Reversible High Affinity Uptake of Apo E-Free High Density Lipoproteins in Cultured Pig Hepatocytes

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We examined the high affinity binding, uptake, and degradation of apo E-free 125I-high density lipoprotein (HDL) in cultured pig hepatocytes. At steady state, the cells degraded 9.4% of cell-associated 125I-HDL/hour, compared with 41.7%/hour for 125I-LDL. Pulse-chase experiments at 4° C revealed that high affinity 125I-HDL binding was reversible. Similar experiments at 37° C revealed that about 70% of the cell-associated 125I-HDL was released as a macromolecule; the remainder was degraded to acid-soluble products. In contrast, over 75% of the 125I-LDL that was released had been degraded to acid soluble products. The amount of macromolecular 125I-HDL released at 37° C was similar to the amount that was bound to the cell surface, as estimated from measurements of trypsin-releasable radioactivity. Density gradient ultracentrifugation and SDS-polyacrylamide gel electrophoretic analysis of macromolecular 125I-HDL released to the medium revealed an increase in density, and the apparent partial proteolysis of apo A-I (Mr 25,000) to products of apparent Mr 12,000-14,000. The findings suggest that high affinity 125I-HDL uptake had a reversible component in which HDL was concentrated temporarily at the cell surface, modified, and then released as a somewhat denser lipoprotein particle. Measurement of 125I-HDL and 125I-LDL degradation in cell homogenates revealed no difference in the inherent susceptibility of the two lipoproteins to proteolysis by lysosomal enzymes. The overall slower rate of degradation of 125I-HDL compared to 125I-LDL was therefore due in part to the smaller fraction of HDL that was committed to irreversible catabolism. The rate of catabolism of this fraction, however, was considerable. Cells pulsed at 4° C and subsequently warmed to 37° C released one-half the acid-soluble products from 125I-HDL within about 4 hours, compared with 2 hours for cells pulsed with 125I-LDL. These findings indicate that HDL was internalized, transported to lysosomes, and degraded at about one-half the rate of LDL. (Arteriosclerosis 5:142-152, March/April 1985)

High density lipoproteins (HDL) are synthesized primarily in the liver and intestine. Certain lipid and apoprotein constituents of HDL participate in cholesterol esterification and the catabolism of triglyceride-rich lipoproteins. HDL stimulates the removal of cholesterol from several kinds of cultured cells. HDL may also accept cholesterol from the tissues in vivo and transport cholesterol from peripheral tissues to the liver for disposal or reutilization.
apo E-free porcine HDL apoprotein in cultured pig hepatocytes. Degradation was mediated by a high affinity site with properties different than either the LDL receptor or the apo E-specific site described in liver microsomes. The most striking difference was that the site recognized both LDL or apo E-free HDL; $^{125}$I-HDL degradation was inhibited by both unlabeled lipoproteins. We called the site the “lipoprotein binding site” because it recognized both lipoproteins. During those studies, we compared clearance rates for $^{125}$I-HDL and $^{129}$I-LDL with that of $^{14}$C-sucrose, which was used to measure the rate of bulk-phase pinocytosis. $^{125}$I-HDL was cleared at least 54-fold faster, and $^{129}$I-LDL at least 309-fold faster than $^{14}$C-sucrose. This observation suggests that although the high affinity catabolism of both lipoproteins occurred readily, $^{125}$I-HDL was degraded more slowly than $^{129}$I-LDL. This might result from differences in the ease with which the two lipoproteins reached the lysosomes or to differences in their rates of proteolysis once they had entered the lysosomes. In the present study, we investigated the relative proportions of cell-associated $^{125}$I-HDL and $^{129}$I-LDL that were degraded and the relative susceptibility of the two lipoproteins to hydrolysis by lysosomal proteases. Our findings indicated: 1) that the cells degraded about 10% of cell-associated $^{125}$I-HDL per hour/permilligram of cell protein, compared with about 40% per hour for $^{129}$I-LDL; 2) that this resulted from the presence of a large component of reversible, high affinity $^{125}$I-HDL uptake by the cells.

**Methods**

**Lipoproteins**

HDL (d = 1.12–1.16 g/ml) and LDL (d = 1.019–1.063 g/ml) were isolated by ultracentrifugation from the plasma of normolipidemic adult swine, and these lipoproteins were dialyzed against 0.15 M NaCl containing 0.5 mg/ml disodium ethylenediamine tetraacetate (EDTA), pH 7.4. The LDL contained no detectable apo A-1. The HDL was treated by heparin-Sepharose affinity chromatography to remove possible traces of apo B and apo E-containing lipoproteins. Heparin-Sepharose-treated HDL was examined by SDS-polyacrylamide gel electrophoresis and contained no apo B or apo E. The major apoprotein was apo A-1, Mr 25,500; the minor apoproteins were a rapidly migrating component, possibly apo C, and traces of two unidentified proteins, Mr 66,500 and 58,000, noted previously. The lipoproteins were sterilized by ultrafiltration (0.45 μm filters) and were stored at 4°C. Porcine lipoprotein-deficient serum (LPDS, d > 1.25 g/ml) was dialyzed, sterilized, and stored at −70°C until used.

Apo E-free HDL and LDL were labeled with $^{125}$I. Generally, about 80% of the radioactivity in $^{125}$I-HDL was associated with apo A-1, and about 95% in $^{125}$I-LDL was in apo B. Labeled lipids accounted for less than 6% of the radioactivity in $^{125}$I-HDL and less than 3% of that in $^{125}$I-LDL.

**Cells**

Young Duroc or Yorkshire pigs (2.5 to 5.0 kg) were pre-anesthetized with ketamine (22 mg/kg) and anesthetized with sodium pentobarbital (7 mg/kg), which was administered intravenously. Hepatocytes were prepared after perfusion of the liver with collagenase, 0.03% (wt/vol) as described previously and were cultured for 20 to 24 hours in Eagle’s minimum essential medium (MEM) containing penicillin (100 units/ml), streptomycin (100 mg/ml) and 10% fetal calf serum (FCS).

**Uptake and Degradation Assays In Cells**

At the time of the experiments, the cells were transferred to MEM containing porcine LPDS (10% vol/vol, 5-8 mg/ml serum protein) and incubated at 4°C or 37°C with $^{125}$I-lipoproteins (5 μg/ml) ± 20- to 100-fold excess of unlabeled lipoproteins. After the incubation, the medium was added to 0.5 ml trichloroacetic acid (50% wt/vol) and allowed to stand at 2°C to 4°C for 30 minutes. Degradation was calculated from the measurements of acid-soluble radioactivity.

**Estimation of Surface-Bound $^{125}$I-HDL by Treatment with Trypsin**

In some experiments, surface binding of $^{125}$I-HDL was estimated after brief treatment of the cells with trypsin under conditions modified from those described by others. Cells were incubated with $^{125}$I-HDL (5 μg/ml) ± unlabeled HDL (150 μg/ml), washed as described above, then transferred to serum-free MEM containing trypsin (30 μg/mg) (Worthington, Freehold, New Jersey); cells were then incubated for 10 minutes at 37°C. In several preliminary experiments, it was determined that under these conditions, maximal trypsin-mediated release was obtained without significant loss of cell protein. The trypsin-treated cell monolayers appeared morphologically intact by light microscopy and the cells’ ability to exclude trypan blue dye was unimpaired.
Density Gradient Ultracentrifugation

After incubation with trypsin, the medium was removed, the cells were washed once with a cold PBS solution of 0.5% BSA, and twice with cold PBS, then dissolved in 1 M NaOH for the measurement of cell-associated radioactivity and cell protein. Cell surface binding was calculated from the reduction in cell-associated radioactivity and was corrected for non-specific binding by subtracting the amount of trypsin-sensitive radioactivity when the cells were pulsed in the presence of unlabeled HDL.

Pulse-Chase Experiments

Cells were pulsed with 129I-lipoproteins as indicated, then washed as described above and transferred to the chase medium. The chase medium was MEM that contained 10% LPDS (5 to 8 mg/ml of serum protein). Unlabeled lipoproteins were omitted from the chase medium unless otherwise specified. Cell-associated radioactivity and acid-soluble radioactivity in the medium were then measured at various times. The total radioactivity released was calculated from the reduction in cell-associated radioactivity during the chase.

Degradation In Cell Homogenates

After 22 hours in 10% FCS-containing medium, cells were washed twice with cold PBS, then scraped from the plates, suspended in PBS, and centrifuged at 3000 g for 10 minutes at 4° C. The cell pellet was suspended in 5 x 10^-3 M Tris-HCl (5.0 ml), pH 7.5, and frozen at -70° C. The suspension was thawed and sonicated twice at 0 to 2° C (20 seconds each, maximum power, Sonifer Model S125, Branson Instruments, Incorporated, Danbury, Connecticut). The cells were disrupted completely as judged by light microscopic examination. The lysosomes also appeared to be disrupted completely inasmuch as there was no initial lag in the proteolysis of 125I-HDL and 125I-LDL at pH 4.0 (see Results). 125I-HDL or 125I-LDL (100 μg/ml protein) was incubated at 37° C for 60 minutes with cell homogenate (500 μg/ml protein) in citrate buffer (0.075-0.080 M), pH 4.0. The reaction was terminated by adding ice-cold trichloroacetic acid (final concentration 10% wt/vol); the acid-soluble radioactivity in the supernatant was measured.

Protein was measured as described by Lowry et al33 using bovine serum albumin as the standard. Polycrylamide gel electrophoresis was performed in 13% gels in the presence of 0.1% sodium dodecyl sulfate (SDS) under reducing conditions.34

Results

Previous measurements20 of 125I-HDL and 125I-LDL clearance that were compared with 14C-sucrose in cultured pig hepatocytes suggested that 125I-HDL was cleared at a rate 5.7-fold slower than 125I-LDL. Here we estimated the relative rates of 125I-HDL and 125I-LDL degradation as the fraction of the amount of the cell-associated lipoprotein that was degraded per hour in the steady state. Cells were incubated at 37° C with 125I-HDL or 125I-LDL, and uptake and degradation were measured as a function of time. Table 1 summarizes for 17 cell preparations the degradation rates that were measured between the 12th and 25th hours after the start of the incubations. In this period, uptake was at a steady state and degradation was linear with time (Figure 1). 125I-HDL degradation averaged 9.4% of cell-associated lipoprotein-protein/hour, compared with 41.7%/hour for 125I-LDL (Table 1). Thus, in terms of the proportion of the steady-state levels of the two degraded lipoproteins, 4.4 times more 125I-LDL was degraded.

Three general mechanisms were considered that might account for the difference. First, HDL might be internalized or transported to the lysosomes more slowly than LDL. Second, a smaller proportion of internalized HDL might be committed to lysosomal degradation even if the rates of transport of both lipoproteins to the lysosomes were equal. Finally, the two lipoproteins might be differentially susceptible to proteolysis after they entered the lysosomes.

To address the first two questions, we performed pulse-chase experiments to examine the fate of the cell-associated lipoproteins. In the first experiment, two sets of cell plates were used in parallel. The first set was incubated with 125I-lipoprotein in 10% LPDS-medium for periods up to 25 hours; this set was used
Table 1. Relative Rates of Degradation of $^{125}$I-HDL and $^{125}$I-LDL in Cultured Pig Hepatocytes

<table>
<thead>
<tr>
<th>Lipoprotein</th>
<th>No. of expts</th>
<th>Cell associated at steady state* (pmol/mg cell protein)</th>
<th>Degradation rate at steady state* (pmol/hr/mg cell protein)†</th>
<th>Relative degradation rate (%)‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{125}$I-HDL</td>
<td>11</td>
<td>2.287 (± 0.277)</td>
<td>0.201 (± 0.027)</td>
<td>9.4 (± 1.5)</td>
</tr>
<tr>
<td>$^{125}$I-LDL</td>
<td>13</td>
<td>0.299 (± 0.047)</td>
<td>0.108 (± 0.015)</td>
<td>41.7 (± 7.5)</td>
</tr>
</tbody>
</table>

Eleven experiments were performed with $^{125}$I-HDL and 13 with $^{125}$I-LDL. Values are means ± SEM.

*The 10% LPDS medium contained $^{125}$I-lipoprotein (5 μg/ml lipoprotein-protein) ± the respective unlabeled lipoprotein (100–500 μg/ml in different experiments).

†Assuming LDL MW 2.5 × 10⁶, 25% protein; HDL MW 2.1 × 10⁵, 44% protein (see references 49–52).

‡Relative degradation rate = degradation rate

To follow the time course of uptake and degradation (Figure 1), the second set was similarly incubated, but unlabeled lipoprotein was substituted for $^{125}$I-lipoprotein. After 24 hours, the unlabeled medium was replaced with fresh medium containing $^{125}$I-HDL or $^{125}$I-LDL and the cells were pulsed for 2 hours at 37°C. For both lipoproteins, therefore, the pulse occurred while uptake was at steady state and the degradation rates were constant. At the end of the pulse, the cells were transferred to the chase medium, and the release of cell-associated radioactivity was followed as a function of time (Figure 2). It should be noted that unlabeled HDL was omitted from the chase medium when the cells were pulsed with $^{125}$I-HDL in order to prevent any possible exchange of labeled apo A-1 between cell-bound $^{125}$I-HDL and the...
unlabeled HDL in the medium. Such an exchange would have mimicked release by removing surface-bound radioactivity, even if HDL itself was not released.

The cells lost 87% of the radioactivity from [125I]-HDL within 5 hours, and of this, 10% to 15% was acid-soluble (Figure 2 A), indicating the release of an appreciable amount of [125I]-HDL in macromolecular form. We previously found that high affinity [125I]-HDL uptake and degradation was mediated by a "lipoprotein binding site" that recognized both apo E-free HDL or LDL. In a control experiment, the cells were pulsed with [125I]-HDL in the presence of LDL (100 µg/ml). Under these conditions, there was no high affinity uptake of [125I]-HDL or subsequent release during the chase period (data not shown).

A similar experiment was performed with [125I]-LDL except that 200 µg/ml of HDL was present during the pulse and chase to prevent [125I]-LDL binding to the "lipoprotein binding site." The cells released 93% of the [125I]-LDL radioactivity within 4 hours (Figure 2 B). In contrast to [125I]-HDL, about 75% of this radioactivity was acid-soluble. High affinity [125I]-LDL uptake, release and degradation were inhibited when unlabeled LDL (200 µg/ml) was present during the pulse.

Similar experiments were performed in which cells were pulsed at 4° C with labeled HDL or LDL and then chased at 37° C. Under these conditions, all of the cell-associated radioactivity would have been surface-bound at the beginning of the chase. As above, unlabeled HDL (100 mg/ml) was included in the pulse medium when the cells were pulsed with [125I]-LDL. In both cases, however, unlabeled lipoproteins were omitted from the chase media. The observed patterns of release and degradation (Figure 3) were virtually identical to those above. Over 75% of the radioactivity from [125I]-HDL was released within 2 hours and about one-third of the released HDL had been degraded to acid-soluble products (Figure 3 A). [125I]-LDL was also released rapidly from the cells, but about 90% of the released radioactivity was degraded to acid-soluble products (Figure 3 B).

The foregoing experiments indicated that the cells released macromolecular HDL apoprotein, but not whether the binding reaction itself was reversible or whether the reversal of the binding reaction accounted for the release of macromolecular [125I]-HDL.

The reversibility of [125I]-HDL binding at 4° C was examined in an experiment in which cells were pulsed at 4° C, then transferred to chase medium and incubated further at 4° C. In this experiment, two parallel sets of cells were used. The first series was incubated with [125I]-HDL for varying periods to follow the time course of binding (Figure 4). The second series was pulsed for 8 hours during which time the binding reaction reached equilibrium. The cells were then washed and transferred to the chase medium, and the cell-associated radioactivity was readily released to the medium (Figure 4). Since both the pulse and the chase were conducted at 4° C, the uptake and release of [125I]-HDL was presumed to be

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Figure 3. Release of bound lipoproteins. Cells were pulsed with 5 µg/ml [125I]-HDL (A) or [125I]-LDL (B) at 4° C for 2 hours. For [125I]-LDL, unlabeled HDL (100 µg/ml) was present in the pulse medium. The cells were transferred to 10% LPDS, warmed to 37° C, and incubated for the times indicated. Specific uptake and degradation were measured as described in Methods.

Figure 4. Reversibility of [125I]-HDL binding. Two parallel series of cells were incubated at 4° C in 10% LPDS containing [125I]-HDL (5 µg/ml) ± unlabeled HDL (150 µg/ml). The first series was incubated for periods up to 23 hours to follow the time course of binding. The second series was incubated for 8 hours, then transferred to 10% LPDS. The decrease in cell-associated radioactivity was measured.
from the cell surface. Unlabeled HDL was again omitted from the chase medium, and the loss of radioactivity from the cells therefore represented the actual release of \( ^{125} \text{I}-\text{HDL} \), rather than the exchange of labeled apo A-1 with unlabeled HDL.

The following experiments were performed to distinguish cell surface binding from internalized \( ^{125} \text{I}-\text{HDL} \) and to compare surface binding at 37°C with that at 4°C. The experiments were conducted at the same time in parallel sets of cells. After 16 hours in culture, the cells were transferred to 10% LPDS and incubated at 4°C or 37°C with \( ^{125} \text{I}-\text{HDL} \) for 21 hours. During this time, uptake reached equilibrium at both temperatures (not shown). At this point, the cells were washed and treated with trypsin as described in Methods. The results are shown in Table 2. The equilibrium binding at 4°C was 43% of the steady state uptake at 37°C. The amount of trypsin-releasable \( ^{125} \text{I}-\text{HDL} \) that was associated with the cells at 37°C was slightly greater than that at 4°C, but both values agreed reasonably well with equilibrium binding at 4°C. Assuming that trypsin-releasable radioactivity provided a reasonably accurate measure of surface-bound lipoprotein, the results indicated that at equilibrium, similar amounts of \( ^{125} \text{I}-\text{HDL} \) were bound at both temperatures. Furthermore, the data strongly suggested that under the conditions of the experiments, surface binding accounted for almost one-half of the steady-state level of cell-associated \( ^{125} \text{I}-\text{HDL} \) at 37°C. These findings are similar to those in suspended rat hepatocytes \(^{22}\) in which surface binding reportedly accounted for about 60% of the steady state uptake of HDL.

In the following experiments, the total amount of trypsin-sensitive radioactivity was compared with the total macromolecular \( ^{125} \text{I}-\text{HDL} \) released. After 16 hours in culture, the cells were transferred to 10% LPDS and pulsed with \( ^{125} \text{I}-\text{HDL} \) (5 mg/ml) for 2.5 hours at 37°C. During this period, uptake reached 95 ng/mg cell protein, or 85% of steady-state level (Figure 5). At this point, some of the cells were used to measure trypsin-sensitive radioactivity, some were transferred to chase medium and incubated further at 37°C, and the remaining cells were continued in the pulse medium to follow the time course of uptake. Trypsin maximally removed 47 ng of \( ^{125} \text{I}-\text{HDL} \)/mg cell protein, or about one-half the radioactivity that associated with the cells during the pulse. As shown in Figure 5, during an 8-hour chase the cells released about 80% of the total cell-associated radioactivity, of which TCA-soluble products accounted for about one-third. At each time point, the amount of internalized HDL that was released was calculated as the difference between the total released radioactivity and the trypsin-sensitive (i.e., surface-bound) radioactivity. As shown in Figure 5, after correcting for surface-bound \( ^{125} \text{I}-\text{HDL} \), the amount of internalized HDL that was released corresponded closely to the amount of TCA-soluble radioactivity in the medium, suggesting that little macromolecular HDL accumulated in the cells.

The \( ^{125} \text{I}-\text{HDL} \) that was released from the cells as a macromolecule was examined by density gradient ultracentrifugation to determine whether it was released as a lipoprotein. Cells were pulsed with \( ^{125} \text{I}-\text{HDL} \) (5 mg/ml) for 2.5 hours at 37°C, then incubated in chase medium for 4 hours at the same temperature. The medium was recovered and subjected to density gradient ultracentrifugation as described in Methods.

Several kinds of controls were performed. First, the pulse and chase were conducted in the absence of cells. The cell-free plates bound 9% as much \( ^{125} \text{I}-\text{HDL} \) as the cell-containing plates. This represented the maximum possible binding to the plastic dishes, and probably overestimated such binding considerably because most of the surface of the cell-containing plates was occupied by the cells and would presumably have been less accessible to the labeled lipoproteins. One-third of the radioactivity that was bound to the control plates was released to the chase medium. Therefore, at most, about 3% of the

### Table 2. Trypsin-Sensitive \( ^{125} \text{I}-\text{HDL} \) Uptake in Cultured Pig Hepatocytes

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Cell--associated ( ^{125} \text{I}-\text{HDL} ) (ng/mg cell protein)</th>
<th>Trypsin releasable ( ^{125} \text{I}-\text{HDL} ) (ng/mg cell protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>37°C</td>
<td>120</td>
<td>51</td>
</tr>
<tr>
<td>4°C</td>
<td>52</td>
<td>42</td>
</tr>
</tbody>
</table>

*Cells were incubated with \( ^{125} \text{I}-\text{HDL} \) (5 μg/ml) ± unlabeled HDL (150 μg/ml) for 21 hours at the indicated temperature, during which the system reached equilibrium. High affinity uptake was calculated as described in Methods.

†Calculated from the reduction in cell-associated \( ^{125} \text{I}-\text{HDL} \) and corrected for the release of nonspecifically bound lipoprotein.

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**Figure 5.** Cells were pulsed at 37°C for 2.5 hours with \( ^{125} \text{I}-\text{HDL} \) (5 μg/ml), then transferred to 10% LPDS, and incubated at 37°C for the times indicated. Trypsin-releasable radioactivity was determined in a parallel set of plates, and the release of internalized \( ^{125} \text{I}-\text{HDL} \) (x-x) was calculated as indicated in the text.
radioactivity released from the cell plates during the chase could have been bound to the plastic dishes.

Controls were also performed in which $^{125}$I-HDL was incubated at 37°C for 4 hours with either 10% LPDS or with cell-conditioned medium. The cell-conditioned medium was prepared by incubating the cells with 10% LPDS for the period of the pulse. The distribution of TCA-precipitable radioactivity in the density gradient is shown in Figure 6. For both controls, about 80% of the $^{125}$I-HDL was in the top quartile of the tubes and another 15% was in the second quartile. The remaining 5% to 7% was in the bottom one-half of the tubes. Of the $^{125}$I-HDL released from the cells, 65% was in the top quartile. There was about a one-third increase in the amount of TCA-precipitable radioactivity in the second quartile, and about 17% of the radioactivity was in the bottom one-half of the tubes.

We concluded first, that $^{125}$I-HDL was released as a lipoprotein in the HDL density range and second, that the lipoprotein had undergone cell-mediated changes in which the density of at least some of the released lipoprotein particles was increased somewhat. It should be noted that unlabeled HDL was omitted from the chase medium and was not added before ultracentrifugation, to avoid possible influences of the unlabeled lipoprotein on the ultracentrifugal behavior of the labeled lipoprotein.

Finally, experiments were performed to determine whether the apoprotein of $^{125}$I-HDL remained intact during its association with the cells. Cells were pulsed with $^{125}$I-HDL for 2.5 hours at 37°C, then transferred to chase medium for 4 hours at 37°C. The medium was recovered, concentrated by pressure filtration, and subjected to SDS-polyacrylamide gel electrophoresis under reducing conditions. Two kinds of controls were used. In the first, the distribution of radioactivity was determined directly in the $^{125}$I-HDL substrate; in the second, it was determined after a 4-hour incubation in cell-conditioned medium. The results are shown in Figure 7. The distribution of radioactivity in the two controls was the same. About 75% of the radioactivity was associated with apo A-1. In contrast, the major peak in $^{125}$I-HDL that was released from the cells had a faster rate of migration. In several experiments, this peak had an apparent $M_r$ of 12,000 to 14,000. A smaller peak also appeared at the origin, perhaps reflecting the presence of aggregates.

Figure 6. Cells were pulsed with $^{125}$I-HDL (5 µg/ml) for 2.5 hours at 37°C, then transferred to 10% LPDS at 37°C for 4 hours. The medium was subjected to density gradient ultracentrifugation as described in the text. The vertical lines at the top of the bars indicate the results of duplicate ultracentrifugal analyses.

Figure 7. Distribution of radioactivity in macromolecular $^{125}$I-HDL released from cells. Macromolecular $^{125}$I-HDL released to the medium was subjected to SDS-polyacrylamide gel electrophoresis in 13% gels as described in the text.
In the experiment described above, 125I-HDL was not recovered from the chase medium before electrophoresis. The results were the same in other experiments (data not shown) in which 125I-HDL was first reisolated from the chase medium at density 1.21 or density 1.25 g/ml. The data were consistent with the cell-mediated conversion of apo A-1 to partially degraded products of about one-half the molecular weight of apo A-1.

Degradation in Cell Homogenates

The degradation of 125I-HDL and 125I-LDL was measured in homogenates of cultured hepatocytes to determine whether there were inherent differences in their susceptibility to proteolysis by lysosomal enzymes. In these experiments, each of the labeled lipoproteins was incubated with cell homogenate at 37°C in 0.075 to 0.080 M citrate buffer of the appropriate pH, and the production of TCA-soluble radioactivity was measured. Control incubations were performed in which the homogenate was first inactivated by heating for 3 minutes in a boiling water bath. Little, if any, degradation was observed in the controls.

At pH 4.0, cell homogenates catalyzed the degradation of both lipoproteins at constant rates for at least 90 minutes (data not shown). There was no initial latent period, suggesting that lysosomes had been disrupted completely during the preparation of the cell homogenates. The pH-dependence of degradation was measured by varying the pH from 3.0 to 6.0 in increments of 0.5 pH unit (Figure 8 A). The degradation rates for both lipoproteins were maximal at pH 4.0; the rates at pH 3.0 were 42% of those at pH 4.0, and degradation was not detected at pH 6.0.

Degradation rates were measured at varying substrate concentrations (10 to 90 µg/ml). The apparent Km for degradation, as calculated from Lineweaver-Burke plots, was 0.77 × 10−6 M for 125I-HDL and 0.51 × 10−6 M for 125I-LDL.

The heat stability of the proteolytic activity was examined. Homogenates were heated to 48.5°C for up to 2 minutes at pH 7.4, after which degradation was initiated by adding the incubation buffer and 125I-HDL or 125I-LDL. There was a similar loss of enzymatic activity with both lipoproteins as the period of heat treatment was increased; about 40% of the activity was lost after 2 minutes (Figure 8 B). Heating for 2 minutes at 60°C destroyed 97% of the activity with either substrate. Pepstatin or leupeptin, two inhibitors of lysosomal proteases, were added to the incubation systems to determine whether they affected the degradation of the two substrates differently. Pepstatin inhibited the degradation of both lipoproteins by approximately 95%, and was maximally effective in concentrations above 50 ng/ml. Leupeptin inhibited the degradation of the two lipoproteins by less than 10% when present at a concentration of 2.5 µg/ml.

Figure 8. Degradation of 125I-HDL and 125I-LDL by porcine hepatocyte homogenates. A. pH dependence of degradation. 125I-lipoprotein (100 µg/ml lipoprotein-protein) was incubated for 60 minutes at 37°C in a mixture containing 0.075 M citrate buffer and cell homogenate (500 µg/ml homogenate protein). B. Heat stability. Cell homogenate was heated at 48.5°C for the times indicated, after which 0.075 M citrate buffer (pH 4.0) containing 125I-lipoprotein (100 µg/ml) was added and the mixture was incubated for 60 minutes at 37°C. ○—○ indicates 125I-LDL; •—• indicates 125I-HDL.
Discussion

We previously found that HDL degradation in cultured pig hepatocytes was mediated by a pronase-insensitive "lipoprotein binding site" that recognized either apo E-free HDL or LDL. This site accounted for virtually all the high affinity 125I-HDL uptake and degradation that were measured. It also accounted for at least one-half of the overall high affinity uptake of LDL, but apparently mediated little LDL degradation; LDL degradation was mediated primarily by an LDL receptor-like site that accounted for one-half or less of the high affinity uptake of LDL. The major finding in the present study was that the "lipoprotein binding site" also mediated the reversible high affinity uptake of apo E-free HDL.

In the course of the earlier studies, we estimated minimum clearance rates for HDL and LDL from measurements of the rates of degradation of the two lipoproteins. Those measurements suggested that although both lipoproteins were cleared considerably faster than could be accounted for by bulk-phase pinocytosis alone, 125I-HDL was cleared about 5.7 times more slowly than 125I-LDL. Others have similarly reported that HDL was degraded much less readily than LDL in cultured human fibroblasts and in rat aortic smooth muscle cells. In the study reported here, we found that, on average, the fraction of cell-associated 125I-LDL that was degraded was about 4.4-fold greater than that of 125I-HDL. This difference agreed well with that reflected by the lipoprotein clearance measurements.

The pulse-chase experiments revealed differences in the fate of the two lipoproteins after high affinity uptake. High affinity 125I-LDL uptake was predominantly unidirectional; the lipoprotein was apparently directed primarily to the lysosomes and was degraded in a fashion similar to that in fibroblasts. It should be noted that since the pulse medium contained sufficient HDL to prevent 125I-LDL binding to the "lipoprotein binding site," high affinity 125I-LDL uptake in this experiment was via sites that did not bind apo E-free HDL.

In contrast, 125I-HDL uptake by the "lipoprotein binding site" was primarily bidirectional. Part of the lipoprotein was internalized and part was bound at the cell surface and then released as a lipoprotein. The cells therefore exhibited high affinity, reversible uptake of HDL. The apparently slower rate of degradation of 125I-HDL was at least partially accounted for by the reversibility of HDL binding and the consequently smaller proportion of cell-associated 125I-HDL that was committed to irreversible degradation. It should be noted, however, that the rate at which the cells internalized and degraded this fraction of the cell-associated HDL was appreciable. This is particularly evident from the data in Figure 3 in which the cells were pulsed at 4°C and then warmed to 37°C. One-half the TCA-soluble degradation products of 125I-HDL were formed within about 4 hours. By comparison, one-half the TCA-soluble degradation products of 125I-LDL were formed within about 2 hours. Thus, the observations suggest that once it was committed to complete degradation, 125I-HDL was transported and degraded at about one-half the rate of 125I-LDL.

HDL was partially modified before being released as macromolecule. The absolute amounts of 125I-HDL recovered were too small to allow compositional analysis, but the increased density of the lipoprotein suggests the possible removal of lipids and subsequent release of lipid-depleted HDL. Recent observations of Glass et al and Stein et al are pertinent to this speculation. These workers, using HDL labeled with nonmetabolizable tracers, reported the selective hepatic uptake in rats of the labeled cholesterol ester analogue, compared to labeled HDL-apoprotein. Similar findings were reported in cultured pig liver cells. We have not yet determined whether a similar process occurs in cultured pig liver cells.

The apoprotein of HDL was also modified before release, as indicated by the disappearance of apo A-1 and the appearance of more rapidly migrating proteins in the SDS-polyacrylamide gel electrophorograms. The redistribution of radioactivity might have resulted either from the cell-mediated limited proteolysis of apo A-1 or from the selective removal of apo A-1, followed by the release of an apo A-1 deficient particle in which other apoproteins constituted a greater fraction of the total radioactivity. The selective removal of apo A-1, however, seems unlikely for several reasons. First, the removal of protein might be expected to produce a less dense particle; the opposite was observed. Second, it can be calculated from the data in Figures 4 and 6, that the loss of intact apo A-1 from HDL was at least 50% greater than could be accounted for by the sum of residual cell-associated radioactivity and TCA-soluble degradation products in the chase medium, even if it is assumed that apo A-1 was the only apoprotein that was taken up and degraded. The findings are thus more consistent with the cell-mediated partial hydrolysis of apo A-1 in which products of approximately one-half the molecular weight of apo A-1 were produced. The significance of this observation is unknown.

Various studies of the fate of HDL or LDL in several other kinds of cultured cells suggested that part of the HDL or LDL that was internalized was subsequently resorbed as a macromolecule. Our findings, however, suggest that HDL was modified at the cell surface, rather than intracellularly. First, the binding reaction itself was readily reversible, and second, assuming that trypsin-releasable radioactivity gave a reasonably accurate measure of surface binding, the release of macromolecular 125I-HDL could be accounted for almost completely by reversal of the binding reaction. While our observations do not absolutely preclude intracellular modification, they do indicate little accumulation of macromolecular HDL inside the cell. If HDL was partially degraded inside the cell, it had to be internalized, modified, and resorbed very rapidly.
Several lysosomal proteases appear to participate in the degradation of the apoproteins. It has been suggested that Cathepsin D may play a role in the initial degradation of apo B and the thiol cathepsins (Cathepsins B, L, H) may be primarily involved in the degradation of the fragments. Other studies also indicate the synergistic participation of Cathepsins B and D in the degradation of apo B.

In the present study, we did not address the question of which lysosomal enzymes were responsible for the degradation of HDL- and LDL-apoproteins but, rather, whether the two lipoproteins might be degraded differently by lysosomal proteases. The results indicated that the apparent Km’s for the degradation of both substrates were similar, and that under various conditions of pH and temperature treatment and in the presence of two inhibitors of lysosomal proteases, the degradation of both substrates was altered to the same extent. There was, therefore, no evidence to suggest that HDL was inherently less susceptible to proteolysis than LDL once the enzymes had access to the lipoproteins.

It is interesting to note that pepstatin inhibited the degradation of both substrates almost completely, while leupeptin inhibited degradation only marginally. We previously found that leupeptin produced only a partial inhibition of HDL and LDL degradation in cultured hepatocytes. Pepstatin is an extremely potent inhibitor of Cathepsin D and it also inhibits Cathepsin B, but at much higher concentrations. Conversely, leupeptin in low concentrations preferentially inhibits Cathepsin B. In the concentrations used in the present study, pepstatin would be expected to inhibit Cathepsin D, but not Cathepsin B; the opposite would be expected with leupeptin. Our findings are consistent with a major role for Cathepsin D in the degradation of both lipoproteins.

In view of the above findings in cultured cells and the apparent ease with which both lipoproteins were degraded in cell homogenates, it is likely that the apparently slower degradation of 125I-HDL in intact cells reflected both the relatively smaller proportion of cell-associated 125I-HDL that was committed to irreversible degradation, and the somewhat slower delivery of HDL to the lysosome.

While the "lipoprotein binding site"-mediated uptake of HDL apparently affords a high affinity mechanism by which the cells can temporarily concentrate HDL at the cell surface, the possible function of such an interaction is not known. Similar findings, however, have been reported by others in cultured bovine and rat aortic smooth muscle cells, and in human fibroblasts. The observations may be relevant to the hypothesis that HDL participates in the removal of cholesterol from peripheral tissues and its subsequent transport to liver for reuse or disposal, since such a process might be facilitated by the transient high affinity association of macromolecular HDL with cells. Indeed, the induction of high affinity HDL binding sites in response to cholesterol loading has been reported in several kinds of peripheral cells, including cultured human fibroblasts and arterial smooth muscle cells, and cultured bovine endothelial cells. The characteristics and reversibility of high affinity HDL binding sites in human fibroblasts and arterial smooth muscle cells were similar to those of the "lipoprotein binding site" in pig hepatocytes. One difference might be noted, however. Biesbroeck et al. noted that essentially all of the HDL that bound to fibroblasts was subsequently released as a macromolecule; none of the bound lipoprotein was degraded to TCA-soluble products and the apoproteins of macromolecular HDL were apparently unaltered following release from the cells. The release of degraded HDL might be expected if the binding site in these peripheral cells served primarily to facilitate the removal of cholesterol from the cells. Our findings indicate that about one-third of the HDL was readily degraded to TCA-soluble products and that apo A-1 in the released lipoprotein was apparently partially degraded. We can speculate that this difference reflects the significant role of the liver in HDL-apoprotein catabolism.
INDEX TERMS: high density lipoprotein • apoprotein catabolism • degradation • cholesterol
Reversible high affinity uptake of apo E-free high density lipoproteins in cultured pig hepatocytes.

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