BSA was omitted from the medium, spontaneous breakdown of 125I-LPL was found in the presence or absence of endothelial cells.

The binding of 125I-LPL in the medium to heparin-agarose gel was assessed. Heparin-agarose (200 μl) previously equilibrated with DMEM-BSA was added to 1 ml of the medium, and incubated at 4 °C for 2 h. The mixture was then centrifuged at 12,800 × g for 10 min and the unbound 125I-LPL was collected. The sedimented gel was washed with DMEM-BSA and the washes were saved. The 125I-LPL bound was then sequentially eluted with 20 mm Tris-HCl buffer, pH 7.4, containing 0.4 M NaCl, 0.75 M NaCl, and finally 2 M NaCl. The eluates were collected and the radioactivity measured.

Metabolic Alterations of Endothelial Cells—The roles of cellular glycosaminoglycans, transport mechanisms, and lysosomal degradations in the metabolism of LPL were examined. Cell surface heparan sulfate molecules were selectively removed by treatment of cells with heparinase (5 units/ml, Sigma). Other agents known to affect LPL function were added to the cells after the internalization of 125I-LPL and release of cell-surface bound 125I-LPL as described above. The cells were washed and then incubated with either monensin, (75 μM), cytochalasin B, (400 μM), chloroquine, (75 μM), or leupeptin, (200 μM), in 1 ml of DMEM-BSA for up to 1 h. The amount of 125I-LPL in the medium, on the cell surface, and in the cell-associated compartments were measured as described above. To study the effect of temperature on the release of internalized LPL-LPL was internalized as usual and then the cells were incubated at 4, 25, and 37 °C.

The effect of pH on dissociation of 125I-LPL bound to cell surface heparan sulfate proteoglycans, was examined by first binding the 125I-LPL to the cells as described and then incubating the cells for 1 h at 4 °C in buffers of various pH. Formic acid, (pH 5.5, 25 mM sodium acetate buffer containing 0.15 M NaCl was used; pH 7.4 and 8.5 buffers were composed of 25 mM Tris and 0.15 M NaCl and the pH was adjusted with HCl.

RESULTS

Binding of 125I-LPL to Endothelial Cells—Our previous studies of LPL associated with endothelial cells were performed at 37 °C (11). However, the current studies were performed at 4 °C to minimize the cellular metabolism of LPL during the binding of LPL to the cells. The binding of purified radioiodinated LPL to endothelial cells was studied by incubating cells with increasing concentrations of LPL at 4 °C for 2 h. The cell surface-bound enzyme was released by addition of heparin. With the addition of increasing amounts of 125I-LPL, a linear increase in surface bound LPL was observed using 0.5 to 15 μg 125I-LPL/ml (Fig. 1), but at higher concentrations (above 15 μg/ml) the slope of the binding curve decreased. The amount of radioactivity associated with the cells after heparin treatment was less than 5% of the radioactivity bound to the cells at all concentrations of 125I-LPL.

The specificity of the binding of 125I-LPL to the cells was examined by release of bound LPL by addition of a 100-fold excess of unlabeled LPL. This addition to cells which had previously been incubated with 6 μg/ml of 125I-LPL reduced the amount of label bound to the cells by 88 ± 4%. A time course of binding of LPL to the endothelial cells showed that binding equilibrium was achieved by 90 min (data not shown); therefore, an incubation time of 2 h was used to bind LPL to the cells in all subsequent experiments. The affinity of the binding sites on the endothelial cell surface for LPL was estimated using Scatchard analysis (24) of the binding data as shown in Fig. 1A. The association constant (Kd) calculated from the slope of the Scatchard plot gave a value of 0.18 × 107 M−1 and the estimated maximum
bound to the cells, the following experiment was performed.

To determine whether endothelial cells can internalize LPL, the effect of heparin addition (to release LPL from the cell surface) was examined. Following the binding of \( {}^{125}\)I-LPL, the cells were incubated at 37 °C in media containing 100 units/ml of heparin. The percentage of the cell surface \( {}^{125}\)I-LPL which was internalized by the heparin-treated cells was markedly reduced compared with control cells (4.7 ± 1.8% versus 30 ± 2%) (Table I). These data demonstrated that LPL must remain bound to the cell surface for it to be internalized.

To examine whether the removal of heparan sulfate proteoglycans on the endothelial cell surface affected the binding and internalization of LPL, the cells were incubated in the presence or absence of heparinase for 2 h at 37 °C and then \( {}^{125}\)I-LPL was allowed to bind to the cells at 4 °C. This heparinase treatment decreased the binding of LPL to the cells by 65 ± 6% compared with control, and during a subsequent incubation at 37 °C, a corresponding decrease in the amount of LPL internalized (50 ± 4%) was observed (Table I). Therefore, the presence of cell surface heparan sulfate proteoglycans was required for the internalization of \( {}^{125}\)I-LPL.

Cellular Metabolism of LPL Internalized by the Endothelial Cells—To investigate the fate of LPL internalized by the cells, \( {}^{125}\)I-LPL was bound at 4 °C (2 h), the bound LPL was treated with heparin to release surface-bound LPL. At every point, the media were saved, and the cells were washed and incubated for 1 h at 37 °C. After this internalization of \( {}^{125}\)I-LPL, the cells received either of three different trypsin treatments, 0.025% for 3 min, 0.025% for 6 min, or 0.25% for 10 min. These treatments did not decrease the amount of cell-associated \( {}^{125}\)I-LPL relative to a control treatment (no trypsin). In parallel experiments, similar trypsin treatments of cells with \( {}^{125}\)I-LPL bound to the surface caused 37, 50, and 82% reductions, respectively, in the amount of cell-surface bound \( {}^{125}\)I-LPL. If the \( {}^{125}\)I-LPL was initially trapped extracellularly or bound to the cell surface, it should have been released by trypsin treatment. This experiment demonstrated that cell-associated LPL which was not released by heparin was also not released by trypsin treatment. The cell-associated \( {}^{125}\)I-LPL most likely represents LPL internalized by the cells.

To investigate whether the internalization was mediated by the binding of LPL to cell surface heparan sulfate proteoglycans, the effect of heparin addition (to release LPL, from the cell surface) was examined. Following the binding of \( {}^{125}\)I-LPL, the cells were incubated at 37 °C in media containing 100 units/ml of heparin. The percentage of the cell surface \( {}^{125}\)I-LPL which was internalized by the heparin-treated cells was markedly reduced compared with control cells (4.7 ± 1.8% versus 30 ± 2%) (Table I). These data demonstrated that LPL must remain bound to the cell surface for it to be internalized.

Fig. 2. Internalization of \( {}^{125}\)I-LPL bound to endothelial cells. Endothelial cells were incubated with \( {}^{125}\)I-LPL (6 µg/ml) for 2 h at 4 °C to bind LPL to the cell surface as described in the legend to Fig. 1. The cells were then incubated for up to 1 h at 37 °C. \( {}^{125}\)I-LPL in the three compartments, 1) medium (●), 2) released from the cell surface by heparin treatment (●, and 3) cell-associated (∆), were measured at each time point as described in the text. Each data point represents the average of results from triplicate dishes. Results are expressed as the percent of the \( {}^{125}\)I-LPL initially bound (226 ng) to the cells.

binding was 1.38 µg of \( {}^{125}\)I-LPL/dish. This is comparable to the binding capacity of 0.95 µg of \( {}^{125}\)I-LPL/dish obtained from the binding curve (Fig. 1A). The dissociation rate constant for LPL bound to the endothelial cell surface was determined from a time course of dissociation of \( {}^{125}\)I-LPL at 4 °C (Fig. 1B). A rate constant of 3.9 \( \times \) 10\(^{-6}\) s\(^{-1}\) was obtained.

Internalization of LPL Bound to the Endothelial Cells—To determine whether endothelial cells can internalize LPL bound to the cells, the following experiment was performed. \( {}^{125}\)I-LPL was bound to the cells at 4 °C, and the cells were incubated at 37 °C for various periods of time. After each time point, the media were saved, and the cells were washed and treated with heparin to release surface-bound LPL. At every time point some LPL was not released by heparin and remained associated with the cells, suggesting that there may have been internalization of \( {}^{125}\)I-LPL. The amount of \( {}^{125}\)I-LPL associated with the cells increased linearly with time and at the end of 60 min, accounted for 28 ± 3% of the \( {}^{125}\)I-LPL initially bound to the cell surface (Fig. 2). This amount of non-heparin-releasable, cell-associated LPL at 37 °C was more than 6-fold greater than that found using an incubation temperature of 4 °C. Most (86 ± 3%) of the cell-associated LPL during the 60-min incubation at 37 °C was found to be precipitated by trichloroacetic acid, suggesting that very little degradation occurred during this time period. The amount of \( {}^{125}\)I-LPL remaining bound to the cell surface (heparin releasable) during the 60-min time course decreased, and there was an increase in the amount of \( {}^{125}\)I-LPL in the medium which may be due to dissociation of some LPL from the cell surface (Fig. 2).

Cells containing internalized LPL were treated with trypsin to eliminate the possibility that the non-heparin-releasable \( {}^{125}\)I-LPL was simply bound to plasma membranes or the plastic culture dishes. \( {}^{125}\)I-LPL was allowed to bind to the cell surfaces (4 °C), the medium was changed, and the cells were incubated for 1 h at 37 °C. After this internalization of \( {}^{125}\)I-LPL, the cells received either of three different trypsin treatments, 0.025% for 3 min, 0.025% for 6 min, or 0.25% for 10 min. These treatments did not decrease the amount of cell-associated \( {}^{125}\)I-LPL relative to a control treatment (no trypsin). In parallel experiments, similar trypsin treatments of cells with \( {}^{125}\)I-LPL bound to the surface induced 37, 50, and 82% reductions, respectively, in the amount of cell-surface bound \( {}^{125}\)I-LPL. If the \( {}^{125}\)I-LPL was initially trapped extracellularly or bound to the cell surface, it should have been released by trypsin treatment. This experiment demonstrated that cell-associated LPL which was not released by heparin was also not released by trypsin treatment. The cell-associated \( {}^{125}\)I-LPL most likely represents LPL internalized by the cells.

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Treatment was carried out by the addition of 100 units of heparin to
of 6 pg LPL/ml as described under “Materials and Methods.” Heparin
was taken up by 1Z51-LPL bound to their surface. The amount of 1Z51-LPL
subsequently internalized was determined. Other cells were treated
with heparinase, 5 units for 2 h at 37 °C, and then 1Z51-LPL was added
to the medium and allowed to bind to the cells. The amount of 1Z51-
LPL bound to the cell surface and the amount of LPL internalized
by endothelial cells after a similar treatment were determined. Results
are mean values of experiments performed in triplicate ± S.E.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>LPL bound to the cells</th>
<th>LPL internalized</th>
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</thead>
<tbody>
<tr>
<td>Control</td>
<td>246 ± 18</td>
<td>72 ± 8</td>
</tr>
<tr>
<td>Heparin</td>
<td>238 ± 26</td>
<td>11 ± 4</td>
</tr>
<tr>
<td>Heparinase</td>
<td>91 ± 11</td>
<td>36 ± 6</td>
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Moreover, as shown in Fig. 3C, the released LPL had a similar
catalytic activity, heparin-binding property, and molecular size of released LPL was examined. As shown in Fig.
3B, after internalization of LPL, the amount of triglyceride hydrolytic activity present in the medium increased over time. The binding of released 1Z51-LPL to heparin-agarose affinity gel in comparison to control 1Z51-LPL (incubated in DMEM-
BSA in the absence of cells) was examined. The 1Z51-LPL appearing in the medium over time was incubated with heparin-agarose, the gel was washed with 0.4 and 0.75 M NaCl solutions, and the amount of 1Z51-LPL bound to the gel and eluting with high salt concentration (2 M NaCl) is shown
in Fig. 3B. At 15 min (release time) 66 ± 4% of the 1Z51-LPL was eluted from the gel with high salt; a comparable amount of 1Z51-LPL from control medium (incubated at 37 °C in the absence of cells for 15 min) was eluted from the gel by high salt (65 ± 4%). This percentage decreased over time, and for the 60-min time point 42 ± 6% of released 1Z51-LPL and 38 ± 2% of control LPL were eluted from the heparin-agarose with 2 M NaCl. The remainder of 1Z51-LPL in both cases was recovered in the unbound and lower salt concentration washes. This decrease in LPL binding to the affinity gel with time may represent a spontaneous conversion of LPL from dimeric to monomeric forms (25) or represent some partial degradation of the molecule (26). However, the similar decrease in binding of LPL in control medium demonstrated that this change in LPL is exclusive of cellular processes. Moreover, as shown in Fig. 3C, the released LPL had a similar
electrophoretic migration to that of control 1Z51-LPL incubated in the absence of cells. These data confirm that internalized LPL is release from the cells without any major modifications.

Recycling of 1Z51-LPL by Endothelial Cells—The results presented so far suggest that LPL bound to the cell surface is internalized and delivered back to the cell surface and the
medium. To examine whether there is recycling of the LPL by the cells, i.e. transport to the cell surface followed by another round of binding, internalization, and release, heparin was used to decrease the rebinding of LPL (if any) to the cell surface. The release of internalized LPL was studied in the presence of heparin. Endothelial cells were allowed to internalize $^{125}$I-LPL as described above (at 37°C for 1 h) followed by removal of residual surface-bound LPL by heparin treatment. The cells were then incubated in the presence of heparin, heparinase, or unlabeled LPL for 30 min. As shown in Fig. 4, the appearance of $^{125}$I-LPL in the medium was greater in the presence of heparin than in control medium. Compared with control, at 15 and 30 min there was a 1.3- and 1.7-fold increase, respectively, in the amount of $^{125}$I-LPL in the heparin-containing medium. The increase of $^{125}$I-LPL in the medium in the presence of heparin resulted in a corresponding decrease in the amount of $^{125}$I-LPL present inside the cells (data not shown). The most likely mechanism for this effect is that heparin displaces LPL from binding sites on the cell surface, soluble enzyme-heparin complexes are formed, and this LPL in the medium is prevented from rebinding and being internalized by the endothelial cells.

Two other treatments which decrease the binding of $^{125}$I-LPL to the endothelial cell surface (heparinase and excess unlabeled LPL) were similarly tested. We hypothesized that loss of cell surface glycosaminoglycans might decrease recycling of LPL and result in more labeled LPL in the medium. Furthermore, we expected that an increase in the amount of $^{125}$I-LPL in the medium would also be found if labeled LPL returning to the surface was allowed to exchange with an excess of unlabeled LPL added to the medium. As shown in Fig. 4, at the end of 30 min in both cases there was an increase in the amount of $^{125}$I-LPL in the medium, although the magnitude of increase was less than that observed with heparin. This may be related to the relative efficiency of heparinase to degrade the heparan sulfate moiety of the cell surface proteoglycans and for the unlabeled LPL to exchange with $^{125}$I-LPL bound to endothelial cell-surface heparan sulfate proteoglycans. These experiments strongly suggest that there is continuous recycling of $^{125}$I-LPL bound to heparan sulfate proteoglycans on the endothelial cell surface.

Effect of Alterations of Endothelial Cell Metabolism on the Release of $^{125}$I-LPL—The above results suggest that $^{125}$I-LPL was internalized and then released from within the cells into the medium. Further support for the hypothesis that LPL is internalized by endothelial cells and subsequently released was sought in other experiments. To examine whether the release of internalized $^{125}$I-LPL is a metabolic process, the effect of temperature was studied. $^{125}$I-LPL was first internalized and then incubated at various temperatures and the $^{125}$I LPL released in the medium was monitored. At 37°C, 36 ± 4% of internalized $^{125}$I-LPL was released from the cells at 60 min, at 25°C the release of $^{125}$I-LPL was 20 ± 7%, and at 4°C only 4 ± 1.8% of $^{125}$I-LPL was released into the medium. These results demonstrate that the release of intracellular LPL is a temperature-dependent process.

To investigate the intracellular route of $^{125}$I-LPL release by the endothelial cells, the effect of altering cellular transport was examined. To exclude any potential effects of the reagents used on $^{125}$I-LPL binding or internalization by the endothelial cells, the agents were added after internalization of LPL. Treatment with monensin, a proton ionophore which raises endocytotic vesicle pH (27-31), resulted in greater accumulation of $^{125}$I-LPL present inside the cells (Fig. 5). Monensin treated endothelial cells had 48% more $^{125}$I-LPL present inside the cells compared with control cells (Fig. 5). Cytochalasin B, a compound which effects the microfilament system and interferes with cellular transport, also blocked the transit of $^{125}$I-LPL from the cells resulting in more than a 100% increase in the amount of LPL in the cells (Fig. 5). The role of lysosomal processing was examined using two lysosomal protease inhibitors, chloroquine a weak base that is concentrated in lysosomes and raises their pH (29) and leupeptin, a lysosomal protease inhibitor that does not affect intracellular pH (30). Both agents had no effect on the release of internalized $^{125}$I-LPL by the endothelial cells (Fig. 5). The temperature dependence and the effects of biochemical agents provided further proof that LPL was present intracellularly and not processed by lysosomes.

Effect of pH on the Association of $^{125}$I-LPL with Endothelial Cells—The results obtained with monensin treatment suggested that raising the pH in the endocytotic vesicle may lead to the accumulation of $^{125}$I LPL inside the cells. The pH in the endocytotic vesicle is acidic (27-29) and the low pH in these vesicles is believed to be an important factor in the dissociation of ligands from their receptors (29, 31). In most cases dissociation enables the receptor to be recycled back to the surface and the ligand is then targeted to the lysosome. The results presented in Table I suggested that internalization
tion and not mere extracellular trapping or plasma membrane binding of LPL was obtained in experiments which showed similar incubation at 4 °C. The trapping of LPL within the amount of internalized LPL compared with that found after intracellular location of this pool. The role of cell surface demonstrated in the experiments using heparinase and heparin treatments (Table I).

The effect of monensin is of special interest. The known effects of monensin on inhibiting proton gradients and increasing endocytic vesicle pH suggest that a step in the pathway for LPL may be mediated via an intracellular compartment that requires acidification, probably the endocytic vesicle, since several receptor-mediated ligands are transferred to such vesicles after internalization (27). The effect of pH on the dissociation of LPL from heparan sulfate proteoglycans on the cell surface provided a likely explanation for effects of monensin on LPL recycling. The LPL-heparan sulfate interactions were dissociated by exposure to alkaline pH relative to pH 5.5. Monensin by raising endocytic vesicle pH may have dissociated LPL from its heparan sulfate receptor. On the other hand, acidic pH, which the LPL receptor complex may encounter in the endocytic vesicle, may actually facilitate the maintenance of the complex (Fig. 5). On the basis of these results a model for the metabolism of LPL in the endothelial cells may be suggested. LPL under conditions appropriate for binding (high LPL concentrations and neutral pH) binds to its heparan sulfate proteoglycan receptors on the cell surface. After binding, LPL is internalized by receptor-mediated endocytosis. The ligand receptor complex then moves to a non-lysosomal acidic compartment (endocytic vesicle) which augments the ligand-receptor interactions. The complex remains intact and either the ligand and receptor are delivered together directly to the medium or to the cell surface where some LPL is dissociated from the heparan sulfate proteoglycan. In either case some LPL may rebind to the surface and be committed into a recycling pathway.

The cell surface heparan sulfate proteoglycans may serve as more than just passive binding sites for LPL. These proteoglycans display several properties required of cell surface receptors, i.e., high affinity, saturability, reversibility, and, as demonstrated here, internalization and targeting of the ligand to a nondegradative pathway. Several other proteins, including thrombin, thrombospordin, basic fibroblast growth factor, diaminoxidase, and antithrombin have been shown to bind to heparan sulfate proteoglycans present on endothelial cell surface. Furthermore, in the case of thrombospordin and basic fibroblast growth factor these proteins are internalized and degraded within the cells (10, 33). Antithrombin is bound but may not be internalized (34). Therefore, a variety of metabolic fates in endothelial cells are exhibited by various heparan sulfate proteoglycan-binding proteins. Several studies (35–37) have demonstrated that cell surface heparan sulfate proteoglycans display a wide range of structural diversity in the core proteins. This raises the possibility that various heparan sulfate proteoglycans may have different ligand binding specificities and therefore may play different functional roles.

The major purpose of this study was to understand the molecular mechanisms underlying the interactions and metabolic fate of LPL associated with endothelial cells. It was

**DISCUSSION**

The experiments presented here demonstrate that the binding of LPL to the endothelial cell surface results in internalization of the enzyme. Evidence in favor of true internalization and not mere extracellular trapping or plasma membrane binding of LPL was obtained in experiments which showed failure of heparin or trypsin treatments to release this pool of LPL. In addition, when cells with LPL bound to their surface were incubated at 37 °C, we observed an increase in the amount of internalized LPL compared with that found after similar incubation at 4 °C. The trapping of LPL within the cells by monensin and cytochalasin B further confirmed the intracellular location of this pool. The role of cell surface heparan sulfate proteoglycans in the internalization was demonstrated in the experiments using heparinase and heparin treatments (Table I).

Results of experiments designed to probe the fate of internalized LPL suggested that this LPL is delivered to the cell surface and the medium. Furthermore, the results obtained with the addition of heparin, heparinase, and excess unlabeled LPL demonstrated that LPL bound to the heparan sulfate proteoglycans is recycled, i.e., there is a continuous process of binding, internalization, and transport to the cell surface. The addition of cytochalasin B led to the accumulation of LPL within the cells, suggesting the involvement of the cellular cytoskeleton. The pathway of internalization and recycling of LPL in the cells does not appear to involve lysosomal processing since the lysosomal enzyme inhibitors showed no effect. This possibility was further supported by the presence of intact, heparin binding, and catalytically active LPL which was released into the medium by the cells.

**Fig. 6. Effect of pH on the association of 125I-LPL with heparan sulfate proteoglycans on endothelial cell-surface.** 125I-LPL (6 μg/ml) was added to DMEM and allowed to bind to endothelial cells at 4 °C for 2 h as described in the legend to Fig. 1. The cells were then incubated with buffers of either pH 5.5, 7.4, or 8.5 for 1 h at 4 °C. The amount of 125I-LPL dissociated from the cell-surface (O) and that remaining bound to the cells (●) was estimated as described in the text. Results are presented as percent of total LPL initially bound. Data presented are the mean value ± S.E. of experiments performed in triplicate dishes.
hoped that elucidation of these mechanisms may help in understanding the distinctive metabolism of LPL in endothelial cells compared with adipocytes, cardiac myocytes, and liver cells where LPL is internalized and degraded in the lysosomes (14–18). Chajek-Shaul et al. (18), on the basis of their model experiments, have proposed that LPL may initially bind to heparan sulfate proteoglycans on liver cells, but is then transferred to another site from which the enzyme would dissociate when the endosome is acidified and LPL loses its catalytic activity. Our studies using endothelial cells suggest that by remaining associated with heparan sulfate proteoglycans even at acidic pH, LPL may be sorted and directed differently in endothelial cells than other cell types.

A question that is raised by these studies is the possible functional significance of recycling of LPL by endothelial cells. One possibility is that it may be a part of the normal turnover of the functional pool of LPL located at the vascular endothelium. A spontaneous release of exogenous and endogenous LPL activity and protein from isolated perfused rat hearts has been demonstrated (17). These and other studies (38) suggest that the main fate of endothelial cell LPL is dissociation of catalytically active LPL into the circulation, followed by liver uptake and degradation. In this regard, catalytically active LPL has been demonstrated in human plasma (23).

Another possible function of the internalization and recycling of endothelial cell-bound LPL pertains to an intracellular regulatable LPL pool within these cells. Since at the end of 60 min about half of the LPL internalized is still present inside the cells, it is tempting to propose that modulation of the recycling process by components of plasma (e.g. other heparan sulfate-binding proteins and lipoproteins) or extracellular matrix (e.g. heparin) of the vascular endothelium could regulate the amount of LPL activity available at the blood vessel wall. It is of interest to note that bovine aortic endothelial cells have been shown to release heparin-like substances in the presence of serum (39). In addition, interruption of this recycling by intravenous administration of heparin to animals and humans would be expected to release both cell surface associated and internalized LPL protein into the circulation (40).

A third possibility is related to the transport of LPL from its site of synthesis, i.e. parenchymal cells, to its site of action, the luminal side of the endothelium. The mechanisms involved in this process remain unknown. Although the present study has demonstrated the internalization and release of LPL from endothelial cells in culture, where the enzyme is internalized and released from the same side of the cell, the data may be indicative of a process of transcytosis, where LPL may be internalized from the basal side and transported to the luminal side of endothelial cells. Receptor-mediated transcytosis of various macromolecules by vascular endothelial cells has been well documented (41–43). The ability of endothelial cells to recycle LPL without degradation, may be an adaptation by these cells to preserve the LPL acquired from the parenchymal cells for LPL function at the vascular endothelium.

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