Hepatic lipase mediates an increase in selective uptake of HDL-associated cholesteryl esters by cells in culture independent from SR-BI

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Abstract Scavenger receptor class B type I (SR-BI) mediates the selective uptake of HDL cholesteryl esters (CEs) by the liver. Hepatic lipase (HL) promotes this lipid uptake independent from lipolysis. The role of SR-BI in this HL-mediated increase in selective CE uptake was explored. Baby hamster kidney (BHK) cells were transfected with the SR-BI cDNA yielding cells with SR-BI expression, whereas no SR-BI was detected in control cells. These cells were incubated in medium containing 125I [3H]cholesteryl oleyl ether-labeled HDL3 (d = 1.125–1.21 g/ml) and HL was absent or present. Tetrahydrodipristatin (THL) blocked lipolysis. In control BHK cells and in BHK cells with SR-BI, HDL3 selective CE uptake ([3H]125I) was detectable and SR-BI promoted this uptake. In both cell types, HL mediated an increase in selective CE uptake from HDL3. Quantitatively, this HL effect was similar in control BHK cells and in BHK cells with SR-BI. These results suggest that HL promotes selective uptake independent from SR-BI. To investigate the role of cell surface proteoglycans on the HL-mediated HDL3 uptake, proteoglycan deficiency was induced by heparinase digestion. Proteoglycan deficiency decreased the HL-mediated promotion of selective CE uptake. In summary, the stimulating HL effect on HDL selective CE uptake is independent from SR-BI and lipolysis. Proteoglycans are a requisite for the HL action on selective uptake. Results suggest that (a) pathway(s) distinct from SR-BI mediate(s) selective CE uptake from HDL—Brundert, M., J. Heeren, H. Greten, and F. Rinninger. Hepatic lipase mediates an increase in selective uptake of HDL-associated cholesteryl esters by cells in culture independent from SR-BI. J. Lipid Res. 2003. 44: 1020–1032.

HDL-associated cholesteryl esters (CEs) are taken up by hepatocytes and steroidogenic cells selectively, i.e., independent from HDL holoparticle internalization (1). Scavenger receptor class B type I (SR-BI), an HDL receptor protein, plays a central role in this pathway in vivo (2). The physiologic function of SR-BI in HDL metabolism is illustrated in genetically modified mice that have no SR-BI expression or a reduced SR-BI expression in the liver (3, 4). SR-BI deficiency in these rodents increases plasma HDL cholesterol and decreases HDL selective CE uptake by the liver (3, 4).

Besides cell membrane receptors, lipoprotein metabolism in plasma is modified by lipolytic enzymes. Lipoprotein lipase (LPL) is abundant in muscle and adipose tissue and hydrolyzes chylomicron- and VLDL-associated triglycerides to provide fatty acids to tissues as an energy source (5). Hepatic lipase (HL) is related to LPL with respect to structure and function. This enzyme is synthesized by hepatocytes (6) and is attached to the vascular endothelium of adrenals, ovaries, and the sinusoids of the liver (7, 8). One metabolic function of HL is the hydrolysis of HDL-associated phospholipids and triglycerides (9).

Distinct from lipolysis, both LPL and HL have been implicated in lipoprotein holo-particle metabolism. LPL promotes the uptake of apolipoprotein B (apoB)-containing lipoproteins independent from lipolysis in cultured cells (10, 11). With respect to the mechanism of this effect, LPL binds to cell surface proteoglycans and associates with lipoproteins as well (5, 12). Presumably, this “bridging” concentrates the lipoprotein particles on the cell surface and thereby facilitates their uptake. This internalization may be mediated by cell surface heparan sulfate proteoglycans (13) and/or by lipoprotein receptors (14, 15).

Analogously, HL stimulates the cellular uptake of holo-lipoprotein particles. Experiments with cultured cells showed that this enzyme mediates the uptake of apoB-
containing triglyceride-rich lipoproteins (13, 15, 16). In this mechanism, cell surface proteoglycans and cellular receptors may be involved as well; similarly, this was proposed for LPL (5). Again, this bridging function of HL is independent from lipolysis (13, 15, 16).

Besides holo-particle internalization, both LPL and HL promote the selective lipid uptake from lipoproteins. Initial evidence established that these enzymes facilitate the selective CE uptake from HDL by perfused rat liver (17, 18), as well as by hepatic and extrahepatic cells in culture (13, 19, 20, 21, 22). This lipase-mediated increase in selective CE uptake is independent from lipolysis but dependent on cell surface proteoglycans (20, 21, 22). Besides HDL, recent investigations provided evidence that LPL mediates an increase in selective CE uptake from LDL as well (23). In summary, these observations show that LPL and HL can modulate the selective lipid uptake pathway.

The LPL- and HL-mediated promotion of selective CE uptake raises the question of whether SR-BI is involved in the mechanism of these lipase actions. Recently the role of SR-BI in the lipase-promoted increase in selective CE uptake was explored (23, 24, 25). In transfected cells with stable SR-BI expression and in SR-BI-deficient cells, the LPL-mediated increase in selective CE uptake from LDL and HDL was independent from SR-BI (23, 24). In contrast, transient expression of HL and SR-BI in human embryonal kidney 293 (HEK 293) cells synergistically facilitated the selective CE uptake from HDL (25). Taken together, the role of SR-BI in the lipase-mediated increase in selective CE uptake from lipoproteins is controversial at present.

In this study, the question was addressed whether SR-BI plays a role in the mechanism of the HL-mediated increase in HDL selective CE uptake. Baby hamster kidney (BHK) cells with no detectable, or with substantial, SR-BI expression were the dominant experimental model (24). In addition, SR-BI-deficient HEK 293 cells were used (23, 25). HDL$_3$ (d = 1.125–1.21 g/ml) was radiolabeled in the protein and lipid moieties (26, 27), and human HL was prepared from plasma or from cell culture media (21, 28). Experiments show that HL stimulates HDL$_3$-selective CE uptake quantitatively to the same extent independent from the cellular SR-BI status, and this effect is independent from lipolysis. In agreement with these results, immunofluorescence studies revealed that HL binding to the cell surface was not affected by the presence or absence of SR-BI (29, 30). Similarly, there was only a minor colocalization of SR-BI and HL in both types of BHK cells. In summary, it is suggested that HL mediates an increase in HDL-selective CE uptake by a mechanism(s) distinct from SR-BI.

### MATERIALS AND METHODS

#### Preparation of unlabeled HDL$_3$, apoA-I, and lipoprotein-deficient serum

Human HDL$_3$ (d = 1.125–1.21 g/ml) was isolated by ultracentrifugation from plasma of healthy donors (27). Heparin-Sepharose (Amersham Pharmacia) affinity chromatography was used to remove any HDL$_3$ particles containing apoE (27). Human apoA-I was prepared as described previously (27). Lipoprotein-deficient serum (LPDS) was isolated by ultracentrifugation as the d $>$ 1.25 g/ml fraction of human plasma (22).

#### Preparation of doubly radiolabeled HDL$_3$

apoA-I was traced with the radioiodinated tyramine cellulose bis(125I-TC-apoA-I) (26). Na$^{125}$I and [1-$\alpha$, 2-$\alpha$ (n-$\alpha$)-cholesteryl oleyl ether ([125I]-CEt) were supplied by Amersham Pharmacia. Doubly radiolabeled HDL$_3$ was prepared as described (22, 26, 27). 125I-TC-apoA-I and [125I]-CEt were incorporated into apoE-deficient HDL$_3$ by exchange. After uptake by cells, both HDL$_3$ tracers are intracellularly trapped (26).

#### Preparation of HL from human HuH7 hepatoma cell culture media

Differentiated HuH7 hepatoma cells secrete HL but no LPL in the culture medium (31). These cells were grown in DMEM (Life Technologies) containing FBS (10% v/v, Life Technologies), penicillin (100 IU/ml, Life Technologies), and streptomycin (100 μg/ml, Life Technologies). For HL preparation, these cells were cultured in Triple Flasks (500 qcm, Nunc) in DMEM, which was supplemented additionally with heparin (5 U/ml, Roche) and l-((1-tosylamido-2-phenyl)ethyl chloromethyl ketone (10 μg/ml, Roche) (21). HL secretion into the media was monitored by enzyme activity determinations as outlined below.

To prepare HL, the HuH7 cell-derived medium was loaded on heparin-sepharose columns (Amersham Pharmacia) after addition of sodium chloride (0.4 M, final concentration). HL was eluted from this column with buffer (pH 7.4) containing NaCl (1 M), Tris (10 mM), and glycerol (10% v/v). This lipase preparation is referred to as “partially purified HL.”

#### Preparation of HL from human postheparin plasma

Highly purified human HL was prepared from postheparin plasma as described (28). This material was a kind gift from Dr. Gunilla Olvecrona, University of Umeå, Umeå, Sweden.

#### HL activity and mass determinations

HL lipolytic activity was measured using a gum arabic-stabilized [14C]trioleyl glycerol emulsion as substrate (21). Briefly, this emulsion was prepared with unlabeled triolein (50 μg, Fluka) and [14C]trioleyl glycerol (2.5 μCi, Amersham Pharmacia), followed by sonication in buffer containing Tris (0.2 M), lysophosphatidylcholine (0.2% v/v, Sigma), and BSA (5 mg/ml, Sigma). To each sample containing HL activity, 0.1 ml of this mixture was added, and an incubation (28°C, 30 min) followed. This reaction was stopped by the addition of chloroform-heptane-methanol (75:60:85, v/v/v). After centrifugation of the samples (20 min), the upper phases were transferred to a vial containing scintillation fluid.

HL mass was analyzed by ELISA (32).

#### Inhibition of the lipolytic activity of HL

Tetrahydrolipstatin (THL; Orlistat(R), Roche), a covalent inhibitor of the catalytically active site of all mammalian lipases, was used to inhibit the lipolytic activity of HL (33, 34). Media for the uptake assays with cells containing radiolabeled HDL$_3$ and HL were prepared, and THL (final concentration 50 μg/ml) dissolved in ethanol (final concentration 0.1% v/v) was added if HL was absent or present. The lipolytic activity of HL is completely blocked under these conditions, as demonstrated earlier (16, 21, 22) and as shown below.

#### Cloning of SR-BI and transfection of BHK cells

BHK cells were transfected with the plasmid pBK-CMV (control, vector) or pBK-CMV-hSR-BI containing the full-length human sequence.
SR-BI cDNA (24). The respective cells stably express SR-BI as demonstrated previously (24).

**Culture of BHK cells**

BHK cells were cultured (37°C) in DMEM containing FBS (5% v/v), penicillin (100 IU/ml), streptomycin (100 μg/ml), glutamine (2 mM, Life Technologies), and G418 sulfate (0.8 mg/ml, Life Technologies). For lipoprotein uptake experiments, both types of BHK cells (500,000 per well) were plated in wells (35 mm, multiwell tissue culture plates, Nunc). Twenty-four hours or 48 h after plating, when the cells were near confluence, the cells were washed (PBS, two times) and the culture medium was replaced by DMEM supplemented with LPDS (5% v/v), antibiotics (see above), and G418 sulfate (0.8 mg/ml). The preincubations and the uptake assays as outlined below followed after culture (37°C, 20 h) in this medium.

**Culture of HEK 293 cells**

HEK 293 cells were cultured (37°C) in DMEM supplemented with FBS (10% v/v), glutamine (2 mM), and antibiotics (see above). For uptake experiments, HEK 293 cells were plated in 35 mm culture wells (Nunc). Twenty hours before the HDL₃ uptake assays, confluent cells were washed (PBS, two times) and the culture medium was replaced by DMEM containing LPDS (5% v/v) and antibiotics (see above).

**Preincubation of the cells**

Before initiating the lipoprotein uptake assays, BHK cells were preincubated in serum-free and lipoprotein-free media (27). These preincubations were performed to allow internalization or dissociation of membrane-associated serum or protein components originating from culture in the presence of LPDS, from BSA, or from cell secretion. After aspiration of the culture medium, the cells were washed with PBS (one time). Preincubation was then performed (37°C, 30 min) in DMEM containing BSA (5 mg/ml) and antibodies (see above).

**Uptake of doubly radiolabeled HDL₃ by cells in culture**

To investigate HDL₃ uptake, BHK cells or HEK 293 cells were incubated (37°C) in DMEM containing BSA (5 mg/ml) and doubly radiolabeled HDL₃; HL was absent or present as indicated in the respective legends (27). The catalytic activity of HL was blocked with THL in these assays as described above. After incubation for the indicated time periods, the medium was aspirated and the cells were washed (PBS, four times). Then DMEM containing BSA (5 mg/ml) and unlabeled HDL₃ (100 μg protein/ml) was added for a chase incubation (37°C, 2.0 h) to remove reversibly cell-associated tracers (35). After this chase period, the medium was aspirated and the cells were washed again (PBS, one time). The cells then were released from the wells with trypsin-EDTA solution (trypsin 0.5 g/l, EDTA 0.2 g/l, 0.5 ml/well; Life Technologies). Trypsin activity was quenched with PBS containing excess BSA (50 mg/ml). The cell suspensions were transferred to tubes with a PBS (4°C) rinse of the wells. The cells were pelleted by centrifugation (2,000 g, 4°C, 15 min) followed by aspiration of the supernatant. The cell pellet was thereafter resuspended in PBS (4°C, 5.0 ml) followed by centrifugation (2,000 g). The final cell pellet was dissolved in NaOH solution (0.1 N, 1.0 ml) and sonicated, and aliquots were used for protein determination, direct 125I radioassay, and 3H radioactivity after lipid extraction (24, 27). In the case of HEK cells, no chase incubation was performed.

**Immunoblot analysis and antibodies**

An anti-SR-BI antisera was prepared by immunization of rabbits with a recombinant human SR-BI fragment (amino acids 495 to 509) (36). Two anti-HL antisera were generated in rabbits by immunization with human HL fusion proteins [amino acids 8 to 174 (N-terminus) or amino acids 148 to 327 (middle)]. These antisera were a kind gift of Dr. Hans Will, University of Hamburg. These anti-HL antisera were mixed (50:50, v/v) and used in immunoblots. The anti-HL antiserum (goat), which was applied for immunofluorescence, was a donation from Hans Jansen, University of Rotterdam, The Netherlands (32).

For immunoblot analysis, postnuclear supernatants were prepared from BHK cells or from HEK 293 cells (24, 37). Samples containing the indicated amount of protein were reduced with 2-mercaptoethanol in gel loading buffer. HL preparations were dissolved in gel loading buffer. All samples were fractionated by SDS-PAGE and the proteins transferred to nitrocellulose membranes. Finally, the membranes were incubated in buffer containing the respective antiserum. The primary antibody was detected using a peroxidase-conjugated goat anti-rabbit IgG (1:5,000; Amersham Pharmacia). Antibody binding was visualized by enhanced chemiluminescence detection (Amersham Pharmacia) and autoradiography. Ponceau S staining of membranes detected molecular mass standards (Amersham Pharmacia).

**Indirect immunofluorescence**

Both types of BHK cells were cultured (48 h) on glass cover slips. Subconfluent cells were incubated (4°C, 60 min) in DMEM containing BSA (2 mg/ml) and apoE-deficient HDL₃ (40 μg protein/ml); highly purified HL (1 μg protein/ml) was absent or present as indicated. Afterwards, nonspecific surface-associated materials were removed by washing with PBS (four times). Indirect immunofluorescence was performed exactly as described (29, 30) using antibodies against human SR-BI (rabbit, dilution 1:100) (36) and against human HL (goat, dilution 1:500) (32). To visualize the primary antibodies, immune-absorbed goat anti-rabbit (Cy2) or donkey (Cy3) anti-goat F(ab')₂ fragments (Dianova, Hamburg, Germany) were used. Finally, cells were washed with PBS containing nucleus stain Hoechst 33342 (Sigma) and embedded in Mowiol (Calbiochem). For confocal scanning microscopy, an inverted Leica TCS microscope (Leica, Heidelberg, Germany) was used.

**Miscellaneous**

Heparinase I (EC 4.2.2.7, from Flavobacterium Heparinum) and heparinase III (EC 4.2.2.8, from Flavobacterium Heparinum) were obtained from Sigma (13). Cholesterol, phospholipids, and triglycerides were determined with enzymatic assays (Roche). Nonesterified fatty acids were analyzed with a colorimetric method (Wako).

**Statistics and calculations**

Data are expressed as means ± SEM. Significance of differences was examined using Student’s t-test.

For cellular uptake of doubly radiolabeled HDL₃, uptake of each tracer is shown in terms of apparent lipoprotein particle uptake. Outlined in this way, uptake of both tracers on a common basis. In the figures, 125I represents apparent lipoprotein particle uptake according to the protein tracer (125I-[LTC apoA-I]) ³H]CEt that, due to the CE tracer and ³H]CEt, shows the difference in apparent HDL₃ particle uptake that is apparent selective CE uptake.

**RESULTS**

Stable cell clones were derived from BHK cells by transfection with an expression plasmid containing the
human SR-BI cDNA (pBK-CMV-hSR-BI) or with the control vector (pBK-CMV) (24). In addition, as an established SR-BI-deficient cell model, HEK 293 cells were used in this study (23, 24, 25). SR-BI expression was explored in postnuclear supernatants that were prepared from BHK or HEK 293 cells. Finally, immunoblots were performed using a human SR-BI-specific antiserum (36) (Fig. 1). In control BHK cells (vector), no signal corresponding to SR-BI could be visualized, whereas a strong band was apparent in SR-BI-transfected cells (Fig. 1). In HEK 293 cells, no SR-BI signal was visible in this analysis (Fig. 1). To verify that the human SR-BI-specific antiserum recognizes this protein in baby hamster tissue as well, a control immunoblot was performed. In this case, purified membranes were isolated from adult baby hamster adrenal glands. This immunoblot showed that the human SR-BI-specific antiserum recognized this HDL receptor protein from baby hamster tissue as well (data not shown). With respect to HDL₃ metabolism, the expression of SR-BI in BHK cells induces a substantial increase in HDL₃-selective CE uptake as presented below (24).

To investigate the effect of HL on HDL₃ metabolism, HL was prepared. In one case, these HL preparations originated from tissue culture media of human HuH7 hepatoma cells, and these partially purified HL preparations were devoid of LPL (21, 31). Alternatively, in many cases, highly purified HL preparations were used that were isolated from human plasma (28). To explore the purity of these HL proteins, immunoblots were performed. In this analysis of both HL preparations, the anti-HL antibody recognized only one band at the appropriate molecular weight (Fig. 2). This immunoreactivity suggests that both HL preparations indeed contain the native enzyme. To block HL-mediated lipolysis during the uptake assays for HDL₃, THL, an active site inhibitor of lipases (33, 34), was added to the medium of the cells. Previous experiments showed that lipolysis is completely inhibited under these experimental conditions (16, 21, 22). However, to investigate under the conditions of this study whether the lipolytic action of HL was indeed blocked by THL, HDL₃ was incubated (37°C, 4.0 h) in medium containing this enzyme or not; THL was absent or present (Table 1). After this incubation, HDL₃ was resolated and the chemical composition was analyzed. Under these conditions, HL and THL had no significant effect on protein, phospholipid, triglyceride, or cholesterol content of the respective HDL₃ (Table 1). This experiment yields no evidence for HL-mediated lipolysis under the experimental conditions of this study. Besides the chemical composition of HDL₃, the lipase-mediated release of nonesterified fatty acids was investigated. HDL₃ was incubated (37°C, 4.0 h) under the same conditions as shown in Table 1. Afterwards, fatty acids in the medium were determined with a colorimetric assay. HL did not increase the fatty acid concentration in the medium (data not shown). These results also argue against lipolysis under these conditions.

To investigate the role of HL and SR-BI in cellular lipoprotein metabolism, apoE-deficient HDL₃ was labeled with ¹²⁵I-TC-apoA-I and [³H]CEt in the protein and lipid moieties (27). The apoE deficiency of this preparation precludes recognition by cellular apoB and apoE (LDL) receptors. Cells were incubated in medium containing this radiolabeled HDL₃. After a chase incubation that removes reversibly cell-associated HDL₃ tracers (35), cell-

![Fig. 1. Immunoblot analysis for scavenger receptor class B type I (SR-BI) in baby hamster kidney (BHK) cells and in human embryonal kidney 293 (HEK 293) cells. Control BHK cells (vector) or BHK cells with SR-BI expression and HEK 293 cells were cultured as described in Materials and Methods. Postnuclear supernatants were prepared from these cells and thereafter 30 μg or 60 μg protein (per lane) were fractionated by SDS-PAGE (10%). Finally, the proteins were transferred to a nitrocellulose membrane. This membrane was immunoblotted with an anti-SR-BI antiserum (human, dilution 1:1,000). IgG binding was visualized as outlined in Materials and Methods. Three similar blots with BHK cells and one with HEK 293 cells yielded qualitatively identical results. MW, molecular weight.](image1)

![Fig. 2. Immunoblot analysis of human hepatic lipase (HL) preparations. Highly purified (lane 1, 40 ng protein; lane 2, 100 ng protein) or partially purified (lane 3, 100 ng protein; lane 4, 300 ng protein) HLs were loaded on SDS-PAGE (10%). After electrophoresis, the proteins were transferred to a nitrocellulose membrane. This membrane was immunoblotted with an anti-HL antiserum (dilution 1:1,000). IgG binding was visualized as outlined in Materials and Methods. One independent immunoblot yielded qualitatively identical results.](image2)
or not as indicated. Afterwards, HDL3 was reisolated by ultracentrifuga-
absent or present as shown. THL (50
els). The difference in uptake ([3H]CEt-125I-TC-apoA-I)
beled HDL3 had virtually no effect on uptake of 125I-TC-
assay medium of both types of BHK cells containing la-
yields apparent selective CE uptake from HDL 3 (1), and
uptake of HDL3-associated [3H]CEt dose dependently. As
in excess of that due to 125I-TC-apoA-I (Fig. 3, lower pan-
panel), and this HL effect was observed in each HDL3
concentration examined. In three independent, very simi-
lar experiments (shown in Fig. 4), very low concentrations
of radiolabeled HDL3 (i.e., 5, 10, or 20 µg protein/ml)
were used. At these low HDL3 concentrations, the stimula-
tory effect of HL on selective CE uptake from HDL3 quan-
titatively was very similar in both types of BHK cells (data
not shown).

To investigate the effect of HL on the kinetics of selec-
tive CE uptake from HDL3, control BHK cells (vector) or
BHK cells with SR-BI expression incubated (37°C) for 30,
120, or 240 min in medium containing doubly radio-
labeled HDL3 and partially purified HL was absent or present.
Finally, cellular tracer content and apparent selective CE
uptake ([3H]CEt-[125I]-TC-apoA-I) were analyzed. Increas-
ing concentrations of radiolabeled HDL3 yielded a dose-
dependent increase in apparent selective CE uptake from
HDL3, and this was true for both types of BHK cells (Fig.
4, lower panels). HL stimulated apparent selective CE up-
take by control BHK cells (vector) and by BHK cells with
SR-BI expression (Fig. 4, lower panels). The absolute in-
crease in apparent HDL3-selective CE uptake induced by
HL is shown in Fig. 4 (top panels) as well. Quantitatively,
this HL-mediated increase in selective CE uptake was similar
in control BHK cells (vector, Fig. 4, top, left panel) and in
BHK cells with SR-BI expression (Fig. 4, top, right panel),
and this HL effect was observed in each HDL3
concentration examined. In three independent, very similar
experiments (shown in Fig. 4), very low concentrations
of radiolabeled HDL3 (i.e., 5, 10, or 20 µg protein/ml)
were used. At these low HDL3 concentrations, the stimula-
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120, or 240 min in medium containing doubly radio-
labeled HDL3 and partially purified HL was absent or present.
Finally, apparent HDL3 selective CE uptake ([3H]CEt-[125I]-TC-apoA-I) was analyzed. In the absence of HL, apparent selective CE uptake by both types of BHK cells was evident after 30 min of incubation, and this uptake increased in a time-dependent manner at fairly linear rates up to 240 min (Fig. 5, lower graphs). Throughout the entire time course, apparent HDL3-selective CE uptake was higher in BHK cells with SR-BI expression compared with control cells (vector, Fig. 5, lower graphs). In parallel, both types of BHK cells were

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**TABLE 1. Hepatic lipase, tetrahydrolipstatin, and the composition of HDL3**

<table>
<thead>
<tr>
<th>Incubation Conditions</th>
<th>–HL</th>
<th>+HL</th>
<th>+THL</th>
</tr>
</thead>
<tbody>
<tr>
<td>% of total mass</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein</td>
<td>63.6 ± 4.8</td>
<td>66.1 ± 0.8</td>
<td>70.9 ± 3.8</td>
</tr>
<tr>
<td>Phospholipid</td>
<td>6.5 ± 0.5</td>
<td>6.9 ± 0.7</td>
<td>8.1 ± 0.4</td>
</tr>
<tr>
<td>Triglyceride</td>
<td>7.8 ± 1.4</td>
<td>5.3 ± 0.7</td>
<td>4.4 ± 0.8</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>22.3 ± 2.8</td>
<td>21.7 ± 0.7</td>
<td>16.5 ± 4.4</td>
</tr>
</tbody>
</table>

HL, hepatic lipase; THL, tetrahydrolipstatin. Apolipoprotein E-deficient HDL3 (80 µg protein/ml) was incubated (37°C, 4.0 h) in medium containing BSA (1 mg/ml), and highly purified HL (200 ng/ml) was absent or present as shown. THL (50 µg/ml) was added to the medium or not as indicated. Afterwards, HDL3 was reisolated by ultracentrifugation at d = 1.21 g/ml followed by extensive dialysis (PBS). Finally, protein, phospholipid, triglyceride, and cholesterol were analyzed as described in Materials and Methods. Values are means ± SEM of four independent determinations. P values were between 0.18 and 0.86 according to Student’s t-test.
incubated in the additional presence of partially purified HL in the medium. HL significantly increased apparent selective CE uptake ($[^3H]CET-[^125I]TC$-apoA-I) from HDL$_3$ by both types of BHK cells, and this effect was evident throughout the entire time course. The absolute increase in apparent HDL$_3$-selective CE uptake, which was mediated by HL, was calculated (Fig. 5, top panels). Quantitatively, this HL-induced increase in apparent HDL$_3$-selective CE uptake was very similar in control BHK cells (vector, Fig. 5, top, left panel) and in BHK cells with SR-BI expression (Fig. 5, top, right panel).

The experiments presented above suggest that the stimulatory effect of HL on HDL$_3$-selective CE uptake is independent from SR-BI. A well-established SR-BI-deficient cell model is HEK 293 cells (23, 24, 25). These cells do not express SR-BI (Fig. 1). To explore the effect of HL on HDL$_3$ metabolism in HEK 293 cells, these cells were incubated (37°C, 4.0 h) in a medium containing several distinct concentrations of doubly radiolabeled HDL$_3$, and partially purified HL was absent (Fig. 6, left panel) or present (Fig. 6, right panel). After this incubation, cellular tracer content was determined. In the absence of HL, apparent HDL$_3$ particle uptake according to $[^3H]CET-[^125I]TC$-apoA-I or $[^3H]CET$ increased dose dependently. This yielded an HDL$_3$ dose-dependent increase in apparent selective CE uptake ($[^3H]CET-[^125I]TC$-apoA-I) by HEK 293 cells. Addition of HL to the incubation medium containing radio-labeled HDL$_3$ had virtually no effect on uptake of $[^125I]TC$-apoA-I by HEK 293 cells (Fig. 6, right panel). However, HL induced an increase in $[^3H]CET$ uptake. As a result, HL addition yielded a dose-dependent increase in apparent HDL$_3$-selective CE uptake ($[^3H]CET-[^125I]TC$-apoA-I) (Fig. 6, right panel). These experiments with HEK 293 cells also suggest that the HL-mediated increase in HDL$_3$-selective CE uptake is independent from SR-BI.

All experiments presented above suggest that the HL-mediated stimulation of HDL$_3$-selective CE uptake is independent from SR-BI. To evaluate this proposal with an alternative methodological approach, this issue was addressed by an immunofluorescence analysis (29, 30) using...
specific antibodies directed against SR-BI or HL (32, 36). Control BHK cells (vector, Fig. 7A) or BHK cells with SR-BI expression (Fig. 7B) were incubated (4°C, 60 min) in medium that contained apoE-deficient HDL3, and highly purified HL was absent or present. Immunofluorescence analysis followed (see Materials and Methods) (29, 30). In control BHK cells (vector), SR-BI immunofluorescence (green) was very low in cells incubated without or with HL (Fig. 7A). In contrast to control BHK cells (vector), substantial amounts of SR-BI protein were visualized in BHK cells with SR-BI expression (Fig. 7B, green fluorescence). Incubation of both types of BHK cells in medium containing no HL yielded no signal for this enzyme in the immunofluorescence analysis (red fluorescence, Figs. 7A, B). However, if the cells were incubated in the presence of highly purified HL, a signal for this enzyme (red fluorescence) was present in control BHK cells (vector, Fig. 7A) and in BHK cells with SR-BI expression (Fig. 7B). These figures show that HL binds to the surface of control BHK cells (vector) and to the surface of BHK cells with SR-BI expression without any difference in localization and binding capacity.

Merged images revealed only a rare appearance of yellow spots in control BHK cells (vector, Fig. 7A) and in BHK cells with SR-BI expression (Fig. 7B). This suggests only a minor colocalization of SR-BI and HL (≤20%). This immunofluorescence analysis proposes that SR-BI and HL mediate selective CE uptake from HDL3 at distinct sites of the plasma membrane.

Cell surface proteoglycan deficiency diminishes the lipase-mediated increase in selective CE uptake from HDL and LDL (13, 21, 22). Therefore, the question was addressed of whether the effect of HL on HDL3-selective CE uptake is dependent on cell surface proteoglycans in control BHK cells (vector) and in BHK cells with SR-BI expression. Both types of BHK cells were incubated in
parallel in the absence or presence of heparinase I and heparinase III in the medium to deplete the cells from surface proteoglycans (Fig. 8). Previous experiments established that the cells are depleted from proteoglycans under these conditions (22). Afterwards followed an incubation of both types of BHK cells in a medium that contained doubly radiolabeled HDL₃, and partially purified HL was absent or present. Apparent HDL₃-selective CE uptake ([³H]CE₆-¹²⁵I-TC-apoA-I) was analyzed. In control BHK cells (vector) or in BHK cells with SR-BI expression, digestion with heparinases per se had no effect on apparent HDL₃-selective CE uptake (Fig. 8). As expected, HL stimulated apparent selective CE uptake from HDL₃ in control BHK cells (vector) or in BHK cells with SR-BI expression. However, preincubation of the cells with heparinases decreased the stimulatory HL action on apparent HDL₃-selective CE uptake ([³H]CE₆-¹²⁵I-TC-apoA-I) significantly, and this was observed in control BHK cells (vector) and in BHK cells with SR-BI expression (Fig. 8). Thus, independent of the cellular SR-BI status, the HL-mediated stimulation of HDL₃-selective CE uptake is dependent from cell surface proteoglycans.

DISCUSSION

Biochemical experiments of this study provide evidence that both partially purified and highly purified HL preparations promote the selective CE uptake from HDL₃ in cultured cells. This HL-stimulated uptake of HDL₃ was explored in the presence of THL, a compound that blocks HL-mediated lipolysis (33, 34). Under the conditions of this investigation, there was no HL-induced lipolysis of HDL₃ detectable, and this is in agreement with previous studies (21, 22). Due to these observations, the stimulatory effect of HL on selective CE uptake is independent from lipolysis. Quantitatively, the HL-mediated increase in selective CE uptake from HDL₃ is very similar in control BHK cells (vector) and in BHK cells with SR-BI expression. This HL effect is time-, HDL₃-, and HL concentration-dependent in both types of BHK cells. In agreement with these cells, this lipase-stimulated selective CE uptake from HDL₃ is observed in SR-BI-deficient HEK 293 cells (23, 24, 25). Taken together, these results suggest that the HL-mediated increase in HDL₃-selective CE uptake is independent from SR-BI and independent from lipolysis. This conclusion is reinforced by an immunofluorescence
Images showed similar binding capacities for HL in both types of BHK cells, and only a minor colocalization of HL and SR-BI. This suggests that SR-BI expression does not alter cellular binding of HL, and subsequently that the HL effect on selective CE uptake is mainly independent from SR-BI. It should be kept in mind that a complete separation of both proteins at the plasma membrane cannot be expected in this analysis due to the limited resolution of fluorescence microscopy. Heparinase-induced proteoglycan deficiency of the cell surface reduced the HL-mediated increase in HDL3-selective CE uptake, and this suggests that these molecules play a role in the HL-promoted increase in selective lipid uptake (13, 21). This lipase-induced and proteoglycan-mediated HDL3 tethering to the cell membrane most likely plays a role in the mechanism of the HL-induced increase in selective CE uptake (13).

The HL-mediated and SR-BI-independent increase in HDL3 selective CE uptake shown here is in disagreement with earlier experiments (25). Transient expression of SR-BI and catalytically active or inactive HL in HEK 293 cells suggested a synergistic role for these molecules in the mechanism of the lipase-induced increase in HDL selective CE uptake, and this suggests that these molecules play a role in the HL-promoted increase in selective lipid uptake (13, 21). This lipase-induced and proteoglycan-mediated HDL3 tethering to the cell membrane most likely plays a role in the mechanism of the HL-induced increase in selective CE uptake (13).

Fig. 6. Dose-response curve for the uptake of doubly radiolabeled HDL3 by HEK 293 cells and the effect of HL. HEK 293 cells incubated (37°C, 4.0 h) in DMEM containing doubly radiolabeled HDL3, and the respective HDL3 concentrations are indicated in the abscissa; partially purified HL (200 ng protein/ml) was absent (left panel) or present (right panel) as shown. After this incubation, cellular tracer content was analyzed. 125I represents apparent HDL3 particle uptake according to 125I-TC-apoA-I; 3H demonstrates apparent particle uptake due to [3H]CEt, and 3H-125I shows the difference, i.e., apparent selective CE uptake. Values are means ± SEM of three incubations. Where no error bars are shown, the respective bars were smaller than the points. One similar experiment using highly purified HL yielded qualitatively identical results.

Besides HL, another member of the lipase gene family, LPL, mediates a lipolysis-independent increase in HDL3-selective CE uptake in vitro (20, 22). Recently, evidence was presented that the mechanism of this LPL-mediated increase in HDL selective CE uptake is independent from SR-BI (24). Besides HDL, LPL induces an enhancement in LDL selective CE uptake as well, and this effect was again independent from SR-BI (23). In summary, both HL and LPL increase selective CE uptake from HDL and LDL, and the receptor protein SR-BI does not play a role in the mechanism. This conclusion is true for the majority of studies presented so far.

Which is (are) the specific molecular mechanism(s) for the SR-BI-independent increase in HDL-selective CE uptake mediated by lipases? LPL and HL bind to cell surface proteoglycans and lipoproteins, and these interactions closely associate these particles with cell membranes (5, 11, 12, 13, 16). For holo-lipoprotein uptake, presumably this “bridging” facilitates particle internalization by cells (5, 10, 16, 38). Analogously, the results of this and of previous studies (22, 23, 24) are in line with the hypothesis that HL and LPL “anchor” lipoproteins in close association with the plasma membrane. This bridging may facilitate the selective lipid transfer from lipoproteins into cells. This model is in agreement with recent results on the molecular mechanism of the selective CE uptake pathway (39, 40). In transfected cells expressing SR-BI or CD36, a class B scavenger receptor that is closely related to SR-BI, both SR-BI and CD36 bind HDL with high affinity (40). For holo-lipoprotein uptake, presumably this “bridging” facilitates particle internalization by cells (5, 10, 16, 38). Analogously, the results of this and of previous studies (22, 23, 24) are in line with the hypothesis that HL and LPL “anchor” lipoproteins in close association with the plasma membrane.
Fig. 7. Localization of SR-BI and HL in BHK cells by immunofluorescence analysis. Control BHK cells (A) or BHK cells with SR-BI expression (B) were grown on glass coverslips. These cells were incubated (4°C, 60 min) in DMEM containing apoE-deficient HDL₃ (40 μg protein/ml), and highly purified HL (1 μg protein/ml) was absent or present as indicated. Thereafter, nonspecific cell-associated materials were removed by washing (PBS, four times). After fixation and permeabilization, cells were immune double-labeled with an anti-SR-BI antibody (dilution 1:100, visualized in green) and an anti-HL antibody (dilution 1:500, visualized in red) as described in Materials and Methods. Confocal images from each fluorochrome were recorded and superimposed to reach yellow colocalization (merge). Images correspond to confocal projections.
close association between HDL and the plasma membrane facilitates the selective CE transfer from lipoproteins into cells.

By which mechanism(s) are the hydrophobic CE molecules finally transferred from the neutral lipid core of the HDL particle and into the cell following the lipase-mediated HDL tethering to the plasma membrane? The molecular mechanism(s) of this lipid transfer is not defined at present. Pittman and coworkers (41) explored the CE transfer from HDL particles to membranes. Experiments using model membranes, purified plasma membranes, native radiolabeled HDL, and recombinant HDL suggested that CE molecules are transferred to membranes in a collision-mediated process. The latter mechanism involves lipid-lipid interactions independent of membrane proteins (41). According to an alternative model, SR-BI forms a nonaqueous “channel” that mediates the CE movement from a lipoprotein into the plasma membrane (39). However in view of the results of this study, it seems unlikely that this “channel” contributes to the lipase-mediated HDL CE transfer. Taken together, future experiments have to define the molecular mechanism(s) that mediate(s) the lipase-induced increase in selective CE transfer from lipoproteins to cells. Besides lipid-lipid interactions,
a plasma membrane protein distinct from SR-BI may play a role in this lipid transfer pathway.

The interaction between HDL, SR-BI, and HL in lipid delivery to cells was investigated in this study in vitro. What are the physiological implications of these results for the HDL-mediated reverse cholesterol transport to the liver in vivo? Relevant in this context are studies with genetically modified animals. SR-BI and HL play physiologic roles in reverse cholesterol transport to the liver in rodents (2–4, 42). Mice with a genetically induced deficiency of HL (43) or SR-BI (3, 4) have an increase in HDL cholesterol and a decrease in selective CE uptake by the liver. In contrast, hepatic overexpression of HL (42, 44, 45) or SR-BI (46) in mice or rabbits decreases this protective HDL lipoprotein fraction in plasma. In murine adrenal glands, an induced HL deficiency yields an increase in SR-BI expression (47). These studies show that, in vivo, both HL and SR-BI have a substantial impact on HDL metabolism. On the other hand, it is generally accepted that this lipoprotein fraction plays a central role in reverse cholesterol transport. This pathway presumably transports excess cholesterol from peripheral cells to the liver and protects from atherosclerosis. The in vitro investigations presented here explain one potential mechanism by which HL modifies HDL metabolism in vivo (48).

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