Lipoprotein lipase mediates an increase in the selective uptake of high density lipoprotein-associated cholesteryl esters by hepatic cells in culture

Franz Rinninger,[1] Tatjana Kaiser, W. Alexander Mann, Nicolette Meyer, Heiner Greten, Ulrike Beisiegel

Universität Hamburg, Krankenhaus Eppendorf, Medizinische Kernklinik und Poliklinik, Martinistrasse 52, 20246 Hamburg, Germany

Abstract In this study the effect of lipoprotein lipase (LPL) on the selective uptake of high density lipoprotein (HDL) cholesteryl esters (CE) by hepatic cells was investigated. Human HDL_3 (d 1.125–1.21 g/ml) was radiolabeled with ^{125}I in the protein moiety and with ^{3}H in the CE moiety. LPL was prepared from bovine milk. Human hepatocytes in primary culture and human Hep3B hepatoma cells were incubated in medium containing doubly radiolabeled HDL_3 with or without LPL. Without LPL, apparent HDL_3 particle uptake according to the lipid tracer (^{3}H) was in excess of that due to the protein label (^{125}I) indicating selective CE uptake from HDL_3. Addition of LPL increased selective CE uptake up to 7-fold. This stimulation of HDL_3 selective CE uptake was independent of the lipolytic activity of LPL as suggested by several experimental approaches. Cell surface heparan sulfate proteoglycan deficiency decreased the LPL-mediated increase in selective CE uptake suggesting an important role for these molecules. In low density lipoprotein (LDL) receptor- or LDL receptor-related protein-(LRP)-deficient cells, LPL increased selective CE uptake as it did in normal cells yielding no evidence that these receptors play a role in the LPL effect on selective CE uptake. In summary, lipoprotein lipase increases the selective uptake of high density lipoprotein-associated cholesteryl ester by hepatic cells in culture. This effect is dependent on cell surface heparan sulfate proteoglycans but independent of lipolysis and of endocytosis mediated by low density lipoprotein receptor-related or low density lipoprotein receptors.— Rinninger, F., T. Kaiser, W. A. Mann, N. Meyer, H. Greten, and U. Beisiegel. **Lipoprotein lipase mediates an increase in the selective uptake of high density lipoprotein-associated cholesteryl esters by hepatic cells in culture. *J. Lipid Res.* 1998.** 39: **1335–1348.**

Supplementary key words HDL • cholesteryl ester • selective uptake • lipoprotein lipase • liver • reverse cholesterol transport

The diffusion of cholesterol from cell membranes to high density lipoprotein (HDL) is thought to initiate the process of reverse cholesterol transport (1). Lecithin:cholesterol acyl transferase (LCAT) esterifies HDL-associated cholesterol, thereby allowing continued efflux of free cholesterol from membranes. Subsequent transfer of cholesteryl esters (CE) between lipoprotein fractions, requiring the activity of cholesteryl ester transfer protein (CETP), occurs in some species (2). Cells can also take up CE from HDL in a process not dependent on holo-particle uptake, designated selective uptake (3, 4). A wide range of cell types from several species exhibit this pathway in vitro (4, 5). The liver and steroidogenic tissues selectively take up HDL-associated CE in vivo in rodents (3). In rats, the selective CE uptake pathway presumably contributes to reverse cholesterol transport (3). Selective uptake is regulated according to the cholesterol status in both cultured cells (6) and in tissues of intact rats (7). With respect to the mechanism, an initial step of this pathway possibly involves the incorporation of HDL-derived CE into the plasma membrane and this transfer apparently is independent from specific membrane proteins (8, 9). However, recent studies proposed that the class B, type I scavenger receptor (SR-BI) mediates HDL selective lipid uptake (10–12). After uptake, these CE are directed intracellularly to a non-lysosomal destination for degradation (13).

Lipoprotein lipase (LPL) is a key enzyme for lipoprotein catabolism (14). Bound to the vascular endothelium, this protein hydrolyzes triglycerides of chylomicrons and very low density lipoproteins (VLDL) (15). However,

Abbreviations: HDL, high density lipoprotein (d 1.063–1.21 g/ml); HDL_3, high density lipoprotein 3 (d 1.125–1.21 g/ml); LDL, low density lipoprotein (d 1.019–1.063 g/ml); apoA-I, apolipoprotein A-I; apoB, apolipoprotein B; ^{125}I-NMTC, radioiodinated N-methyl-tyramine-cellobiose ligand; ^{125}I-LDL, conventionally radioiodinated LDL; CE, cholesteryl ester; [^{3}H]CEt, [^{3}H]cholesteryl oleyl ether; [^{3}H]CO, [^{3}H] cholesteryl oleate; BSA, bovine serum albumin; CHO cells, Chinese hamster ovary cells; DMEM, Dulbecco's modified Eagle's medium; FH fibroblasts, fibroblasts derived from a patient with familial hypercholesterolemia; LPDS, lipoprotein-deficient serum; LRP, low density lipoprotein receptor-related protein; MEF cells, murine embryonic fibroblasts; PBS, phosphate-buffered saline; SR-BI, scavenger receptor class B, type I: THL, tetrahydrolipstatin.

[1] To whom correspondence should be addressed.

there is growing evidence that LPL is a multifunctional protein in lipoprotein metabolism. In addition to lipolysis, LPL mediates the uptake and degradation of lipoproteins by cells (16–19). Initially Felts, Itakura, and Crane (20) suggested that lipoprotein-associated LPL directs these particles for catabolism to the liver. Recently, Beisiegel, Weber, and Bengtsson-Olivecrona (16) showed an LPL-induced increase in binding of apoE-rich lipoproteins to cells in vitro. LPL also promoted the binding of apoE-containing liposomes to the low density lipoprotein receptor-related protein (LRP) (16, 21). Direct binding of LPL to LRP has been demonstrated as well and this interaction apparently mediates lipoprotein degradation (16, 22, 23). Based on these observations it was suggested that LPL stimulates the cellular catabolism of triglyceride-rich lipoproteins and the specific mechanism involves an interaction between LPL, lipoproteins, and LRP.

Besides triglyceride-rich particles, LPL also increases the cellular uptake of cholesterol-rich lipoproteins (17–19, 24, 25). In hepatic and nonhepatic cells this enzyme promoted a significant increase in uptake and degradation of LDL and Lp[a]. This effect is independent of the lipolytic action of LPL (16, 17, 26). Controversy exists with respect to the cellular mechanism that mediates this lipoprotein uptake. Mulder and coworkers (18) suggested that the LPL-induced increase in LDL degradation is primarily due to an enhanced LDL receptor-mediated endocytosis (27). In contrast, other investigators (19) concluded that the LDL receptor-related protein (LRP) plays an essential role in the LPL-mediated stimulation of LDL degradation. However, an alternative mechanism involving heparan sulfate proteoglycans was proposed as well (17, 25, 28).

LPL binds to cell surface heparan sulfate proteoglycans with high affinity (29). Proteoglycan deficiency is associated with a diminished effect of LPL on lipoprotein uptake, whereas proteoglycan overexpression in transfected cells increases uptake of LPL-enriched lipoproteins (17, 28). These observations suggested that the binding of LPL to proteoglycans is an essential step for the LPL-mediated lipoprotein delivery to cells. With respect to the mechanism of the LPL-mediated LDL uptake, it was proposed that LPL, LDL, and cell surface proteoglycans form a complex, and here LPL brings the lipoprotein in close proximity with the cell membrane (25). Finally, LPL and lipoprotein particles are internalized along with cell surface proteoglycans, and this process is independent of endocytosis involving coated pits (17, 25). Such a mechanism evidently can deliver lipoproteins for catabolism to lysosomes (28).

In contrast to the LPL effect on catabolism of apoB-containing lipoproteins, the potential role of this enzyme for HDL metabolism has not been well explored. In human postheparin plasma, LPL mass and activity elute on gel filtration at positions similar to HDL and LDL (30, 31). Electron microscopy of immunostained gel filtration fractions showed reaction for LPL, apolipoprotein A-I, and apolipoprotein B, i.e., major apolipoproteins of HDL and LDL, respectively, in identical fractions (30). Therefore, in plasma, considerable amounts of LPL evidently are associated with HDL (30, 31). With liposomes, LPL mediated an increase in cellular uptake of radiolabeled cholesteryl ether in excess of that of phospholipids, and this suggested that LPL stimulates CE uptake independent of phospholipid internalization (32). Recently, in mouse peritoneal macrophages, this enzyme increased the selective uptake of HDL_3-associated CE even though the mechanism has not been explored (33).

The current investigation addresses the question of whether LPL modifies the selective uptake of HDL-associated CE. In addition, the mechanism of such a potential effect was investigated. The tissue of predominant interest was the liver and therefore human hepatocytes in primary culture and human Hep3B hepatoma cells were used (5). Results demonstrate a substantial LPL-mediated increase in selective CE uptake and this effect is independent of the lipolytic action of this enzyme. For this LPL effect on selective CE uptake, cell surface heparan sulfate proteoglycans play an essential role, whereas no evidence was obtained for an involvement of LDL receptors or LRP.

METHODS

Preparation of unlabeled lipoproteins

Human HDL_3 (d 1.125–1.21 g/ml) was isolated by ultracentrifugation from pooled plasma of healthy donors (34). Heparin-Sepharose (Pharmacia) affinity chromatography was used to remove HDL_3 particles containing apolipoprotein E (apoE) (35). No apoE could be detected by sodium dodecyl sulfate polyacrylamide gel electrophoresis in the final HDL_3 preparation. Lipoprotein-deficient serum (LPDS) was prepared by ultracentrifugation as the $d > 1.25$ g/ml fraction of human plasma.

Preparation of labeled lipoproteins

Human apolipoprotein A-I was traced with ^{125}I-NMTC–apoA-I (^{125}I-labeled N-methyl-tyramine cellobiose–apoA-I) (36). Na^{125}I, [1-alpha, 2-alpha (n)-^{3}H]cholesteryl oleyl ether ([^{3}H]CEt) and [1-alpha, 2-alpha (n)-^{3}H]cholesteryl oleate ([^{3}H]CO) were supplied by Amersham.

Doubly radiolabeled HDL_3 was prepared as described previously (4, 5). Briefly, apoE-deficient HDL_3 was labeled with [^{3}H]CEt by exchange from donor liposomal particles that contained [^{3}H]CEt using human plasma cholesteryl ester transfer protein (CETP). Donor particles were removed from labeled HDL_3 by flotation. Thereafter, ^{125}I-NMTC–apoA-I was associated with [^{3}H]CEt-labeled HDL_3 by exchange (37°C, 24 h). Finally, the doubly radiolabeled HDL_3 fraction was separated from unbound ^{125}I-NMTC–apoA-I by ultracentrifugation.

ApoE-depleted HDL_3 was labeled with [^{3}H]cholesteryl oleate ([^{3}H]CO) by exchange similarly as this was done for doubly radiolabeled HDL_3 (5).

LDL was conventionally radioiodinated (^{125}I-HDL_3) using IODO-GEN (Pierce) (37).

Throughout the preparative procedure and during storage, all labeled HDL_3 preparations were kept in the presence of sodium azide (0.2%, w/v), EDTA (1.0 mm) and DTNB (1.5 mm, Sigma); the latter was used to inhibit lecithin:cholesterol acyltransferase (LCAT) (36). All lipoproteins were sterile-filtered (0.45 μm, Sartorius) before use in experiments and inhibitors were removed from the preparations by dialysis (PBS, EDTA 1 mm, pH 7.4).

Preparation of lipoprotein lipase (LPL)

LPL was purified from bovine milk by heparin-Sepharose affinity chromatography (Pharmacia) and stored at −70°C (15). Tetrahydrolipstatin (THL, Orlistat®) was used to covalently inhibit the active site and thus the lipolytic activity of LPL (26, 38). Media for the uptake assays with cells containing doubly radiolabeled HDL_3 and LPL were prepared and THL (final concentration 50 μg/ml) dissolved in ethanol (final concentration 0.1%, v/v) was added to these media with or without LPL. Under these conditions, the lipolytic activity of LPL is almost completely inhibited (23, 26).

Human hepatocytes in primary culture

Human hepatocytes were isolated from normal human liver tissue and cultured as described (5). The culture medium (35-mm wells, Nunc) was replaced 48 or 72 h after plating by Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with lipoprotein-deficient serum (LPDS, 20%, v/v), penicillin (100 IU/ml, Gibco) and streptomycin (100 μg/ml, Gibco). After culture (24 h, 37°C) in medium containing LPDS, the preincubations and the uptake assays were initiated as outlined below.

Human Hep3B hepatoma cells

Human Hep3B hepatoma cells were grown in DMEM containing fetal bovine serum (10%, v/v, Gibco), penicillin (100 IU/ml) and streptomycin (100 μg/ml) (6). Cells were plated in wells on multiwell tissue culture plates (35 mm, Nunc). When the cells were close to confluency, usually 48 h after plating, they were incubated at 37°C for 2 × 24 h in DMEM containing LPDS (10%, v/v) and antibiotics. In some cases LDL was also present during the second 24 h of culture in medium containing LPDS. Finally, the preincubations and the uptake and degradation assays were initiated as outlined below.

Chinese hamster ovary cells (CHO cells)

Wild type Chinese hamster ovary cells (CHO-K1) and Chinese hamster ovary cell mutants defective in xylosyltransferase, i.e., in glycosaminoglycan synthesis (CHO-745), were grown in Ham's F12 medium (Gibco) supplemented with fetal bovine serum (10%, v/v), penicillin (100 IU/ml), and streptomycin (100 μg/ml) (39). After plating (35-mm wells), the cells were cultured (37°C) for 2 × 24 h in Ham's F12 medium containing LPDS (10%, v/v) and antibiotics. Thereafter, the preincubations and the uptake assays were carried out as described below.

Murine embryonic fibroblasts (MEF cells)

Wild type murine embryonic fibroblasts (MEF 1), fibroblasts homozygous for the disrupted LRP allele (MEF 2), fibroblasts homozygous for the disrupted LDL receptor allele (MEF 3), and fibroblasts carrying two defective alleles for both LRP and LDL receptors (MEF 4) were grown in DMEM supplemented with fetal bovine serum (10%, v/v) (40). After plating (35-mm wells), the cells were cultured (37°C) for 24 h in DMEM containing fetal bovine serum (10%, v/v). Thereafter this medium was replaced by DMEM that contained LPDS (10%, v/v) and cells were incubated (37°C) in this medium for 24 h. Finally, the preincubations and the uptake assays were carried out as outlined below. The absence or presence of LRP or LDL receptors on each cell line was detected by immunoblots using specific antibodies (40).

Human skin fibroblasts

Normal human skin fibroblasts and skin fibroblasts originating from a patient deficient in LDL (apoB, E) receptors were grown in DMEM containing fetal bovine serum (10%, v/v), penicillin (100 IU/ml), and streptomycin (100 μg/ml) (6, 41). Fibroblasts were plated in wells (35 mm). When the cells were near confluency, the medium was replaced by DMEM that contained LPDS (2.5 mg protein/ml) and antibiotics. After incubation (37°C) for 2 × 24 h in this medium, the preincubations and the uptake assays were carried out as described below.

Preincubation of the cells

Before initiating the uptake or degradation assays, all cells were preincubated in serum-free and lipoprotein-free medium to allow internalization or dissociation of membrane-associated lipoproteins or serum components. After aspiration of the culture medium, cells were washed with PBS (2×). Thereafter this preincubation (37°C, 0.5 h) was initiated in DMEM containing bovine serum albumin (BSA, 5 mg/ml, Sigma) and antibiotics. Chloroquine, monensin, or Sandoz compound 58-035 supplemented this medium in some cases as indicated.

Uptake of doubly radiolabeled HDL_3 by cells in culture

To investigate HDL_3 uptake, cells were incubated in DMEM containing doubly radiolabeled HDL_3 and BSA (5 mg/ml) with or without LPL as shown (5). After incubation at 37°C for the indicated time periods, the medium was aspirated and cells were washed with PBS (4×). Then DMEM containing BSA (5 mg/ml) and unlabeled HDL_3 (100 μg HDL_3 protein/ml) was added for a chase incubation (37°C, 2.0 h) to remove reversibly cell-associated tracers (8). After this chase period, the medium was aspirated and the cells were washed again (PBS, 1×). Cells were finally released from the wells with trypsin/EDTA solution (0.5 g/l trypsin, 0.2 g/l EDTA, 1.0 ml/well, Gibco). Trypsin activity was quenched by addition of 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 then pelleted by centrifugation (2,000 *g*, 15 min, 4°C) followed by aspiration of the supernatant. The cell pellet was resuspended in PBS (5.0 ml) followed by centrifugation (2,000 *g*, 15 min, 4°C). The final cell pellet was dissolved in NaOH solution (0.1 N, 1.0 ml) and sonicated and aliquots were used for protein determination (42), direct ^{125}I radioassay and ^{3}H radioassay after lipid extraction (43). As indicated in the respective legends, in some cases cells were not chase incubated before harvest.

^{125}I-labeled LDL degradation by cells in culture

Briefly, cells incubated (37°C) in DMEM containing BSA (5 mg/ml) and ^{125}I-labeled LDL; chloroquine, monensin, and excess unlabeled LDL (300 μg LDL protein/ml) were absent or present during these incubations as indicated in the legends (5, 44). ^{125}I-labeled LDL degradation was assayed as the amount of ^{125}I-labeled trichloroacetic acid-soluble (non-iodide) material formed by cells and excreted into the medium. ^{125}I-labeled LDL degraded in the absence of unlabeled LDL minus the amount of ^{125}I-labeled LDL degraded in the presence of excess unlabeled LDL represents specific ^{125}I-labeled LDL degradation.

Hydrolysis of HDL_3-associated [^{3}H]cholesteryl oleate by cells in culture

Cells were incubated (37°C, 2.0 h) in DMEM containing BSA (5 mg/ml) and [^{3}H]CO-labeled HDL_3 (5). To inhibit reesterification of hydrolyzed [^{3}H]CO, Sandoz compound 58-035 (5 μg/ml), an inhibitor of acyl-CoA:cholesterol acyltransferase, was present in this medium as well (45). Finally, the medium was aspirated and the cells were washed with PBS (4×) and harvested by treatment with trypsin/EDTA solution as described above. The cell pellet was dissolved in water followed by sonication. Aliquots of the cell suspension were assayed for cell protein (42) or used for extraction of cellular lipids (46) with 0.05 mg cholesteryl oleate (Sigma) and 0.08 mg cholesterol (Sigma) added as carrier. The distribution of radioactivity among lipid classes was determined by thin-layer chromatography on silica gel-coated plates (Merck).

Endogenous labeling of newly synthesized heparan sulfate proteoglycans with [^{35}S]Na_2SO_4

Human Hep3B hepatoma cells were cultured (37°C, 48 h) in DMEM containing LPDS (10%, v/v), [^{35}S]Na_2SO_4 (12.5 μCi/well, Amersham), and antibiotics (47). Thereafter the cells were washed with PBS (12×) and incubated (37°C, 2.0 h) in DMEM containing heparinase I and heparinase III. After these incubations, the media were harvested. The wells were washed with PBS (2×, 4°C) and finally the cells were released from the wells by scraping the cells into NaOH solution (0.1 N, 4°C). Cell-associated radioactivity, radiotracer released in the medium, and cell protein (42) were determined.

Miscellaneous

Protein analysis followed the procedure described by Lowry and coworkers (42). Phospholipid, triglyceride, unesterified and total cholesterol were determined using enzymatic assays (Boehringer). Esterified cholesterol represents the difference between total and unesterified cholesterol.

Non-denaturing polyacrylamide gradient gel electrophoresis (3–30% polyacrylamide) was carried out as described (48). Fast protein liquid chromatography (FPLC) using a Superose 6B column (Pharmacia) was performed as outlined previously (49).

Monensin (Sigma) was dissolved in ethanol and control cells were incubated in the presence of ethanol alone; the final ethanol concentration never exceeded 0.5% (v/v).

Heparinase I (EC 4.2.2.7, from *Flavobacterium heparinum*) and heparinase III (EC 4.2.2.8, from *Flavobacterium heparinum*) were supplied by Sigma (50).

Statistics and calculations

Data are expressed as mean ± SEM. Significance of differences was examined using Student's *t*-test for paired data.

For cellular uptake of doubly radiolabeled HDL_3, uptake of each tracer is shown in terms of apparent HDL_3 particle uptake, expressed as lipoprotein protein (4, 6). This is done to compare uptake of both tracers on the same basis. In figures and tables, ^{125}I represents apparent HDL_3 particle uptake according to the protein tracer (^{125}I-NMTC–apoA-I), [^{3}H] that due to the CE tracer ([^{3}H]CEt) and [^{3}H]CEt–^{125}I shows the difference in HDL_3 particle uptake, i.e., apparent selective CE uptake (5). Uptake of HDL_3 holo-particles is represented by equal rates of uptake for both tracers. HDL_3 selective CE uptake results from lipid uptake in excess of that due to HDL_3 particle uptake (4).

RESULTS

To test the hypothesis that LPL modifies the cellular metabolism of HDL_3, human hepatocytes in primary culture were incubated in the presence of doubly radiolabeled HDL_3 with and without LPL (**Fig. 1**). In the absence of LPL, hepatocytes took up [^{3}H]CEt as well as ^{125}I-NMTC–apoA-I from HDL_3. However, apparent HDL_3 particle uptake according to the lipid tracer ([^{3}H]CEt) was in substantial excess of that due to ^{125}I-NMTC–apoA-I. Previously it has been shown that ^{125}I-NMTC–apoA-I represents HDL_3 holo-particle uptake (4). If the difference in uptake ([^{3}H]CEt − ^{125}I) is calculated, then apparent selective CE uptake is obtained. This result obtained in the experiment shown in Fig. 1 agrees with previous investigations (5). Addition of LPL to the incubation medium increased the uptake of HDL_3-associated [^{3}H]CEt in a dose-dependent manner and the latter was close to saturation at approxi-

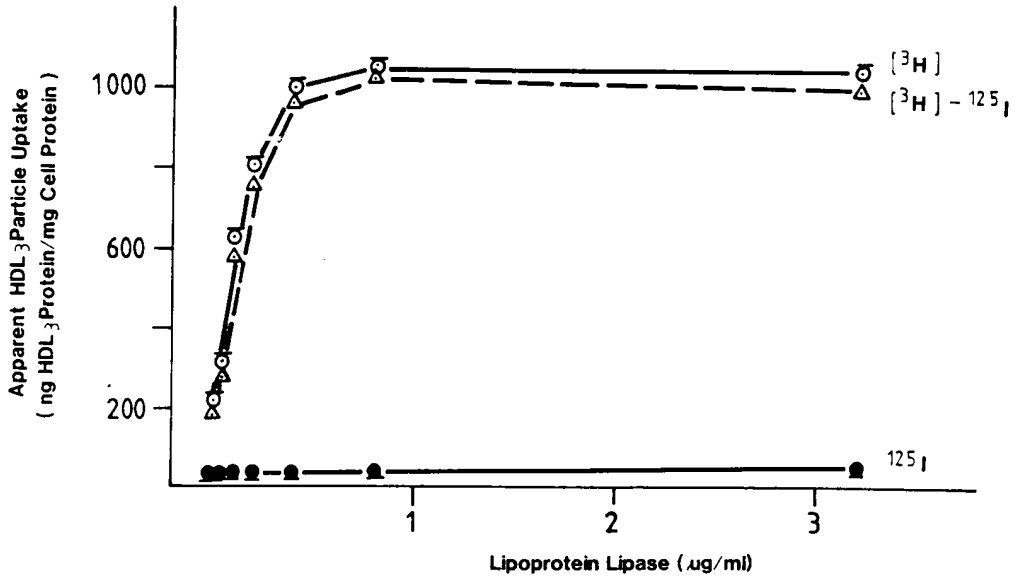

Fig. 1. Dose-response curve for the effect of LPL on uptake of doubly radiolabeled HDL_3 by human hepatocytes in primary culture. Hepatocytes incubated (37°C, 2.0 h) in DMEM containing doubly radiolabeled HDL_3 (40 μg HDL_3 protein/ml) and LPL was absent or present as indicated in the abscissa. After a chase incubation (see Methods), cellular tracer content was determined and apparent HDL_3 particle uptake was calculated as described in Methods. ^{125}I (●) represents apparent HDL_3 particle uptake according to ^{125}I-NMTC–apoA-I; [^{3}H] (○) demonstrates apparent HDL_3 particle uptake due to [^{3}H]cholesteryl oleyl ether and [^{3}H]−^{125}I (△) shows the difference, i.e., apparent HDL_3 selective CE uptake. Values are means ± SEM of n = 6 (without LPL) or n = 3 (with LPL) incubations; one similar experiment yielded qualitatively identical results.

mately 0.5 μg LPL protein/ml (Fig. 1). In contrast, LPL had only a minor effect on ^{125}I-NMTC–apoA-I uptake. The difference in HDL_3 particle uptake between [^{3}H]CEt and ^{125}I-NMTC–apoA-I yields apparent selective CE uptake and the latter is stimulated by LPL in a concentration-dependent manner. Compared to the absence of LPL (= 100%), this enzyme increased HDL_3 selective CE uptake by 522 ± 2.8% (mean ± SEM, n = 3) at an LPL concentration of 3.2 μg/ml. Thus LPL promotes the selective uptake of HDL_3-associated CE by human hepatocytes.

With human Hep3B hepatoma cells, HDL_3 uptake experiments were performed under very similar conditions as shown in Fig. 1 (data not shown). These cells were incubated (37°C, 2.0 h) in medium containing doubly radiolabeled HDL_3 (40 μg HDL_3 protein/ml) and with or without various concentrations of LPL (0–1.8 μg/ml). In the absence of LPL, apparent HDL_3 selective CE uptake was 329.9 ± 14.2 ng HDL_3 protein/mg cell protein (mean ± SEM, n = 6). LPL stimulated selective CE uptake in a dose-dependent manner; at an LPL concentration of 1.8 μg/ml, apparent HDL_3 selective CE uptake increased to 1,283.5 ± 27.0 ng HDL_3 protein/mg cell protein (mean ± SEM, n = 3) and this corresponds to an increase of 389 ± 8% (mean ± SEM, n = 3). Thus LPL had a qualitatively identical effect on HDL_3 uptake in Hep3B hepatoma cells and in human hepatocytes in primary culture.

Selective CE uptake from HDL is a time-dependent process (6). To investigate the effect of LPL on the kinetics of tracer uptake from HDL_3, Hep3B hepatoma cells were incubated in medium containing doubly radiolabeled HDL_3 (40 μg HDL_3 protein/ml) for 0.16, 1.0, 2.0, and 4.0 h (data not shown). In the absence of LPL, uptake of HDL_3-associated [^{3}H]CEt and ^{125}I–NMTC–apoA-I increased in a time-dependent manner at linear rates. Apparent HDL_3 particle uptake according to [^{3}H]CEt was in excess of that due to ^{125}I-NMTC–apoA-I throughout the time course, yielding a time-dependent increase in selective CE uptake. In parallel, Hep3B cells were incubated in the additional presence of LPL (0.4 μg/ml). LPL significantly increased the uptake of HDL_3-associated [^{3}H]CEt throughout the time course with only a very minor effect on ^{125}I-NMTC–apoA-I internalization; apparent HDL_3 selective CE uptake ([^{3}H]CEt − ^{125}I) increased due to the presence of LPL between 301 ± 18% and 531 ± 15% (n = 3, mean ± SEM). Thus LPL time-dependently stimulated HDL_3 selective CE uptake by Hep 3B hepatoma cells, at least up to 4 h.

In the experiments presented above, the lipolytic activity of LPL was inhibited by tetrahydrolipstatin (THL) (26, 38). Therefore these results imply that the LPL effect on HDL_3 selective CE uptake is independent of lipolysis. However, the question was raised whether LPL stimulated selective CE uptake in the absence of THL. In addition, the issue was addressed whether this compound itself had an effect on selective CE uptake. Human Hep3B hepatoma cells were incubated in medium containing doubly radiolabeled HDL_3 with or without LPL and/or THL (**Table 1**). THL had no effect on tracer uptake from HDL_3 in the absence of LPL; thus this compound did not modify apparent HDL_3 selective CE uptake (Table 1). As expected, LPL increased HDL_3 selective CE uptake by Hep3B cells up to 622%. This stimulatory LPL effect was quantitatively very similar whether the inhibitor was absent or present during the uptake assay. These results suggest that the action of LPL on selective CE uptake is independent of the lipolytic activity of this enzyme. Similarly, THL has no effect on HDL_3 selective CE uptake per se.

TABLE 1. Effect of tetrahydrolipstatin (THL) on uptake of tracers from doubly radiolabeled HDL_3 by human Hep3B hepatoma cells in culture

Experiment	Apparent HDL_3 Selective CE Uptake ([^{3}H]CEt − ^{125}I)	
	−THL	+THL
	ng HDL_3 protein/mg cell protein	
−LPL		
1	87.0 ± 13.3	96.4 ± 1.5
2	37.3 ± 1.8	35.9 ± 4.5
3	692.0 ± 19.7	670.3 ± 14.4
4	673.3 ± 15.6	721.8 ± 25.3
+LPL		
1, 0.4 μg/ml	496.0 ± 35.2	599.3 ± 74.4
2, 0.4 μg/ml	228.8 ± 12.0	187.6 ± 9.1
3, 1.0 μg/ml	1,899.5 ± 101.0	2,404 ± 61.4
4, 1.0 μg/ml	2,156.0 ± 97.0	1,958.3 ± 108.0

Hep3B hepatoma cells were incubated in DMEM that contained doubly radiolabeled HDL_3 (40 μg HDL_3 protein/ml); LPL and THL (50 μg/ml) were absent or present as indicated. In experiments 1 and 2, cells were incubated for 0.5 h; in experiments 3 and 4 cells were incubated for 2.0 h at 37°C. There was no chase incubation in experiments 1 and 2, whereas a chase incubation was performed in experiments 3 and 4 (see Methods). Finally, cells were harvested and apparent HDL_3 selective uptake was determined as described in Methods. Values are means ± SEM of n = 3 (experiment 1) or n = 4 (experiments 2, 3, 4) independent incubations.

To investigate directly whether the lipolytic action of LPL was inhibited by THL, HDL_3 was incubated at 37°C in medium containing this enzyme and with or without THL (**Table 2**). After incubation, HDL_3 was reisolated by ultracentrifugation and the chemical composition was analyzed. In these assays THL had no effect on protein, phospho-

TABLE 2. LPL and the composition of HDL_3: effect of tetrahydrolipstatin (THL)

Parameter	Incubation conditions	
	+ LPL − THL	+ LPL + THL
	% of total mass	
Protein	62.3	61.7
Phospholipid	18.9	20.6
Triglyceride	8.2	7.6
Unesterified cholesterol	2.9	2.9
Esterified cholesterol	7.6	7.2

HDL_3 (40 μg HDL_3 protein/ml) was incubated (37°C, 2 h) in DMEM containing BSA (5 mg/ml) and LPL (0.4 μg/ml); THL (50 μg/ml) was absent or present as indicated. HDL_3 was thereafter reisolated by ultracentrifugation at d 1.21 g/ml followed by extensive dialysis (PBS). Finally, protein, phospholipid, triglyceride, unesterified and esterified cholesterol were determined as described in Methods. Values are means of n = 2 independent incubations in each case.

lipid, triglyceride, unesterified and esterified cholesterol content of the respective HDL_3 preparation (Table 2). This experiment yielded no evidence for lipolysis under these experimental conditions.

In a related experiment, HDL_3 was incubated at 4°C or at 37°C in medium supplemented with LPL and again with or without THL (**Fig. 2**). After HDL_3 reisolation by ultracentrifugation, these preparations were loaded on gradient gels and subjected to electrophoresis under native conditions (48). All HDL_3 preparations migrated identically on these gels (Fig. 2). In parallel, each of the HDL_3 fractions presented in Fig. 2 was subjected to fast protein liquid chromatography (FPLC) (49). The elution profiles for each of these HDL_3 preparations were identical (data not shown). In addition, no HDL_3 aggregates could be detected by this analytical approach. Thus electrophoresis and chromatography did not indicate a change in HDL_3 particle size due to these incubations with LPL. Also, these experiments yielded no evidence that LPL was catalytically active under the experimental conditions applied.

LPL binds to cell surface heparan sulfate proteoglycans (17, 29). Next, the question was addressed as to whether these molecules played a role in the LPL-mediated stimulation of HDL_3 selective CE uptake. Heparinases degrade the glycosaminoglycan side chains of cell surface heparan sulfate proteoglycans (29, 50). Human hepatocytes in primary culture were preincubated in the absence or presence of heparinase I and heparinase III; the uptake assay in medium containing doubly radiolabeled HDL_3 and with or without LPL was then carried out (**Fig. 3**). In hepatocytes preincubated without heparinases, LPL significantly increased the uptake of [^{3}H]CEt from HDL_3 whereas the internalization of ^{125}I-NMTC–apoA-I was only marginally modified (Fig. 3A). Thus LPL substantially stimulated HDL_3 selective CE uptake ([^{3}H]CEt − ^{125}I). In contrast, in hepatocytes preincubated in the presence of heparinases, the LPL-mediated increase in uptake of HDL_3-associated [^{3}H]CEt was significantly reduced, yielding a diminished rate of apparent selective CE uptake (Fig. 3B).

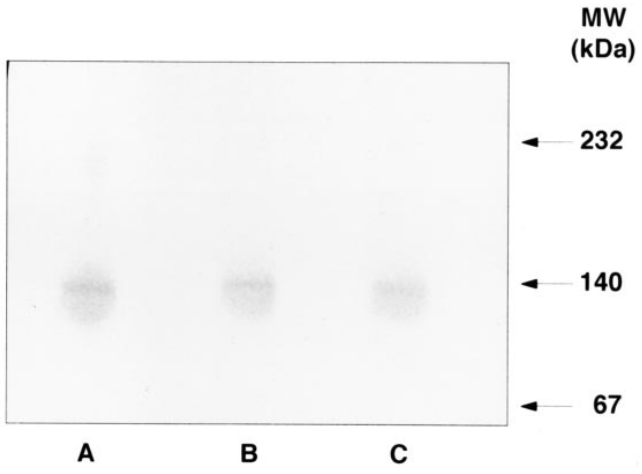

Fig. 2. Non-denaturing polyacrylamide gradient gel electrophoresis of HDL_3. HDL_3 (40 μg HDL_3 protein/ml) was incubated (2 h) at 4°C (A) or at 37°C (B and C) in DMEM containing BSA (5 mg/ml) and LPL (0.4 μg/ml); THL (50 μg/ml) was also present in (C). Thereafter HDL_3 was reisolated by ultracentrifugation at d 1.21 g/ml, followed by extensive dialysis (PBS). Finally, the respective HDL_3 preparation (60 μg protein/lane) and high molecular weight standard proteins (Pharmacia) were loaded on a gradient gel. Following electrophoresis under non-denaturing conditions, the gel was fixed and stained with Coomassie brilliant blue. Molecular masses of 67, 140 and 232 kDa correspond to diameters of 7.1, 8.2 and 10.4 nm, respectively. Shown is a typical experiment of a total of three.

Experiments were also performed with human Hep3B hepatoma cells and human skin fibroblasts under experimental conditions very similar to those shown in Fig. 3 (data not shown). In these cells pretreatment with heparinases also significantly reduced the stimulatory effect of LPL on HDL_3 selective CE uptake. These results suggest that cell surface proteoglycans play an essential role in the LPL-mediated increase in selective CE uptake by cultured cells.

The effect of heparinases on cell surface heparan sulfate proteoglycans was directly examined (47). Hep3B hepatoma cells were incubated (48 h, 37°C) in DMEM supplemented with LPDS (10%, v/v) and [^{35}S]Na_2SO_4 as described in Methods (data not shown). Thereafter followed an incubation (37°C, 2.0 h, DMEM) in the absence or presence of different concentrations of heparinase I (0–6.0 U/ml) and heparinase III (0–1.8 U/ml). Cell-associated and medium-released [^{35}S]Na_2SO_4 were finally analyzed. Treatment with heparinases dose-dependently reduced cell-associated radioactivity (up to 48%) and increased the tracer content of the incubation medium (up to 202%). Thus heparinases removed significant amounts of proteoglycans from the cell surface under these conditions.

The role of cell surface proteoglycans in the LPL-mediated stimulation of HDL_3 selective CE uptake was addressed using Chinese hamster ovary cells (CHO cells) as well (39). In contrast to wild type cells (CHO-K1), mutant cells (CHO-745) are defective in proteoglycan synthesis. Both cell types were incubated in parallel in medium containing doubly radiolabeled HDL_3 and with or without LPL (**Fig. 4**). In wild type CHO cells (K1), LPL dose-dependently stimulated the uptake of HDL_3-associated [^{3}H]CEt whereas the effect on ^{125}I-NMTC–apoA-I was marginal (Fig. 4A); thus in CHO-K1 cells also LPL significantly increased the rate of apparent HDL_3 selective CE uptake. In contrast, in proteoglycan-deficient CHO cells (745), LPL had only a minor effect on uptake of HDL_3-associated [^{3}H]CEt and of ^{125}I-NMTC–apoA-I; thus, in these mutant cells, virtually no LPL-mediated increase in selective CE uptake was detected (Fig. 4B).

In an alternative approach, wild type CHO cells were depleted of cell surface proteoglycans. CHO-K1 cells were pre-incubated (37°C, 2.0 h) in medium and with or without heparinase I (1.25 U/ml) and heparinase III (0.42 U/ml) (data not shown). Thereafter followed an incubation (37°C, 0.5 h) in DMEM containing doubly radiolabeled HDL_3 (40 μg HDL_3 protein/ml) and with or without LPL (0.4 μg/ml). In the absence of heparinases during prein-

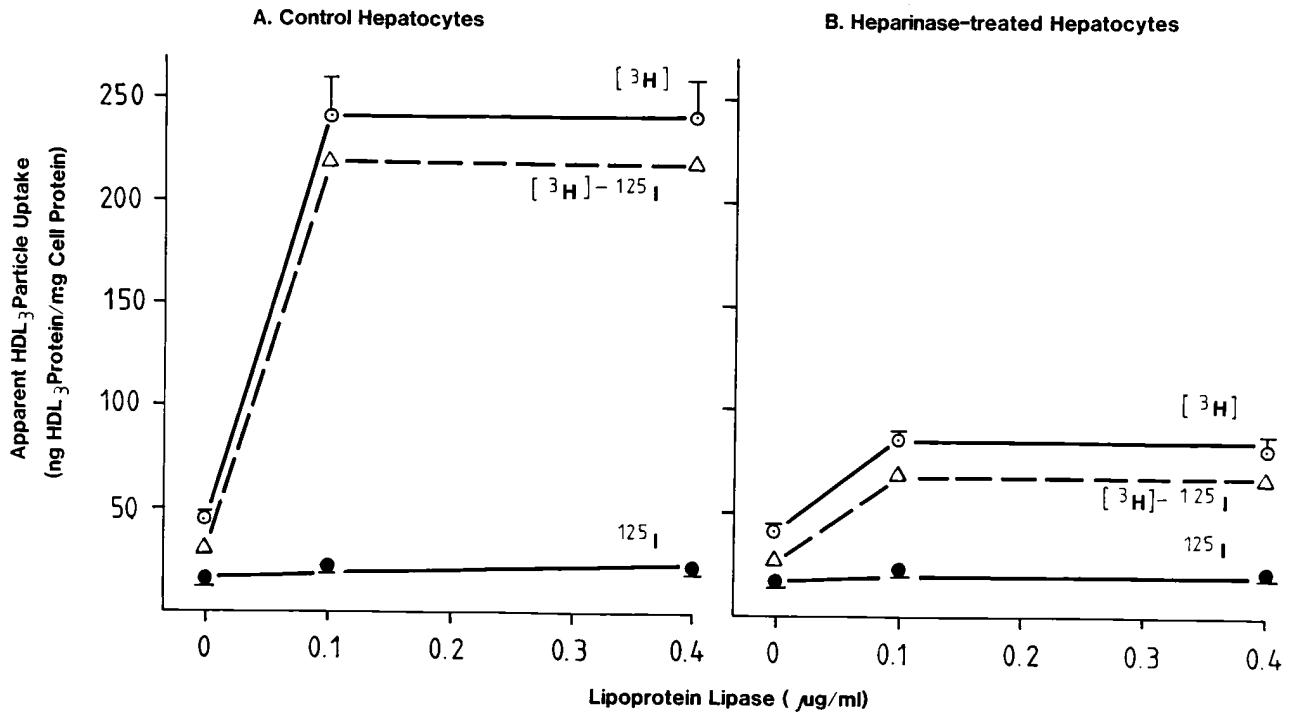

Fig. 3. Effect of preincubation with heparinases on the LPL-mediated increase in tracer uptake from doubly radiolabeled HDL_3 by human hepatocytes in primary culture. After preincubation (see Methods), hepatocytes incubated (37°C, 2.0 h) in DMEM in the absence (A) or presence (B) of heparinase I (1.25 U/ml) and heparinase III (0.42 U/ml). Thereafter this medium was aspirated and both groups of cells were washed (PBS, 2×) followed by an incubation (37°C, 0.5 h) in DMEM containing doubly radiolabeled HDL_3 (40 µg HDL_3 protein/ml); LPL was absent or present as indicated in the abscissa. Finally, the cells were harvested and apparent HDL_3 particle uptake was determined as described in Methods. ^{125}I (●) represents apparent HDL_3 particle uptake according to ^{125}I-NMTC–apoA-I; [^3H] (○) demonstrates apparent HDL_3 particle uptake due to [^3H]cholesteryl oleyl ether; and [^3H]-^{125}I (△) shows the difference, i.e., apparent HDL_3 selective CE uptake. Values are means ± SEM of n = 4 incubations; an independent experiment yielded qualitatively identical results.

cubation and in the presence of LPL during the uptake assay, apparent HDL_3 selective CE uptake was 439.0 ± 26.2 ng HDL_3 protein/mg cell protein; preincubation of the cells with heparinases reduced the respective value to 277.2 ± 11.1 (means ± SEM, n = 4 independent incubations; an independent experiment yielded qualitatively identical results). These experiments with CHO cells suggest that heparan sulfate proteoglycans of the cell surface play a significant role for the LPL-mediated stimulation of HDL_3 selective CE uptake.

HDL selective CE uptake is independent of endocytosis mediated by LDL receptors or low density lipoprotein receptor-related protein (LRP) (4, 5). However, a role for these structurally related receptors for the LPL-mediated stimulation of cellular lipoprotein uptake has been suggested (16, 18, 19, 22, 23). To address the issue of whether these receptors are involved in the LPL-mediated increase in selective CE uptake, murine embryonic fibroblasts (MEF cells) were used (40). Wild type MEF cells have LRP and LDL receptors. Fibroblasts with a homozygous deficiency for LRP or LDL receptors and cells with homozygous deficiency for both LRP and LDL receptors are also available. These four cell strains were incubated in parallel in medium containing doubly radiolabeled HDL_3 and with or without LPL (**Fig. 5**). In the absence of LPL, for each cell type, apparent HDL_3 particle uptake according to [^3H]CEt was in excess of that due to ^{125}I-NMTC–apoA-I; thus all MEF cells demonstrated apparent HDL_3 selective CE uptake and quantitatively this lipid uptake was similar in each case. These cells were incubated in parallel in the additional presence of LPL in the uptake medium (Fig. 5). LPL stimulated the uptake of HDL_3-associated [^3H]CEt significantly in all strains of MEF cells; this yielded an increase in apparent selective CE uptake of up to 404%. These experiments yielded no evidence that LRP or LDL receptors play a role in the LPL-mediated increase in HDL_3 selective CE uptake by cells.

As a more general strategy to address the issue of whether LDL receptors, LRP, and endocytosis were involved in the LPL effect on HDL_3 selective CE uptake, the carboxylic ionophore monensin, an inhibitor of receptor recycling and endocytosis, was used (5, 51). Human Hep3B hepatoma cells were preincubated in the absence or presence of monensin and thereafter an uptake assay in medium containing doubly radiolabeled HDL_3 and with or without LPL or monensin was performed (**Fig. 6B**). In the absence of LPL, monensin reduced the uptake of HDL_3-associated [^3H]CEt marginally, whereas this compound had no effect on uptake of ^{125}I-NMTC–apoA-I; therefore apparent HDL_3 selective CE uptake ([^3H]CEt − ^{125}I) was reduced to some degree, a result that is consistent with previous observations (4). In the absence of

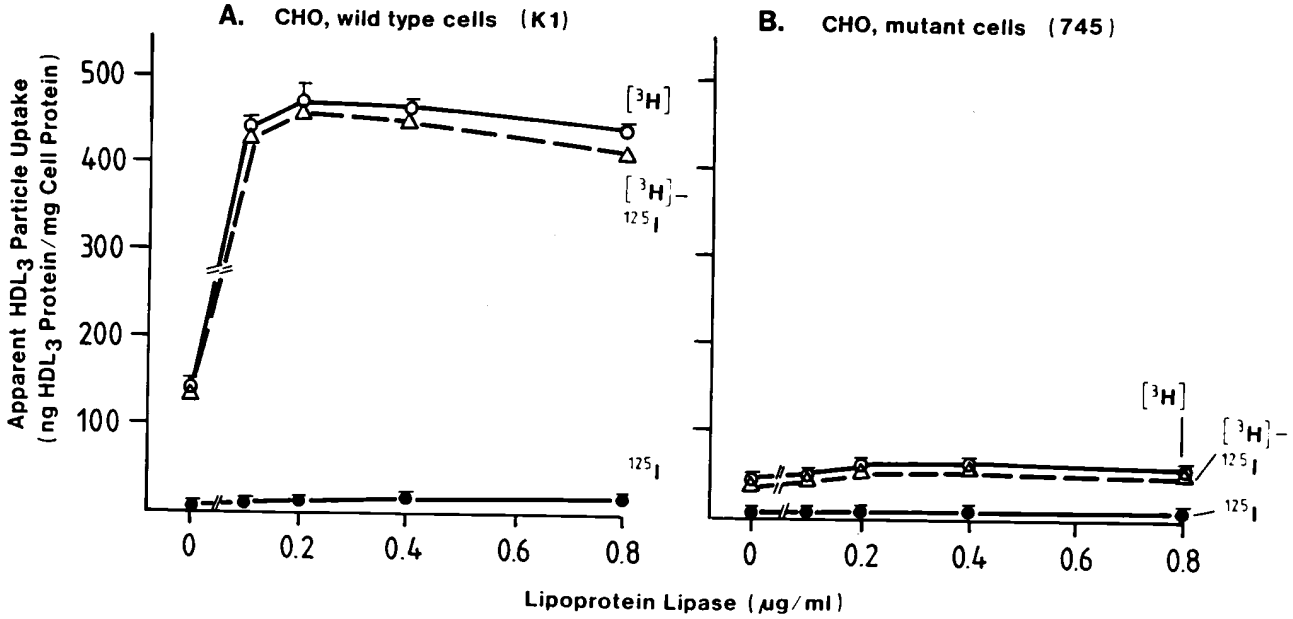

Fig. 4. Effect of LPL on the uptake of doubly radiolabeled HDL_3 by wild type (K1) and mutant (745) Chinese hamster ovary (CHO) cells in culture. Wild type (CHO-K1, (A)) and mutant (CHO-745, (B)) Chinese hamster ovary cells incubated (37°C, 0.5 h) in medium containing doubly radiolabeled HDL_3 (40 μg HDL_3 protein/ml) and LPL was absent or present as indicated in the abscissa. After a chase incubation (see Methods), cellular tracer content was determined and apparent HDL_3 particle uptake was calculated as described in Methods. ^{125}I (●) represents apparent lipoprotein particle uptake according to ^{125}I-NMTC–apoA-I; $[^3H]$ (○) demonstrates apparent lipoprotein particle uptake due to [^{3}H]cholesteryl oleyl ether and $[^3H]-^{125}I$ (△) shows the difference, i.e. apparent HDL_3 selective CE uptake. Values are means ± SEM of n = 6 (without LPL) or n = 3 (with LPL) independent incubations. One independent experiment yielded qualitatively identical results.

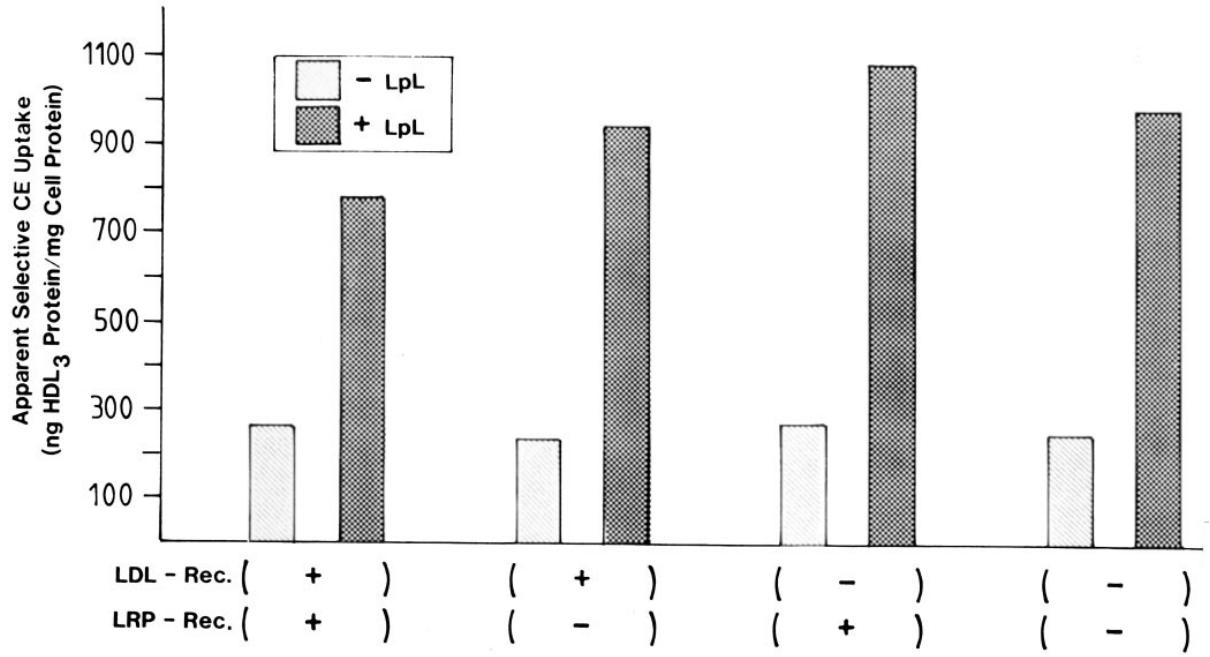

Fig. 5. Effect of LPL on the uptake of doubly radiolabeled HDL_3 by murine embryonic fibroblasts (MEF cells) in culture. Wild-type murine embryonic fibroblasts (MEF 1), murine embryonic fibroblasts with a homozygous deficiency for LRP (MEF 2) or LDL receptors (MEF 3), and murine embryonic fibroblasts with a homozygous deficiency for both LRP and LDL receptors (MEF 4) as indicated in the abscissa were cultured in parallel. After preincubation (see Methods), all strains of cells were incubated (37°C, 2.0 h) in medium containing doubly radiolabeled HDL_3 (20 μg HDL_3 protein/ml) and LPL (0.2 μg/ml) was absent or present during these incubations as indicated. After a chase incubation (see Methods) cellular tracer content was determined and apparent HDL_3 selective CE uptake was calculated as described in Methods. Open bars represent HDL_3 selective CE uptake in the absence of LPL and cross-hatched bars represent HDL_3 selective CE uptake in the presence of LPL. Values are means ± SEM of n = 6 (without LPL) or n = 2 (with LPL) independent incubations. Maximum variation from the mean was 7% (without LPL) and 3% (with LPL). Three independent experiments yielded qualitatively identical results.

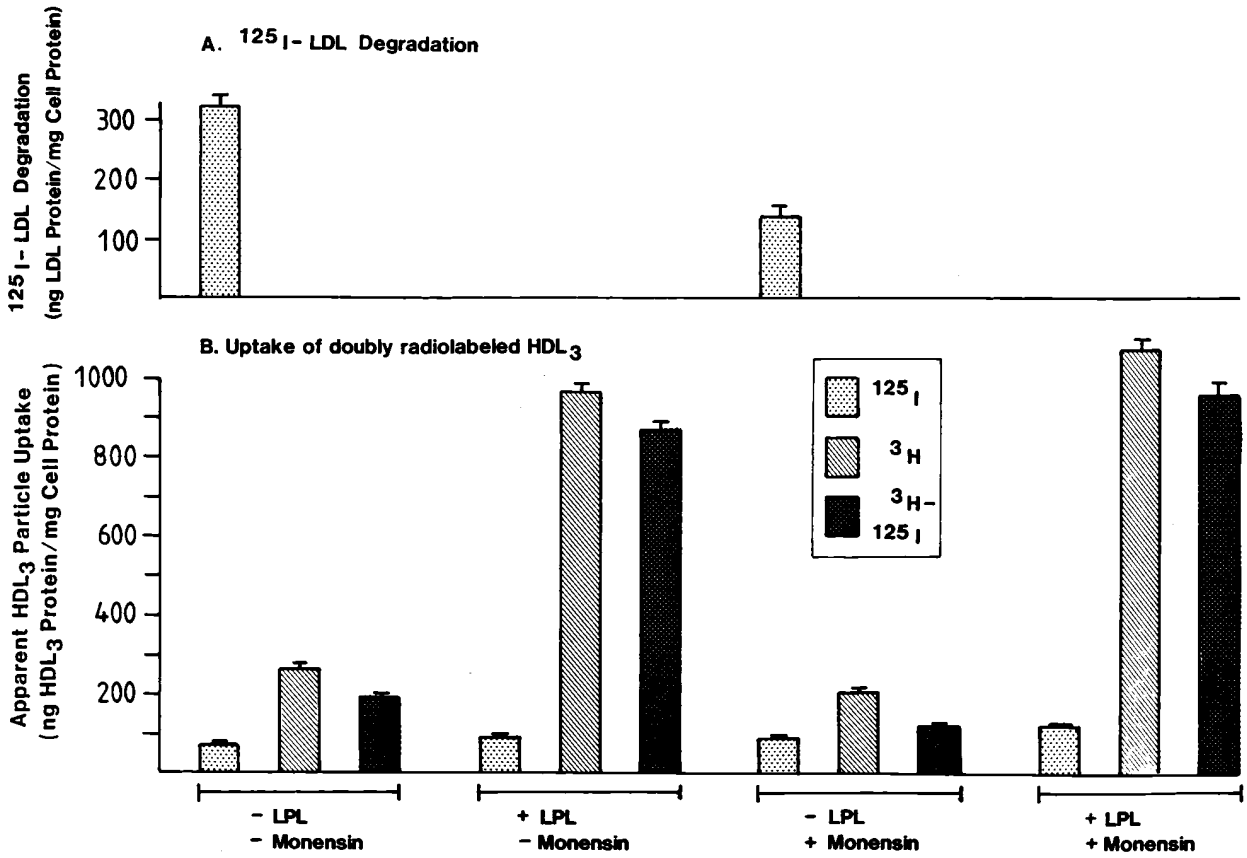

Fig. 6. Effects of monensin and LPL on tracer uptake from doubly radiolabeled HDL_3 and on degradation of ^{125}I-labeled LDL by human Hep3B hepatoma cells in culture. Hep3B hepatoma cells preincubated (37°C, 0.5 h, see Methods) in DMEM in the absence or presence of monensin (10 μM) (B). Thereafter cells were incubated (37°C, 2.0 h) in DMEM containing doubly radiolabeled HDL_3 (40 μg HDL_3 protein/ml); LPL (0.4 μg/ml) and monensin (10 μm) were absent or present as indicated in the abscissa. After a chase incubation (see Methods) in the absence or presence of monensin (10 μm) apparent HDL_3 particle uptake was determined as described in Methods. ^{125}I represents apparent HDL_3 particle uptake according to ^{125}I-NMTC–apoA-I; [^3H] demonstrates apparent HDL_3 particle uptake due to [^3H]cholesteryl oleyl ether; and [^3H]−^{125}I shows the difference, i.e., apparent HDL_3 selective CE uptake. (A) In parallel, Hep3B cells were incubated (37°C, 4.0 h) in DMEM containing ^{125}I-labeled LDL (10 μg LDL protein/ml) and monensin (10 μm) was absent or present as indicated in the abscissa (below "B"). Total ^{125}I-labeled LDL degradation was determined as described in Methods. Values are means ± SEM of n = 6 (HDL_3 uptake) or n = 4 (^{125}I-labeled LDL degradation) incubations. An independent experiment yielded qualitatively identical results.

monensin, LPL increased the uptake of [^3H]CEt from doubly radiolabeled HDL_3 yielding an enhanced rate of apparent selective CE uptake. In one experimental group, LPL and monensin were simultaneously present during the uptake assay for HDL_3. In the presence of monensin LPL mediated a significant increase in [^3H]CEt uptake from HDL_3; therefore selective CE uptake was stimulated by LPL to a similar extent whether or not monensin was present (Fig. 6B). To ensure that monensin in fact inhibited endocytosis, degradation of ^{125}I-labeled LDL by Hep3B cells was investigated (Fig. 6A). As expected, monensin decreased ^{125}I-labeled LDL degradation to 47 ± 6% (control = 100%, mean ± SEM, n = 4).

LDL receptor-deficient human skin fibroblasts (FH fibroblasts) were used as well to address the role of these receptors in the LPL-related increase in HDL_3 selective CE uptake (41). FH fibroblasts were incubated in medium containing doubly radiolabeled HDL_3 with or without LPL (**Fig. 7**). LPL increased the uptake of HDL_3-associated [^3H]CEt by fibroblasts significantly whereas this enzyme had only a marginal effect on uptake of ^{125}I-NMTC–apoA-I. The difference in apparent HDL_3 particle uptake according to both tracers ([^3H]CEt − ^{125}I) yielded apparent selective CE uptake, and LPL dose-dependently increased the rate of this process in these LDL receptor-deficient cells (Fig. 7).

In summary, these experiments suggest that LDL receptors, LRP, and endocytosis play no role in the LPL-mediated increase in HDL_3 selective CE uptake.

Selective CE uptake from HDL is a regulated pathway (6, 7). Cellular cholesterol-loading down-regulates selective uptake whereas sterol deprivation does the opposite (6). The question was addressed whether the LPL effect on this pathway is regulated as well. In addition, in cholesterol-loaded cells LDL receptors are down-regulated and thus the expected LPL effect is essentially independent of these membrane proteins (27). Hep3B hepatoma cells were cholesterol-depleted or cholesterol-loaded by culture under appropriate conditions (**Table 3**) (6). Preliminary experiments showed that the magnitude of LDL receptor

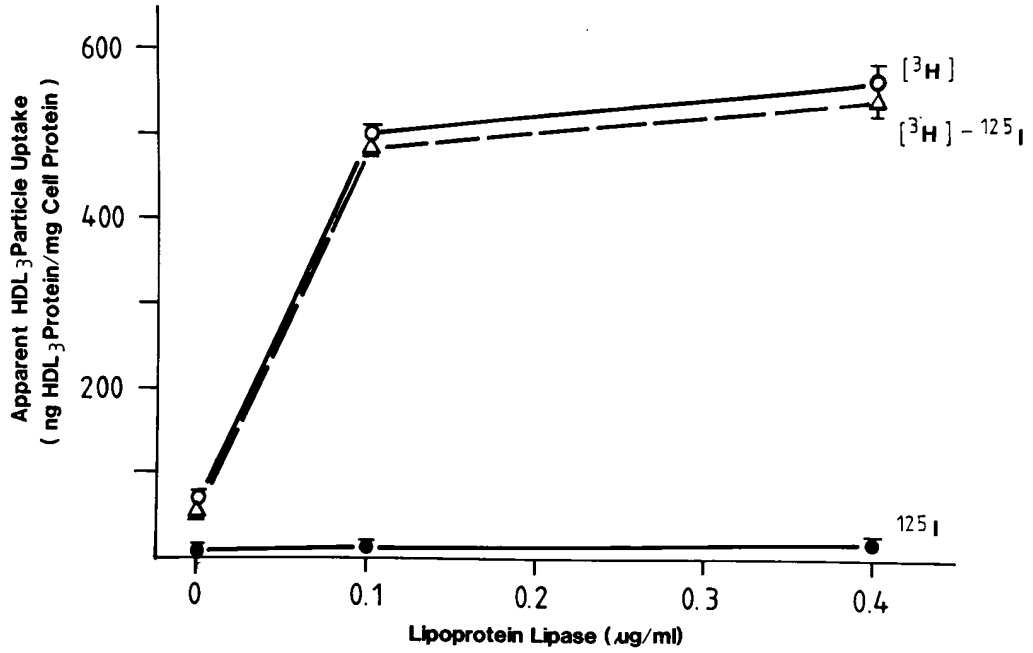

Fig. 7. Effect of LPL on the uptake of doubly radiolabeled HDL_3 by human LDL receptor-deficient skin fibroblasts. LDL receptor-deficient skin fibroblasts (FH fibroblasts) incubated (37°C, 1.0 h) in DMEM containing doubly radio-labeled HDL_3 (40 µg HDL_3 protein/ml) and LPL was absent or present as indicated in the abscissa. After a chase incubation (see Methods), cellular tracer content was determined and apparent HDL_3 particle uptake was calculated as described in Methods. ^{125}I (●) represents apparent HDL_3 particle uptake according to ^{125}I-NMTC–apo A-I, [^3H] (○) demonstrates apparent HDL_3 particle uptake due to [^3H]cholesteryl oleyl ether, and [^3H]−^{125}I (△) shows the difference, i.e. apparent HDL_3 selective CE uptake. Values are means ± SEM of n = 6 (without LPL) or n = 3 (with LPL) incubations; a similar independent experiment yielded qualitatively identical results.

down-regulation achieved in Hep3B cells was more limited compared to fibroblasts (data not shown). After culture, cells were incubated in the presence of doubly radiolabeled HDL_3 and with or without LPL. Compared to non-loaded Hep3B cells, cholesterol-loading decreased the uptake of HDL_3-associated [^3H]CEt, yielding a significantly decreased rate of apparent HDL_3 selective CE uptake (Table 3). In unloaded and in cholesterol-loaded Hep3B cells, the presence of LPL during the uptake assay for radiolabeled HDL_3 stimulated uptake of [^3H]CEt and this yielded an increased rate of apparent selective CE uptake. Remarkably, quantitatively this LPL-mediated increase in selective CE uptake was almost identical in cholesterol-deficient and cholesterol-loaded cells. In parallel experiments, degradation of ^{125}I-labeled LDL was examined (Table 3). As expected, cholesterol-loading down-regulated the rate of ^{125}I-labeled LDL degradation. Thus, despite a significant down-regulation of HDL_3 selective CE uptake by cholesterol-loading of Hep3B cells, the stimulatory effect of LPL on HDL_3 lipid uptake was not modified. These results suggest that CE selectively taken up from HDL_3 may enter two cellular compartments: one compartment is regulated by cholesterol-loading of the cells whereas the other is not. On the other hand, this experiment also yields no evidence for a role of LDL receptors in the LPL-mediated increase in HDL_3 selective CE uptake.

HDL CE that are selectively internalized are processed intracellularly by a mechanism distinct from the endosomal and lysosomal catabolic pathway and therefore the fate of these lipids is different from those associated with other lipoprotein fractions, for example, LDL (13, 27). The question was investigated whether CE, taken up due to the action of LPL, are hydrolyzed in a lysosomal cellular compartment or not. Hep3B hepatoma cells were incubated in medium containing [^3H]cholesteryl oleate-labeled HDL_3 ([^3H]CO-HDL_3) and with or without LPL and the lysosomotropic agent chloroquine (**Table 4**). After cell harvest, labeled unesterified and esterified cholesterol were analyzed as described in Methods. In the absence of LPL and chloroquine, the vast majority of internalized [^3H]CO was hydrolyzed by Hep3B cells to yield unesterified [^3H]cholesterol (5). Supplementation of the assay medium with chloroquine alone or LPL alone had no effect on the rate of hydrolysis of initially HDL_3-associated [^3H]CO. However, when Hep3B cells were incubated in medium containing LPL, chloroquine and [^3H]CO-labeled HDL_3, the fraction of unesterified [^3H]cholesterol decreased significantly and the fraction of esterified [^3H]cholesterol increased; thus hydrolysis of [^3H]CO was inhibited by chloroquine in these experiments.

To make sure under these conditions (Table 4) that LPL stimulated HDL_3 selective CE uptake, Hep3B cells were incubated in parallel in the presence of doubly radiolabeled

TABLE 3. Regulation of the uptake of doubly radiolabeled HDL_3 by human Hep 3B hepatoma cells in culture and the effect of LPL

Addition of LDL during Culture	Addition of LPL during Assay	Apparent HDL_3 Selective CE Uptake ($[^3H]CEt - {}^{125}I$)	^{125}I-LDL Degradation
		ng HDL_3 protein/ mg cell protein	*ng LDL protein/ mg cell protein*
None	None	107.4 ± 4.0[a]	531.1 ± 60.9[b]
None	0.8 μg/ml	297.5 ± 38.1	
Increase due to LPL		190.1	
200 μg LDL protein/ml	None	62.3 ± 5.8[a]	245.7 ± 10.8[b]
200 μg LDL protein/ml	0.8 μg/ml	256.9 ± 18.3	
Increase due to LPL		194.6	

Human Hep3B hepatoma cells cultured (37°C) for 2 × 24 h in DMEM containing LPDS (20 %, v/v) as described in Methods. During the second 24-h period, unlabeled LDL (200 μg LDL protein/ml) was added to the medium as indicated. After preincubation (37°C, 0.5 h, without lipoproteins), the cells were incubated (37°C) in medium containing doubly radiolabeled HDL_3 (40 μg HDL_3 protein/ml, 2.0 h) or ^{125}I-labeled LDL (5 μg LDL protein/ml, 4.0 h); LPL (0.8 μg/ml) was absent or present during this incubation as indicated. In the case of HDL_3 uptake, a chase incubation followed as described in Methods. Finally, cells (HDL_3 uptake) and media (^{125}I-labeled LDL degradation) were harvested. Apparent HDL_3 selective CE uptake and specific ^{125}I-LDL degradation were determined as described in Methods. Values are means ± SEM of n = 3 – n = 6 incubations performed in one experiment. Two similar independent experiments yielded qualitatively identical results.

[a] $P < 0.0001$.

[b] $P < 0.001$.

HDL_3 and with or without LPL or chloroquine (**Table 5**). As expected, LPL increased apparent HDL_3 selective CE uptake by these cells and chloroquine had no effect on this pathway (Table 5) (4). In chloroquine-treated cells, LPL promoted apparent HDL_3 selective CE uptake similarly as in the absence of chloroquine. To verify the inhibitory action of chloroquine on lysosomes, ^{125}I-labeled LDL degradation by Hep3B cells was explored in parallel (Table 5). As expected, chloroquine significantly reduced ^{125}I-labeled LDL degradation, thus establishing lysosomal inhibition.

Finally, under conditions of lysosomal inhibition, LPL increased HDL_3 selective CE uptake similarly as it did in the absence of chloroquine. HDL_3-derived CE taken up due to the stimulatory action of LPL evidently are directed to a lysosomal cellular compartment for hydrolysis.

TABLE 4. Hydrolysis of HDL_3-associated $[^3H]$cholesteryl oleate by human Hep3B hepatoma cells in culture and the effects of LPL and chloroquine

Additions during Assay		Unesterified Cholesterol	Esterified Cholesterol
LPL	Chloroquine		
		% of total [3H] cpm recovered	
None	None	86	12
0.4 μg/ml	None	73	24
None	50 μm	80	18
0.4 μg/ml	50 μm	40	56

Hep 3B hepatoma cells preincubated (37°C, 0.5 h) in DMEM containing BSA (5 mg/ml) in the absence or presence of chloroquine (50 μm) as indicated. In addition, Sandoz compound 58-035 (5 mg/ml) supplemented the medium. After pre-incubation, all assays were initiated in parallel. Hep 3B cells were incubated (37°C, 2.0 hr) in DMEM containing $[^3H]$CO-labeled HDL_3 (40 μg HDL_3 protein/ml) and Sandoz compound 58-035 (5 mg/ml); LPL (0.4 μg/l) and chloroquine (50 μm) were absent or present as indicated. The cells were harvested and unesterified and esterified $[^3H]$cholesterol were analyzed as described in Methods. Values are means ± SEM of n = 2 independent determinations. Two independent experiments yielded qualitatively identical results.

DISCUSSION

In human hepatocytes in primary culture and in human hepatoma cells, LPL mediates an approximately 7-fold increase in HDL_3 selective CE uptake. This LPL effect was also established in Chinese hamster ovary (CHO) cells (39), murine embryonic fibroblasts (MEF cells) (40), normal human skin fibroblasts (6), and human skin fibroblasts deficient in LDL receptors (FH fibroblasts) (41). Remarkably, all experiments with liver cells were in close qualitative agreement with studies performed with nonhepatic cell types. These observations are consistent with a previously reported LPL-induced increase in HDL selective CE uptake by mouse peritoneal macrophages (33).

TABLE 5. Uptake of doubly radiolabeled HDL_3 and degradation of ^{125}I-labeled LDL by Hep3B hepatoma cells in culture

Additions during Assay		Apparent HDL_3 Selective CE Uptake ($[^3H]CEt - {}^{125}I$)	^{125}I-Labeled LDL Degradation
LPL	Chloroquine		
		ng HDL_3 protein/ mg cell protein	*ng LDL protein/ mg cell protein*
None	None	68.4 ± 4.7	2,815 ± 84
0.4 μg/ml	None	146.1 ± 5.1	nd
None	50 μm	61.0 ± 0.6	91 ± 3
0.4 μg/ml	50 μm	140.8 ± 3.4	nd

Hep 3B hepatoma cells were incubated (37°C, 2.0 h) in DMEM containing doubly radiolabeled HDL_3 (40 μg HDL_3 protein/ml); LPL (0.4 μg/ml) and chloroquine (50 μm) were absent or present as indicated. After a chase incubation (see Methods), cells were harvested and apparent HDL_3 selective CE uptake was determined as described in Methods. Values are means ± SEM of n = 3 incubations. Two independent experiments yielded qualitatively identical results. In parallel, Hep 3B hepatoma cells were incubated (37°C, 4.0 h) in medium containing ^{125}I-labeled LDL (10 μg LDL protein/ml) and chloroquine (50 μm) was absent or present as indicated. Thereafter ^{125}I-LDL degradation was analyzed as detailed in Methods. Values are means ± SEM of n = 3 independent determinations. One similar experiment yielded qualitatively identical results; nd, not determined.

Therefore, it is suggested that the mechanism of the LPL-mediated increase in selective CE uptake is a general one that is not restricted to liver cells.

The LPL-mediated increase in lipoprotein degradation is well established for apoB-containing particles (16–18, 24, 25). However, this study demonstrates that LPL can modify the catabolism of yet another lipoprotein fraction, i.e., HDL_3 with apoA-I as the major apolipoprotein. Here this enzyme increased selective CE uptake substantially. LPL also induced a marginal increase in cellular HDL_3 holo-particle uptake. However, substantially more CE are delivered to cells by selective CE uptake from HDL_3 compared to particle uptake, and the fractional increase in selective uptake due to LPL exceeds the fractional increase mediated by particle uptake. For cholesterol homeostasis, therefore, the LPL-induced increase in selective CE uptake probably is physiologically more relevant. For cellular lipoprotein catabolism independent of lipolysis, at least two distinct LPL actions can thus be differentiated: *a*) promotion of holo-particle uptake and *b*) stimulation of selective CE uptake. Which of these LPL effects predominates for a given lipoprotein fraction may be determined by the size or the composition of the respective particle.

Tetrahydrolipstatin (THL), an inhibitor of the lipolytic action of LPL (23, 26), was used in HDL_3 uptake experiments; LPL stimulated selective CE uptake in the absence or presence of THL at identical rates. HDL_3 was incubated in medium containing LPL and the inhibitor THL was absent or present. Analysis of HDL_3 composition and size using electrophoresis and fast protein liquid chromatography (FPLC) yielded no evidence for an LPL-induced modification of HDL_3. With respect to the kinetics, the LPL effect on selective CE uptake was apparent immediately (10 min) after initiation of the HDL_3 uptake assay and this stimulation persisted up to 4 h at a linear rate. LPL requires the presence of an activator, i.e., apolipoprotein C-II (apoC-II), to express substantial lipolytic activity (52). Compared to triglyceride-rich lipoproteins, HDL_3 is deficient in apoC-II (14). In summary, several lines of evidence suggest that the action of LPL on HDL_3 selective CE uptake by cells is a structural effect that is independent of lipolysis. This conclusion is in agreement with the action of LPL on catabolism of apoB-containing lipoproteins (16, 17, 24, 25).

The role of cell surface heparan sulfate proteoglycans in the LPL-mediated stimulation of HDL_3 metabolism was explored in proteoglycan-deficient CHO cells and alternatively proteoglycan deficiency was induced by heparinase digestion (39, 50). A deficiency of these molecules significantly reduced the stimulation of selective CE uptake by LPL. Therefore it is proposed that the effect of LPL on HDL_3 selective CE uptake is dependent on cell surface heparan sulfate proteoglycans. This conclusion is consistent with the proteoglycan dependency of the LPL effect on degradation of apoB-containing lipoproteins by cells (17, 18, 25).

Several investigations have concluded that LDL receptors or LRP play a role in the LPL-mediated catabolism of apoB-containing lipoproteins (16, 18, 19). HDL selective CE uptake per se is independent of these receptors (4, 5). The role of LRP and of LDL receptors for the LPL effect on selective CE uptake was addressed here with several experimental approaches. These included LDL receptor- or LRP-deficient cells and treatment of cells with monensin, an inhibitor of endocytosis (51). Alternatively, LDL receptors were down-regulated by cellular cholesterol-loading. All experiments provided no evidence for a function of LDL receptors or LRP in the LPL-mediated increase in HDL_3 selective CE uptake. In line with this conclusion are studies on LPL-promoted catabolism of LDL which may be receptor-independent as well (17, 25).

In agreement with previous investigations, HDL_3 selective CE uptake was down-regulated by cholesterol-loading of the cells (6). However, the magnitude of the LPL-mediated stimulation of selective CE uptake was unchanged. This suggests that CE selectively taken up from HDL_3 may enter two distinct cellular pools: *A*) an "LPL-independent" compartment that is regulated by cell cholesterol and *B*) an "LPL-dependent" cellular CE compartment that is not affected by cell cholesterol. One explanation for this observation may be two kinetically distinct cellular CE pools that were previously defined (9). Alternatively it can be speculated that two cellular mechanisms contribute to selective CE uptake, for example one involving SR-BI and one involving lipid–lipid interactions, and both might be affected by cellular cholesterol loading differentially (see below).

For the LPL-mediated cellular lipoprotein uptake, a novel mechanism has recently been suggested (17, 25, 28, 53). Accordingly, LPL binds to cell surface heparan sulfate proteoglycans and interacts with lipoproteins as well (14, 29). Promoted by LPL, multimolecular complexes are formed that are composed of lipoproteins, proteoglycans, and LPL. LPL in this model forms a "bridge" between the membrane and the lipoprotein (25). These complexes can be internalized along with cell surface heparan sulfate proteoglycans and are finally degraded in lysosomes (28).

Two mechanisms potentially mediate HDL selective CE uptake. *1*) For the lipid transfer from HDL to the plasma membrane, an essential role of lipid–lipid interactions was suggested and the latter are independent of membrane proteins or apolipoproteins (8, 9). *2*) Alternatively, the class B, type I scavenger receptor (SR-BI) may constitute a cell surface receptor that mediates selective CE uptake (10, 11). SR-BI increases HDL association with cells but not HDL holo-particle degradation and in parallel this molecule enhances selective CE uptake (10). This protein is expressed most abundantly in liver, adrenal gland, and ovary, i.e., tissues that display high rates of selective CE uptake in vivo (3). Functionally, hepatic SR-BI overexpression in mice yields a decrease in plasma HDL and an increase in biliary cholesterol (54). In contrast, in mice with a targeted null mutation in the SR-BI gene, HDL cholesterol is increased (12). Within the plasma membrane, SR-BI apparently is localized in cholesterol-rich microdomains designated caveolae (55). These results provide strong evidence that SR-BI plays an essential role in HDL

metabolism in vivo (10). Considering both potential mechanisms for selective CE uptake together, a new hypothesis emerges. Accordingly, SR-BI enhances the HDL association with the plasma membrane (10). Due to this increased presence of particles close to the cell surface, selective CE transfer from HDL to the membrane is stimulated and here lipid–lipid interactions might play a role (9).

The question should be raised as to whether SR-BI or lipid–lipid interactions (or both) are involved in the LPL-induced CE transfer from HDL to cells (9, 10). This issue was not addressed here. However, in analogy to the effect of LPL on the uptake of apoB-containing lipoproteins (17) and under consideration of the results of this investigation, it can be hypothesized that LPL, HDL, and proteoglycans form a complex. In this complex, LPL "anchors" HDL close to the cell membrane. Here LPL might facilitate the interaction between HDL and SR-BI and thereby increase selective CE uptake. This model is consistent with the enhanced cell association of HDL mediated by SR-BI (10). Alternatively, LPL might stimulate the lipid transfer from HDL to the cell membrane independently from a membrane protein and thus independently of SR-BI as well; in this case lipid–lipid interactions might play a physiologic role (9). One possibility is that SR-BI as well as LPL promote selective CE uptake by a similar mechanism, i.e., by "anchoring" HDL to the cell membrane, which is followed by increased CE transfer. Caveolae could be those membrane microdomains that are involved in this lipid uptake (55). Future investigations should explore the molecular events that occur at the plasma membrane.

Intracellularly lipoproteins can be degraded in lysosomes (27). However, hydrolysis of selectively taken up HDL CE is independent of these organelles even though the specific compartment involved has not yet been defined (13). Previously, biochemical and morphological studies showed a lysosomal degradation of lipoproteins whose uptake is enhanced by LPL (17, 25, 28, 53). Similarly, LPL itself is catabolized in these organelles. In the present study, CE that are selectively taken up due to the action of LPL are evidently catabolized in lysosomes. In line with several other investigations (17, 53) these results also imply that all lipoprotein holo-particles as well as individual components whose uptake is mediated by LPL are finally catabolized in lysosomes. However, future studies should define in more detail the mechanisms that determine the specific site of intracellular catabolism of lipoproteins and their components.

Which are the physiological implications of the LPL-mediated increase in HDL selective CE uptake observed here in vitro for reverse cholesterol transport in vivo (1)? In rats, the vast majority of plasma HDL CE is degraded by the liver (3). Similarly, radioiodinated LPL is primarily catabolized in this organ as well (56). In plasma, substantial amounts of LPL are associated with HDL (30, 31). The structural association between this lipase and HDL and the predominant catabolism of both molecules by the liver raise the possibility that LPL can indeed modify HDL metabolism in vivo. To the extent that uptake of HDL-associated CE by the liver mediates reverse cholesterol transport, then the LPL-mediated increase in selective CE uptake observed in vitro may play an anti-atherogenic role in vivo (1).

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